DESCRIPTION

TITLE OF THE INVENTION

ANTIBODY GENE EXPRESSION-SECRETION SYSTEM

Technical Field

[0001]

The present invention relates to a system for expressing an antibody gene and/or secretion, and more specifically to a DNA encoding a specific signal peptide used for secretion of antibodies such as Trastuzumab or a signal peptide-linker conjugate, a DNA insert comprising an antibody gene linked to the DNA, a vector having the DNA insert inserted thereinto, intestinal bacteria such as microorganisms of genus Bifidobacterium (bifidobacteria), which are transformed with the vector, etc.

Background Art

[0002]

Signal peptide is a sequence peptide, which comprises, dominantly, short hydrophobic amino acids (approximately 3 to 60 amino acids) in protein molecules and instructs secretion (transportation of the protein to endoplasmic reticulum). Such a signal peptide is also referred to as a "signal sequence," "localization signal," "transport (transfer) signal," etc.

[0003]

As signal sequences of bifidobacteria, the signal sequences of secretory proteins, such as amylase of Bifidobacterium adolescentis, or Sec1, Sec2 and Sec3 of

Bifidobacterium breve, have been reported, for example. In addition, the present inventors have proposed a signal sequence that can be applied to a plasmid for transformation of bifidobacteria (see, for example, Patent Documents 1 and 2).

[0004]

Other than these, the genomic analysis of Bifidobacterium longum has also been reported (see, for example, Patent Document 3).

[0005]

Moreover, there have been proposed: a method which comprises screening for an antibody gene expressed in cancer patient-derived B cells from a cancer antigen library derived from cultured cancer cells, so as to identify a more universal novel antibody gene against cancer antigens, without limitation of a source for collecting B cells (see, for example, Patent Document 4); a method of providing a gene library consisting of combinations of immunoglobulin light-chain variable region genes and heavy-chain variable region genes (see, for example, Patent Document 5); and a method of producing an antibody, which is capable of efficiently preparing a highly-versatile monoclonal antibody in a short time (see, for example, Patent Document 6), etc.

Prior Art Documents

Patent Documents

[0006]

Patent Document 1: WO 2011/093465

Patent Document 2: WO 2011/093467

Patent Document 3: EP1227152A1

Patent Document 4: Japanese unexamined Patent Application Publication No. 2010-35472

Patent Document 5: Japanese unexamined Patent Application Publication No. 2011-87586

Patent Document 6: Japanese unexamined Patent Application Publication No. 2006-180708

Summary of the Invention

Object to be Solved by the Invention

[0007]

It is an object of the present invention to provide signal sequence information capable of secreting an antibody to the outside of cells in generation of the antibody by bifidobacteria, an antibody expression vector capable of secreting an antibody to the outside of cells by utilizing the signal sequence information, and bifidobacteria which are transformed with the antibody expression vector and are capable of secreting an antibody.

Means to Solve the Object

[0008]

It has been well known that a HER2 gene product (a cell division-stimulating receptor HER2 that is present on a cell surface) is overproduced by gene amplification in breast cancer, stomach cancer, prostate cancer and the like, and thus that such overproduction becomes a cancer exacerbation factor. In antibody therapy, and particularly, in antibody therapy for cancer, it is important to allow an antibody to locally express and/or

secrete in cancer. Thus, the present inventors have used a plasmid for transformation of bifidobacteria having the signal sequence disclosed in the aforementioned Patent Document 1 or 2, with regard to a signal sequence that can be applied to a plasmid vector for transformation of bifidobacteria. As a result, it was found that secretion of antibodies is not sufficient. Hence, the inventors have studied novel signal sequences, and have found that Bifidobacterium longum transformed with a vector having inserted thereinto a DNA insert comprising a Trastuzumab single-chain antibody (scFv) gene linked to the 3'-terminus of a DNA encoding signal sequences named as SP27 and SP7 or of a signal peptide-linker conjugate having a linker sequence linked to such a signal sequence, efficiently secretes Trastuzumab to the outside of the cells, thereby completing the present invention.

[0009]

Specifically, the present invention relates to the following:

[1] A DNA encoding a signal peptide consisting of an amino acid sequence described in the following a) or b):

a) an amino acid sequence shown in SEQ ID NO: 1 (SP27) or SEQ ID NO: 107 (SP7); or

b) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids, with respect to the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107, wherein a peptide consisting of the amino acid sequence functions as a signal peptide in Bifidobacterium longum,

[2] The DNA according to the above [1], consisting of a

nucleotide sequence shown in SEQ ID NO: 2 (DNA encoding SP27) or SEQ ID NO: 108 (DNA encoding SP7),

[3] A DNA encoding a signal peptide-linker conjugate having a linker consisting of an amino acid sequence linked to the C-terminus of a signal peptide consisting of an amino acid sequence described in the following a) or b):

a) an amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107; or

b) an amino acid sequence comprising a deletion, substitution or addition of one or two amino acids, with respect to the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107, wherein a peptide consisting of the amino acid sequence functions as a signal peptide in Bifidobacterium longum,

[4] The DNA according to the above [3], wherein the signal peptide-linker conjugate consists of an amino acid sequence shown in SEQ ID NO: 3 (SP27L6) or SEQ ID NO: 109 (SP7L20),

[5] The DNA according to the above [4], consisting of a nucleotide sequence shown in SEQ ID NO: 4 (DNA encoding SP27L6) or SEQ ID NO: 110 (DNA encoding SL7L20),

[6] A DNA insert, wherein the 5'-terminus of an antibody gene is linked to the 3'-terminus of the DNA according to any one of the above [1] to [5],

[7] The DNA insert according to the above [6], wherein the antibody gene is the gene of an antibody having an anticancer activity,

[8] The DNA insert according to the above [7], wherein the antibody having an anticancer activity is Trastuzumab,

[9] The DNA insert according to the above [8], wherein the Trastuzumab is a Trastuzumab single-chain antibody,

[10] A vector having the DNA insert according to any one of the above [6] to [9] inserted thereinto,

[11] An intestinal bacterium transformed with the vector according to the above [10],

[12] The intestinal bacterium according to the above [11], wherein the bacterium is a microorganism belonging to genus Bifidobacterium,

[13] The intestinal bacterium according to the above [12], wherein the microorganism belonging to genus Bifidobacterium is Bifidobacterium longum,

[14] An antibody drug composition comprising, as an active ingredient, the intestinal bacterium according to any one of the above [10] to [13], and

[15] The antibody drug composition according to the above [14], which is an anticancer agent composition.

Effect of the Invention

[0010]

According to the present invention, by utilizing a DNA encoding a signal peptide or a signal peptide-linker conjugate, which is excellent in secretion of antibodies, bifidobacteria capable of efficiently secreting antibodies to the outside of the cells can be obtained. When the above described antibody is an antibody having an anticancer activity, such as Trastuzumab, such bifidobacteria is useful as an anticancer agent.

Brief Description of Drawings

[0011]

[Figure 1] Figure 1 shows the structure of a Trastuzumab scFv secretion plasmid. Trastuzumab scFv-coding sequence was inserted into a shuttle plasmid for protein expression to produce recombinant bifidobacteria. For use in secretion, a secretion signal and a linker sequence were fused with the head of Trastuzumab scFv, and for use in detection of a Trastuzumab scFv protein, a histidine tag was fused with the C-terminus thereof. The structure of the plasmid is shown in Figure 1. Using such a plasmid, bifidobacteria, Bifidobacterium longum 105A strain, was transformed.

[Figure 2] Figure 2 shows the whole nucleotide sequence of a Trastuzumab scFv antibody.

[Figure 3] Figure 3 shows the structure of a plasmid pSP1B-9.

[Figure 4] Figure 4 shows the structure of a PCR product by using a plasmid pHuSP1-Trastuzumab scFv as a template.

[Figure 5] Figure 5 shows the structure of Trastuzumab scFv in recombinant bifidobacteria detected by Western blot analysis. Using a culture supernatant of recombinant bifidobacteria, Western blot analysis was carried out. Detection was carried out using a histidine tag as an indicator. Bands were detected in three types of recombinant bifidobacteria (plasmid names: HuSP27L0-Trastuzumab scFv, HuSP27L6-Trastuzumab scFv, and HuSP3L22-Trastuzumab scFv).

[Figure 6] Figure 6 shows the results of analysis of the expression of Trastuzumab scFv in two types of bifidobacteria (HuSP27L6-Trastuzumab scFv and HuSP3L22-

Trastuzumab scFv) by Western blotting.

[Figure 7] Figure 7 shows the results of SDS-PAGE for His tag-purified proteins. The above described three strains of recombinant bifidobacteria were cultured, and proteins were purified from culture supernatants, using a histidine tag-fused protein purification kit (TALON Metal Affinity Resin, manufactured by TAKARA BIO INC.). The purified proteins were electrophoresed in 4% to 20% polyacrylamide gel and were then stained, and bands were then cut out.

[Figure 8] Figure 8 shows the results of analysis of His tag-purified proteins by LC-MS/MS. The cut gel was destained, and was then subjected to cystine reduction by DTT, an alkylation treatment and a trypsin treatment, so that a peptide fragment was extracted from the gel. Using this peptide fragment solution, an LC-MS/MS analysis was carried out. The detected peptide was checked against database (the Trastuzumab scFv-coding sequence of the above described plasmid had previously been registered). Some peptide fragments matched with the amino acid sequence of Trastuzumab scFv were detected.

[Figure 9] Figure 9 shows peptide fragments matched with the amino acid sequence of Trastuzumab scFv as a result of the LC-MS/MS analysis.

[Figure 10] Figure 10 shows the construction of a Trastuzumab scFv secretion vector and the expression thereof in E. coli.

[Figure 11] Figure 11 shows the results of measurement of the affinity of Trastuzumab scFv with a HER2 extracellular domain by Biacore X100.

[Figure 12] Figure 12 shows the results of measuring the

affinity of a Trastuzumab full-body antibody with a HER2 extracellular domain by Biacore X100.

[Figure 13] Figure 13 shows the results of a FACS analysis of the binding ability of Trastuzumab scFv and Trastuzumab full-body antibodies to human breast cancer cell lines.

[Figure 14] Figure 14 shows the in vivo dynamics of Cy5.5-labeled Trastuzumab scFv.

[Figure 15] Figure 15 shows the in vivo dynamics of a Cy5.5-labeled Trastuzumab full-body antibody.

[Figure 16] Figure 16 shows the antiproliferative effect of Trastuzumab scFv on an orthotropic grafted tumor of human breast cancer MDA-MB-361 cells.

[Figure 17] Figure 17 shows the results of an SDS-PAGE analysis of the expression of Trastuzumab scFv in bifidobacteria, B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv.

[Figure 18] Figure 18 shows the binding of Trastuzumab scFv purified from B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv to human breast cancer cell lines (HER2-positive line: SK-BR-3; and BT-474/HER2-negative line: SK-MEL-28) by immunostaining.

[Figure 19] Figure 19 shows the binding of Trastuzumab scFv purified from B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv to human breast cancer cell lines (HER2-positive line: SK-BR-3; and BT-474/HER2-negative line: SK-MEL-28) by a flow cytometric method.

[Figure 20] Figure 20 shows that Trastuzumab scFv purified from B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv has a dose-dependent antiproliferative activity on BT474 breast cancer cells.

[Figure 21] Figure 21 shows the antitumor effect of B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv on an orthotropic grafted tumor of a human stomach cancer cell line NCI-N87.

[Figure 22] Figure 22 shows localization of bacteria of genus Bifidobacterium in an orthotropic grafted tumor of a human stomach cancer cell line NCI-N87 by Gram staining.

[Figure 23] Figure 23 shows localization of Trastuzumab scFv in an orthotropic grafted tumor of a human stomach cancer cell line NCI-N87 by immunohistostaining using an anti-His-tag antibody.

Mode of Carrying Out the Invention

[0012]

The signal peptide of the present invention is not particularly limited, as long as it is a signal peptide consisting of a) an amino acid sequence shown in SEQ ID NO: 1 (SP27) or SEQ ID NO: 107 (SP7), or a signal peptide (mutant signal peptide) consisting of b) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids, with respect to the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107, wherein a peptide consisting of the amino acid sequence functions as a signal peptide in Bifidobacterium longum. The above described "amino acid sequence comprising a deletion, substitution or addition of one or several amino acids" means an amino acid sequence in which, for example, 1 to 5, preferably 1 to 3, more preferably 1 or 2, and even more preferably one or any given number of amino acids are deleted, substituted or added. The above

described mutant signal peptide has sequence identity of 90% or more, preferably 95% or more, and more preferably 98% or more with the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107.

[0013]

The DNA of the present invention encoding the above described signal peptide consisting of a) an amino acid sequence shown in SEQ ID NO: 1 (SP27) or SEQ ID NO: 107 (SP7) is not particularly limited, as long as it is a DNA having a nucleotide sequence corresponding to the above described amino acid sequence. Thus, the DNA of the present invention also includes a DNA that is different due to degeneracy of a codon. A specific example of the present DNA is a DNA consisting of a nucleotide sequence shown in SEQ ID NO: 2 (DNA encoding SP27) or SEQ ID NO: 108 (DNA encoding SP7). These DNAs can be produced by any given method known to a person skilled in the art, such as chemical synthesis or a genetic engineering method.

[0014]

The DNA of the present invention encoding the mutant signal peptide described in the above b) (mutant DNA) can also be produced by any given method known to a person skilled in the art, such as chemical synthesis, a genetic engineering method, or mutagenesis. Specifically, such mutant DNA can be obtained by introducing a mutation into a DNA consisting of the nucleotide sequence shown in SEQ ID NO: 2 (DNA encoding SP27) or SEQ ID NO: 108 (DNA encoding SP7) according to a method of allowing an agent serving as a mutagen to come into contact with the DNA, so that the drug is allowed to act on the DNA, or a method of

irradiating the DNA with ultraviolet ray, or a genetic engineering method, etc. A genetic engineering method, site-directed mutagenesis, is useful because this is a method capable of introducing a specific mutation into a specific site, and the site-directed mutagenesis can be carried out according to the method described in Molecular Cloning: A laboratory Mannual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989. (hereinafter abbreviated as "Molecular Cloning 2nd Edition "), Current Protocols in Molecular Biology, Supplement 1 to 38, John Wiley & Sons (1987-1997), etc.

[0015]

A linker (peptide) is preferably linked to the C-terminus of the signal peptide of the present invention. A linker that constitutes a signal peptide-linker conjugate having the linker linked to the C-terminus of the signal peptide means a peptide consisting of an amino acid sequence linking between the C-terminus of the above described signal peptide and the N-terminus of an antibody as a target protein. Such a linker can be appropriately selected from, for example, peptides existing in the C-terminus of the signal peptide. Moreover, a linker consisting of 0 to 30, preferably 3 to 25, and more preferably 5 to 15 amino acid residues is preferable. A linker consisting of an amino acid sequence shown in SEQ ID NO: 3 (SP27L6) or SEQ ID NO: 109 (SP7L20) is particularly preferable.

[0016]

The DNA encoding the signal peptide-linker conjugate of the present invention is not particularly limited, as

long as it is DNA having a nucleotide sequence corresponding to the amino acid sequence of the signal peptide-linker conjugate. Thus, it also includes a DNA that is different due to degeneracy of a codon. A specific example is a DNA consisting of a nucleotide sequence shown in SEQ ID NO: 4 (DNA encoding SP27L6) or SEQ ID NO: 110 (DNA encoding SP7L20). These DNAs can be produced by any given method known to a person skilled in the art, such as chemical synthesis, a genetic engineering method, or mutagenesis.

[0017]

The DNA insert of the present invention is not particularly limited, as long as it is a DNA, wherein the 5'-terminus of an antibody gene is linked to the 3'-terminus of a DNA encoding the above described signal peptide of the present invention or a DNA encoding a signal peptide-linker conjugate. Such a DNA insert is inserted into an expression plasmid vector. A preferred example of the above described antibody gene is a DNA encoding a chimeric antibody-, humanized antibody-, or completely humanized antibody-type full-body antibody, Fc, Fab, Fab', $F(ab')_2$, a single-chain antibody (scFv), a disulfide stabilized antibody (dsFv), or the like. Such an antibody gene can be produced by a known method such as chemical synthesis or a genetic engineering method, based on its amino acid sequence information or nucleotide sequence information (see, for example, Patent Documents 4 to 6).

[0018]

Among the above described antibody genes, the gene

of an antibody having an anticancer activity is preferable. Herein, examples of the antibody having an anticancer activity include monoclonal antibodies produced against a molecule called a tumor-related antigen, which is expressed on a cancer cell membrane, receptors of various types of growth factors, molecules of a cluster of differentiation of leukocytes (CD), or the like. As a main mechanism of antitumor action, the reinforcement of the cancer-killing ability of NK cells by the enhancement of ADCC (antibody-dependent cytotoxicity) cellular activity caused by an antibody-antigen bond has been known. Specific examples of the antibody having an anticancer activity include: an anti-human CD20 human-mouse chimeric monoclonal antibody, rituximab; an anti-HER2 humanized monoclonal antibody, Trastuzumab; an anti-human CD52 humanized monoclonal antibody, alemtuzumab; an anti-human epithelial growth factor receptor (EGFR) chimeric monoclonal antibody, cetuximab; and an anti-human vascular endothelial growth factor (VEGF) humanized monoclonal antibody, bevacizumab. The single-chain antibodies of these antibodies, and in particular, a Trastuzumab single-chain antibody is preferable.

[0019]

The vector of the present invention is not particularly limited, as long as it is an expression plasmid vector suitable for host cells, having the above described DNA insert of the present invention inserted thereinto. Intestinal bacteria, preferably microorganisms belonging to genus Bifidobacterium, and among others, Bifidobacterium longum, which is able to express the DNA

insert of the present invention, is preferable. Moreover, a shuttle vector autonomously replicating in two or more different types of organism hosts is preferable. More preferred examples of such a shuttle vector include: a shuttle vector pBLES100 constructed from pTB6 of Bifidobacterium longum BK51 disclosed in the publication of Matsumura et al. [Matsumura et al., Biosci. Biotechnol. Biochem., 61, 1211-1212 (1997)] and pBR322 of E. coli; shuttle vectors pAV001 and pBRASTA101 disclosed in the publication of Tanaka et al. [Tanaka et al., Biosci Biotechnol Biochem.; 69(2): 422-425 (2005, Feb)]; and a pBE-S DNA shuttle vector (manufactured by TAKARA BIO INC.), which is constructed from E. coli and B. subtilis used in a BIC method (Brevibacillus In vivo Cloning method) and which is capable of expressing in Gram-positive bacteria a secretory protein derived from a eukaryote, having an S-S bond in molecules. Furthermore, the above described expression plasmid vector optionally comprises a promoter, a terminator, and a drug resistance gene as a marker gene.

[0020]

In addition, utilizing a screening system for a signal peptide used for a target antibody high secretion-expression system, the above described expression plasmid vector can be selected and/or evaluated as follows. A vector, which comprises a promoter such as a Bifidobacterium longum hup promoter (Hu promoter) or a Bifidobacterium longum 105A P30 promoter (P30 promoter), and also comprises a DNA encoding a secretion signal peptide, a multicloning site (MCS) and a His tag sequence downstream of the promoter, and into the MCS of which a

target antibody gene is inserted, and which is thereafter cleaved with restriction enzyme to be linearized, and the In-Fusion cloning system of Clontech, etc. are used, and the amount of an antibody secreted from bifidobacteria transformed with a vector, into which the DNA of the present invention encoding a secretion signal peptide suitable for the target antibody, etc. has been inserted, is measured, so that an available expression plasmid vector can be selected.

[0021]

The intestinal bacteria of the present invention are not particularly limited, as long as they are intestinal bacteria transformed with the above described vector of the present invention. The intestinal bacteria as host cells are normal bacteria mainly comprising obligate anaerobic bacteria living in the intestinal portions of humans or animals. Specific examples of the intestinal bacteria include Gram-positive lactic acid bacteria such as bacterium of the genus Lactobacillus or bacterium of the genus Bifidobacterium, and Gram-negative bacteria such as bacterium of the genus Clostridium, E. coli, or bacterium of the genus Bacteroides. Among others, bifidobacteria are preferable.

[0022]

Specific examples of the above described bifidobacteria include Bifidobacterium longum, B. breve, B. adolescentis, B. bifidum, B. pseudolongum, B. thermophirum, B. infantis, B. animalis, B. angulatum, B. asteroides, B. boum, B. catenulatum, B. choerinum, B. coryneforme, B. cuniculi, B. denticolens, B. dentium, B. gallicum, B.

gallinarum, B. globosum, B. indicum, B. inopinatum, B. lactis, B. lactentis, B. magnum, B. merycicum, B. minimum, B. Mongolia Enns, B. parvulorum, B. pseudocatenulatum, B. psychraerophilum, B. pullorum, B. ruminale, B. ruminantium, B. saeculare, B. scardovii, B. subtile, B. suis, and B. thermacidophilum. Among others, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium bifidum, and Bifidobacterium infantis, which have been known to normally exist in human intestine, regardless of age, are preferably used as host cells, and further, Bifidobacterium longum is more preferably used. All of these bacteria are commercially available, or can be easily obtained from depositary institutions. For example, Bifidobacterium longum ATCC-15707, Bifidobacterium bifidum ATCC-11863, and Bifidobacterium infantis ATCC-15697 can be used.

[0023]

Moreover, the strains of such bifidobacteria are not particularly limited, either. Preferred examples of the Bifidobacterium longum strain include Bifidobacterium longum 105A, Bifidobacterium longum aE-194b, Bifidobacterium longum bs-601, and Bifidobacterium longum M101-2. Among these strains, Bifidobacterium longum 105A is preferable. Examples of the Bifidobacterium breve strain include a Bifidobacterium breve standard strain (JCM1192), Bifidobacterium breve aS-1, and Bifidobacterium breve I-53-8W. Among these strains, the Bifidobacterium breve standard strain and Bifidobacterium breve aS-1 are preferable. Examples of the Bifidobacterium infantis strain include a Bifidobacterium infantis standard strain

(JCM1222) and Bifidobacterium infantis I-10-5. Among these strains, the Bifidobacterium infantis standard strain and Bifidobacterium infantis I-10-5 are preferable. An example of the Bifidobacterium lactentis strain is a Bifidobacterium lactentis standard strain (JCM1210).

[0024]

Examples of the method of introducing a vector into intestinal bacteria in the present invention include an electroporation method, an In-Fusion cloning system (Clontech), a liposome method, a lipofection method, a microinjection method, a DEAE-dextran method, and a calcium phosphate method. Among these methods, the electroporation method is preferable. Moreover, methods of using commercially available transfection reagents, such as Lipofectin Reagent (registered trademark), Lipofectamine (registered trademark), Lipofectamine (registered trademark) 2000 Reagent (manufactured by Invitrogen), SuperFect (registered trademark) Transfection Reagent (manufactured by QIAGEN), FuGENE (registered trademark) HD Transfection Reagent (manufactured by Roche Diagnostics), and FuGENE (registered trademark) 6 Transfection Reagent (manufactured by Roche Diagnostics), which have been widely applied in the art, can be applied.

[0025]

The above described intestinal bacteria of the present invention can be used as an antibody drug, and the intestinal bacteria of the present invention transformed with a DNA insert comprising the gene of an antibody having an anticancer activity can be used as an anticancer agent. Accordingly, the antibody drug composition of the

present invention is not particularly limited, as long as it comprises, as an active ingredient, the above described intestinal bacteria of the present invention that is capable of secreting an antibody, and preferably, an antibody having an anticancer activity. The present antibody drug composition optionally comprises any given components, such as a pharmacologically acceptable carrier, an excipient, and a diluent, unless these components impair the action and/or effect of a secreted antibody.

[0026]

The dosage form of the antibody drug composition of the present invention includes a liquid agent or a solid preparation. Such a liquid agent can be produced by purifying a culture solution of the intestinal bacteria of the present invention, then adding, as necessary, a suitable physiological saline or fluid replacement, or pharmaceutical additives, and then filling an ampule or vial bottle, etc. with the obtained mixture. On the other hand, such a solid preparation can be produced by adding a suitable protective agent to the liquid agent, then filling an ampule or vial bottle, etc. with the obtained mixture, and then freeze-drying it, or adding a suitable protective agent to the liquid agent, then freeze-drying it, and then filling an ampule or vial bottle, etc. with the resultant. As a method of administering the antibody drug composition of the present invention to a subject, both oral administration and parenteral administration are applicable. Of these, parenteral administration is preferable, and examples of the parenteral administration include intravenous injection, subcutaneous injection,

local injection, and intraventricular administration. Among these, intravenous injection is most preferable.

[0027]

The dose of the antibody drug composition of the present invention is not particularly limited, as long as it is an amount sufficient for growth in diseased site and the expression of an effective therapeutic amount of active antibody. The dose of the present antibody drug composition is selected, as appropriate, depending on the degree of disease, and the body weight, age and sex of a patient, and the dose can be increased or decreased, as appropriate, depending on the degree of amelioration. From the viewpoint of economic efficiency and from the viewpoint of prevention of side effects as much as possible, the applied dose of the present antibody drug composition is preferably as low as possible within a range in which necessary therapeutic effects can be obtained.

[0028]

For example, in particular, since intravenous administration is required to reduce the risk of embolism caused by cell masses, etc., it is preferable that a possible lowest concentration of injection preparation be dispensed dividedly over several administrations, or that the injection be diluted with a suitable fluid replacement and the thus diluted solution be continuously injected. In the case of an adult for example, the cells of the intestinal bacteria of the present invention, which are in an amount of 10^6 to 10^{12} cfu per kg of body weight, are administered once a day, or dividedly over several

administrations a day, for one to several days, continuously or at appropriate intervals. More specifically, 1 to 1000 mL of a preparation comprising the cells of the microorganisms of genus Bifidobacterium of the present invention at a concentration of 10^4 to 10^{10} cfu/mL is administered to an adult, directly or by being diluted with a suitable fluid replacement, once a day or divided over several administrations a day, continuously for one to several days.

[0029]

Moreover, in the case of local administration in which the intestinal bacteria are directly administered to diseased tissues, it is required for the bacteria to engraft to the entire diseased tissues as much as possible and to grow there. Thus, it is desired to administer a high concentration of injection to several sites in diseased tissues. In the case of an adult for example, the cells of the microorganisms of genus Bifidobacterium of the present invention are administered at a dose of 10^6 to 10^{12} cfu per kg of body weight once or several times a day, as necessary for one to several days, continuously or at appropriate intervals. More specifically, 1 to 1000 mL of a preparation comprising the cells of the bifidobacteria of the present invention at a concentration of 10^4 to 10^{10} cfu/mL is directly administered to an adult, several times a day, as necessary, continuously for one to several days.

[0030]

When the antibody drug composition of the present

invention is an anticancer agent composition, it can be applied, for example, to large bowel cancer, brain tumor, head and neck cancer, breast cancer, lung cancer, esophageal cancer, stomach cancer, liver cancer, gallbladder cancer, bile duct cancer, pancreatic cancer, islet cell carcinoma, choriocarcinoma, colon cancer, renal cell carcinoma, adrenal cortex cancer, bladder cancer, testicular cancer, prostate cancer, testicular tumor, ovary cancer, uterine cancer, choriocarcinoma, thyroid cancer, malignant carcinoid tumor, skin cancer, malignant melanoma, osteosarcoma, soft tissue sarcoma, neuroblastoma, Wilms' tumor, retinoblastoma, melanoma, squamous cell carcinoma, etc.

Examples

[0031]

Hereinafter, the present invention will be more specifically described in the following examples. However, these examples are not intended to limit the technical scope of the present invention.

Example 1

[0032]

1. Construction of secretory Trastuzumab scFv expression plasmid (pHuSPx-Trastuzumab scFv)

A plasmid for expressing secretory Trastuzumab scFv (anti-HER2 low molecular weight single-chain antibody [scFv]) in a histone-like promoter (Hu promoter) of bifidobacteria was constructed. First, a plasmid pHuSP1-Trastuzumab scFv was produced by linking Trastuzumab scFv

to a signal peptide 1 (SP1), and thereafter, pHuSPx-Trastuzumab scFv was produced by the replacement of the signal peptide portion. A summary of the production of the plasmid is shown in Figure 1. The details are as follows.

[0033]

2. Preparation of insert in production of pHuSP1-Trastuzumab scFv

A Trastuzumab scFv insert was prepared as follows. First, the amino acid sequence of a Trastuzumab full-body antibody was downloaded from RCSB Protein data bank (PDB) (http://www.pdb.org/pdb/home/home.do) (PDB 1N8Z). The DNAs of VH, VL and a linker sequence were determined based on the cDNA sequences registered in the PDB database (Figure 2) (SEQ ID NOS: 5 to 11). Using, as a template, a plasmid pOZ Trastuzumab scFv-His prepared by inserting this Trastuzumab scFv-coding sequence (comprising a His-tag sequence at the terminus thereof) into a pOZ1 vector, PCR was carried out. As primers, Trastuzumab scFv_ins_F3 primer (SEQ ID NO: 14) and Trastuzumab scFv_ins_R2 primer (SEQ ID NO: 15) (Table 1; 15 nucleotides on the 5'-terminal side of each primer have a sequence homologous to a vector as shown below) were used, and a Trastuzumab scFv-coding region was amplified to obtain a 777-bp insert PCR product. Using 2.0% agarose gel (1 × TEB buffer, containing ethidium bromide), the insert PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0034]

[Table 1]

SEQ ID NO:		Name of primer	Sequence (5' → 3')	Template plasmid
12	Primers for preparation of vector	SP1_Vec_F3	CCTTCTGCTCGTAGCGATTAC	SP1B-9 (SEQ ID NO: 16)
13		SP1_Vec_R2	TTCCACGCGCTCCTTGG	
14	Primers for preparation of Trastuzumab insert (In-Fusion)	Trastuzumab scFv_ins_INF_F3	aaggagcgcgtggaaGAAGTTCAGCTGGTTGAAAGC	pOZ Trastuzumab scFv-His
15		Trastuzumab scFv_ins_INF_R2	gctacgagcagaaggTTAATGATGGTGATGATGATGTTTAATTTC	

Small letters indicate 15-nucleotide sequence complementary to vector 5'-terminus added to target gene amplification primer for In-Fusion cloning

[0035]

3. Preparation of vector in production of pHuSP1-Trastuzumab scFv

A vector was prepared as follows. Using, as a template, a plasmid pSP1B-9 (comprising a GFPuv gene, a replication origin of E. coli, a replication origin of bifidobacteria, and a spectinomycin resistance gene (Figure 3) (SEQ ID NO: 16)), PCR was carried out. Using, as primers, SP1_Vec_F3 primer (SEQ ID NO: 12) and SP1_Vec_R2 primer (SEQ ID NO: 13) (Table 1), a region, from which the GFPuv gene was excluded, was amplified to obtain a 3983-bp vector PCR product (PrimeSTAR; registered trademark: HS Premix, manufactured by TAKARA BIO INC.). Using 0.8% agarose gel (1 × TEB buffer, containing ethidium bromide), the vector PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0036]

4. Fusing of insert to vector by In-Fusion reaction in production of pHuSP1-Trastuzumab scFv

Using In-Fusion (registered trademark) HD Cloning Kit with Cloning Enhancer (manufactured by Clontech), the insert PCR product was fused to the vector PCR product. First, referring to the website of Clontech, In-Fusion

(registered trademark) Molar Ratio Calculator (http://bioinfo.clontech.com/infusion/molarRatio.do), necessary amounts of the insert and the vector were calculated. Two μL of 5 × In-Fusion HD Enzymes premix, 1 μL of Cloning Enhancer, and necessary amounts of the insert and the vector were mixed with one another, and sterilized water was then added to the obtained mixture, so that a total amount of the reaction system was adjusted to 10 μL. After completion of a reaction at 37°C for 15 minutes, the reaction product was treated at 50°C for 15 minutes and was then left at rest at 4°C.

[0037]

5. Transformation of E. coli, plasmid extraction and sequencing in production of pHuSP1-Trastuzumab scFv

Using 2 μL of In-Fusion reaction solution, E. coli TOP10 chemically Competent Cell (manufactured by Invitrogen) was transformed, and the resultant was then transferred onto an LB (75 μg/mL, containing spectinomycin) plate, followed by performing a culture at 37°C overnight. Conditions for the transformation were as described in the product instruction. The transformed E. coli colonies were cultured in an LB (75 μg/mL, containing spectinomycin) liquid medium at 37°C overnight, and a plasmid was then extracted from the culture (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). It was confirmed that the whole nucleotide sequence of this plasmid was as designed, and the plasmid was designated as a plasmid pHuSP1-Trastuzumab scFv.

[0038]

6. Preparation of inserts (SP2 to SP10, SP12 to SP16, SP19,

and SP21 to SP27) in production of pHuSPx-Trastuzumab scFv (x = 2 to 10, 12 to 16, 19, and 21 to 27), involving replacement of signal peptide

PCR was carried out using, as a template, a plasmid comprising each signal peptide, so as to prepare an insert. Using the primers (wherein 15 nucleotides on the 5'-terminal side of each primer have a sequence homologous to a vector as shown below) and the templates shown in Table 2, PCR was carried out to obtain each insert PCR product. Using 2.0% agarose gel, the insert PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0039]

[Table 2]

SEQ ID NO:		Name of primer	Sequence (5' -> 3')	Template plasmid
17	Primers for preparation of vector	Hu-Trastuzumab_vec_F1	GAAGTTCAGCTGGTTGAAAGCGG	pHuSP1-Trastuzumab (SEQ ID NO: 67)
18		Hu-Vec_R1	AAAGCATCCTTCTTGGGTCAGG	
19	Primers for preparation of SP insert (In-Fusion)	SP2B_ins_INF_F1	caagaaggatgctttGTGGGTATGACTGAGAACGC	SP2B-3 (SEQ ID NO: 68)
20		SP2B-Trastuzumab_ins_INF_R1	aaccagctgaacttcCAAAAACAGCACGCGG	
21		SP3B_ins_INF_F1	caagaaggatgctttATGTTCAATAAGCGACACATCG	SP3B-4 (SEQ ID NO: 69)
22		SP3B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGCGATGGTCAGCTTGC	
23		SP4B_ins_INF_F1	caagaaggatgctttATGACCACTCACAACAGCCAG	SP4B-1 (SEQ ID NO: 70)
24		SP4B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGCCGAACAGACGCGG	
25		SP5B_ins_INF_F1	caagaaggatgctttATGACCGCGATTGACGAG	SP5B-2 (SEQ ID NO: 71)
26		SP5B-Trastuzumab_ins_INF_R1	aaccagctgaacttcTTGGTCGATGATGGCCTTG	
27		SP6B_ins_INF_F1	caagaaggatgctttATGAAGATTGCGGTTGCAG	SP6B-1 (SEQ ID NO: 72)
28		SP6B-Trastuzumab_ins_INF_R1	aaccagctgaacttcATCGACAATAGGACTTTTCCCATTG	
29		SP7B_ins_INF_F1	caagaaggatgctttATGTTTGCGTGCGTAGCC	SP7B-1 (SEQ ID NO: 73)
30		SP7B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGTGGAGGTGGATTCGG	
31		SP8B_ins_INF_F1	caagaaggatgctttATGGTTGGTGACGACACCG	SP8B-1 (SEQ ID NO: 74)
32		SP8B-Trastuzumab_ins_INF_R1	aaccagctgaacttcCATCGTTGCCTCGCC	
33		SP9B_ins_INF_F1	caagaaggatgctttATGGGCACCATGATGCG	SP9B-1 (SEQ ID NO: 75)
34		SP9B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGACGATCTGATGCGCCAG	
35		SP10B_ins_INF_F1	caagaaggatgctttATGATGACTGGTGCACAGGC	SP10B-1 (SEQ ID NO: 76)
36		SP10B-Trastuzumab_ins_INF_R1	aaccagctgaacttcTCGCTGCTTGAGTTTGCC	
37		SP12B_ins_INF_F1	caagaaggatgctttATGGTGTCTTTCAATAAACTGACC	SP12B-2 (SEQ ID NO: 77)
38		SP12B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGAACGGGTCCACAGGGT	
39		SP13B_ins_INF_F1	caagaaggatgctttATGGTCGCCGTCCTCAG	SP13B-1 (SEQ ID NO: 78)
40		SP13B-Trastuzumab_ins_INF_R1	aaccagctgaacttcAGACTCGCTAGCACAGCACAG	
41		SP14B_ins_INF_F1	caagaaggatgctttTTGCCGGGACCTATATGTCC	SP14B-3 (SEQ ID NO: 79)
42		SP14B-Trastuzumab_ins_INF_R1	aaccagctgaacttcTTGGGCCACTATTGTCTTCTCG	
43		SP15B_ins_INF_F1	caagaaggatgctttATGAAACGTAGCGATTATATGTTGG	SP15B-2 (SEQ ID NO: 80)
44		SP15B-Trastuzumab_ins_INF_R1	aaccagctgaacttcCTTGCCTGAGGCATCTTGAATC	
45		SP16B_ins_INF_F1	caagaaggatgctttATGAGCAATAGTGCATCATCGTTTAC	SP16B-2 (SEQ ID NO: 81)
46		SP16B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGCCAACGGAGTCGTCTC	
47		SP19B_ins_INF_F1	caagaaggatgctttTTGGCAAGATGGGTCACTC	SP19B-4 (SEQ ID NO: 82)
48		SP19B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGCCCATGACCGGCATG	
49		SP21B_ins_INF_F1	caagaaggatgctttATGGCATTGACTGATGAACAGG	SP21B-1 (SEQ ID NO: 83)
50		SP21B-Trastuzumab_ins_INF_R1	aaccagctgaacttcACGTGCAGTGGTATGGATGATT	
51		SP22B_ins_INF_F1	caagaaggatgctttTTGGTGTCTATGAGAAGCCCAC	SP22B-2 (SEQ ID NO: 84)
52		SP22B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGATGCGCTCACGCTTGG	
53		SP23B_ins_INF_F1	caagaaggatgctttATGAACAAGCGATGGAACAAAC	SP23B (SEQ ID NO: 85)
54		SP23B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGATCGTCTTGAGAATCTTCAGACG	
55		SP24B_ins_INF_F1	caagaaggatgctttATGGTCGGCATGCGC	SP24B-4 (SEQ ID NO: 86)
56		SP24B-Trastuzumab_ins_INF_R1	aaccagctgaacttcGTTGGTGCGGTTCCGG	
57		SP25B_ins_INF_F1	caagaaggatgctttGTGATGTTATCCACACCCTCCA	SP25B-3 (SEQ ID NO: 87)
58		SP25B-Trastuzumab_ins_INF_R1	aaccagctgaacttcCTGCTCATGATCGGCCCA	
59		SP26_HU_ins_INF_F1	caagaaggatgctttATGAAGAAGAAAGCTCTTGCTTTCG	SP26-1 (SEQ ID NO: 88)
60		SP26_L0-Trastuzumab_ins_INF_R1	aaccagctgaacttcAGCGTTGCTGTTGGAGCC	
61		SP26_HU_ins_INF_F1	caagaaggatgctttATGAAGAAGAAAGCTCTTGCTTTCG	SP26-2 (SEQ ID NO: 89)
62		SP26_L5-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGTGTCACCGGAGGCAG	
63		SP27_HU_ins_INF_F1	caagaaggatgctttATGAACACCATTCGTCGCATC	SP27-3 (SEQ ID NO: 90)
64		SP27_L0-Trastuzumab_ins_INF_R1	aaccagctgaacttcCGCTTGTGCGGTTTGAC	
65		SP27_HU_ins_INF_F1	caagaaggatgctttATGAACACCATTCGTCGCATC	SP27-4 (SEQ ID NO: 91)
66		SP27_L6-Trastuzumab_ins_INF_R1	aaccagctgaacttcGGTGCGGTTAGCCGTG	

Small letters indicate 15-nucleotide sequence complementary to vector 5'-terminus added to target gene amplification primer for In-Fusion cloning

[0040]

7. Preparation of vectors (SP2 to SP10, SP12 to SP16, SP19, and SP21 to SP27) in production of pHuSPx-Trastuzumab scFv (x = 2 to 10, 12 to 16, 19, and 21 to 27), involving replacement of signal peptide

PCR was carried out using the plasmid pHuSP1-Trastuzumab scFv as a template, so as to prepare a vector. Using, as primers, Hu-Trastuzumab_vec_F1 primer (SEQ ID NO: 17) and Hu-vec_R1 primer (SEQ ID NO: 18) (Table 2), a region, from which the signal peptide portion (SP1) was excluded, was amplified to obtain a 4589-bp vector PCR product (Figure 4; comprising a Trastuzumab scFv gene, a replication origin of E. coli, a replication origin of bifidobacteria, and a spectinomycin resistance gene). Using 0.8% agarose gel, the vector PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0041]

8. Linking of inserts to vectors by In-Fusion reaction (SP2 to SP10, SP12 to SP16, SP19, and SP21 to SP27) in production of pHuSPx-Trastuzumab scFv (x = 2 to 10, 12 to 16, 19, and 21 to 27), involving replacement of signal peptide

Using In-Fusion (registered trademark) HD Cloning Kit with Cloning Enhancer (manufactured by Clontech) in the same manner as that described above, the insert PCR products were linked to the vector PCR products.

[0042]

9. Transformation of E. coli, plasmid extraction and sequencing (SP2 to SP10, SP12 to SP16, SP19, and SP21 to SP27) in production of pHuSPx-Trastuzumab scFv (x = 2 to

10, 12 to 16, 19, and 21 to 27), involving replacement of signal peptide

Using 1 μL of In-Fusion reaction solution, E. coli TOP10 chemically Competent Cell (manufactured by Invitrogen) was transformed, and the resultant was then transferred onto an LB (75 μg/mL, containing spectinomycin) plate, followed by performing a culture at 37°C overnight. Conditions for the transformation were as described in the product instruction. The transformed E. coli colonies were cultured in an LB (75 μg/mL, containing spectinomycin) liquid medium at 37°C overnight, and a plasmid was then extracted from the culture (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). It was confirmed that the nucleotide sequence of this plasmid ranging from the vicinity of a Hu promoter to the vicinity of a terminator was as designed. The produced plasmids are shown in Table 3.

[0043]

[Table 3]

Produced plasmids

No.	Plasmid *1	Cleavage site by Signal P *2
1	pHuSP1-Trastuzumab scFv	Non
2	pHuSP2-Trastuzumab scFv	Non
3	pHuSP3L22-Trastuzumab scFv	33/34
4	pHuSP4-Trastuzumab scFv	Non
5	pHuSP5-Trastuzumab scFv	Non
6	pHuSP6-Trastuzumab scFv	Non
7	pHuSP7-Trastuzumab scFv	Non
8	pHuSP8-Trastuzumab scFv	Non
9	pHuSP9-Trastuzumab scFv	Non
10	pHuSP10-Trastuzumab scFv	Non
11	pHuSP12L20-Trastuzumab scFv	37/38
12	pHuSP13-Trastuzumab scFv	Non
13	pHuSP14L20-Trastuzumab scFv	42/43
14	pHuSP15L20-Trastuzumab scFv	30/31
15	pHuSP16-Trastuzumab scFv	Non
16	pHuSP19L43-Trastuzumab scFv	22/23
17	pHuSP21-Trastuzumab scFv	Non
18	pHuSP22-Trastuzumab scFv	Non
19	pHuSP23L27-Trastuzumab scFv	33/34
20	pHuSP24-Trastuzumab scFv	Non
21	pHuSP25-Trastuzumab scFv	Non
22	pHuSP26L0-Trastuzumab scFv	21/22
23	pHuSP26L5-Trastuzumab scFv	21/22
24	pHuSP27L0-Trastuzumab scFv	52/53
25	pHuSP27L6-Trastuzumab scFv	52/53

*1: Regarding names of plasmids

e.g.) pHuSP3L22-Trastuzumab scFv

- p indicates p of plasmid
- Hu indicates Hu promoter
- SP indicates signal peptide, having original serial number
- L indicates the number of linkers, namely, the number of amino acids between signal peptide cleavage prediction site and target protein (in this case, there are 22 amino acids between cleavage prediction site and Trastuzumab scFv)
- Finally, name of target protein (in this case, Trastuzumab scFv)

*2: Cleavage prediction site analyzed with secretion signal cleavage site prediction software SignalP Ver4.0

When there are no cleavage prediction sites, plasmid name does not include Lxx because the number of linkers cannot be determined

[0044]

10. Transformation of bifidobacteria (SP2 to SP10, SP12 to SP16, SP19, and SP21 to SP27) in production of pHuSPx-Trastuzumab scFv (x = 2 to 10, 12 to 16, 19, and 21 to 27), involving replacement of signal peptide

Using 1.5 to 3 μL of plasmid DNA extracted from the transformed E. coli (Table 3), bifidobacteria, Bifidobacterium longum 105A, was transformed according to an electroporation system (Gene Pulser II, manufactured by Bio-Rad Laboratories). After completion of the electric shock, a mixed solution of 800 μL of IMR liquid medium and 50 μL of vitamin C-added solution was immediately added to a cuvette, and it was then recovered into a 2-mL sterilized microtube. The same operations as described above were carried out on each tube, and the cap of each 2-mL tube was then loosened. The resulting solution, together with AneroPack, was placed in a hermetically sealed vessel, it was then placed in an incubator that had been set at 37°C, and it was then incubated for 3 hours. After completion of the incubation, each suspension was fully mixed, and 100 μL of the mixed suspension was taken and was then each applied to one IMR agar medium (containing 75 μg/mL SPCM). Such an agar medium, together with a deoxidant/carbon dioxide generator (AneroPack-Kenki, manufactured by Mitsubishi Gas Chemical Company), was placed in a hermetically sealed vessel, and was then cultured in an incubator that had been set at 37°C for 2 to 3 days. The colonies growing on the plate were picked up with a disposable stick, and were then streaked on a

BL-bS agar medium (containing 75 μg/mL SPCM). The medium, together with a deoxidant/carbon dioxide generator (AneroPack-Kenki, manufactured by Mitsubishi Gas Chemical Company), was placed in a hermetically sealed vessel, and was then cultured in an incubator that had been set at 37°C for 1 day, so as to obtain a streaked culture.

[0045]

11. Western blot analysis of culture supernatant and intracellular protein

The above-obtained streaked culture of recombinant bifidobacteria (Bifidobacterium longum 105A/pHuSPx-Trastuzumab scFv (x = 2 to 10, 12 to 16, 19, and 21 to 27)) was inoculated into an APS-2S-2.5SE (75 μg/mL, spectinomycin) liquid medium, and it was then subjected to an anaerobic culture at 37°C for 24 hours (activated culture solution). Subsequently, 0.5% activated culture solution was inoculated in a medium prepared by adding spectinomycin to a DMEM (low glucose, pyruvic acid, and HEPES) culture medium for cell culture (manufactured by Life Technologies cat #12320-032) : APS-2S-2.5SE (= 9 : 1) to a concentration of 75 μg/mL. The thus obtained mixture was subjected to an anaerobic culture at 37°C for 15 hours. Using this culture solution, a culture supernatant and an intracellular protein were prepared as follows. After the culture solution had been centrifuged, a culture supernatant was recovered. The protein in this culture supernatant was precipitated with trichloroacetic acid (TCA), was then washed with acetone, and was then dissolved in an electrophoretic buffer, so that the protein in the culture supernatant was concentrated.

Separately, an intracellular protein was extracted as follows. 1 mL of the culture solution was mixed with 4 mL of PBS, and the mixed solution was then centrifuged at 12,000 rpm for 5 minutes at 4°C, and a supernatant was then removed. 5 mL of PBS was added to suspend this precipitate, and the obtained mixture was then centrifuged to remove a supernatant. This operation was carried out twice. PBS was added to the cells after completion of washing, to a total amount of 1 mL, and thereafter, the cells were disrupted by an ultrasonic treatment. After the disrupted cells had been centrifuged, a supernatant was recovered. The supernatant was designated as an intracellular extract. The above described culture supernatant concentrate (corresponding to 1 mL of the intracellular protein extract culture solution) was electrophoresed with Mini-PROTEAN TGX GEL 4-20% (manufactured by Bio-Rad). The resultant was transferred on a PVDF membrane (manufactured by Invitrogen, iBlot Transfer Stacks), using iBlot Transfer Device (manufactured by Invitrogen). After completion of blotting, the membrane was blocked, and it was then treated with Anti-His antibody (manufactured by GE Healthcare) used as a primary antibody and then with anti-mouse Ab-HRP (manufactured by GE Healthcare) used as a secondary antibody. Thereafter, luminescence was produced using Western Lightning Ultra (manufactured by Perkin Elmer). This resultant was analyzed using an imaging analyzer (Fluor S Max, manufactured by Bio-Rad). As a result, secretion was observed in three types of bacteria (B. longum 105A/pHuSP3L22-Trastuzumab scFv; B. longum

105A/pHuSP27L0-Trastuzumab scFv; and B. longum 105A/pHuSP27L6-Trastuzumab scFv) (Figure 5). The results of Western blotting on B. longum 105A/pHuSP27L6-Trastuzumab scFv and B. longum 105A/pHuSP3L22-Trastuzumab scFv are shown in Figure 6. A band was detected around the size of Trastuzumab scFv (approximately 25 kDa).

[0046]

12. SDS-PAGE of product purified from bifidobacteria culture supernatant

A streaked culture of recombinant bifidobacteria (Bifidobacterium longum 105A) that express secretory Trastuzumab scFv was inoculated into an APS-2S-2.5SE (75 µg/mL, spectinomycin) liquid medium, and it was then subjected to an anaerobic culture at 37°C for 24 hours (activated culture solution). Subsequently, 0.5% activated culture solution was inoculated in a medium prepared by adding spectinomycin to a DMEM (low glucose, pyruvic acid, and HEPES) culture medium for cell culture (manufactured by Life Technologies cat #12320-032) : APS-2S-2.5SE (= 9 : 1) to a concentration of 75 µg/mL. The thus obtained mixture was subjected to an anaerobic culture at 37°C for 16 hours. After the culture solution had been centrifuged, ammonium sulfate (for enzyme purification, manufactured by Wako Pure Chemical Industries, Ltd.) was added to the culture supernatant, resulting in 80% saturated solution, and the solution was then stirred at 4 °C overnight. After the reaction solution had been centrifuged, 1 × PBS buffer (pH 7.4) was added to the precipitate to dissolve it in the buffer. The thus obtained solution was purified using a histidine

tag protein purification kit (TALON Metal Affinity Resin, manufactured by TAKARA BIO INC.). The purified solution was concentrated by ultrafiltration (Amicon Ultra 10K, manufactured by Merck Millipore). Into the concentrated protein solution, an equal amount of 2 × SDS sample buffer was mixed, and the obtained mixture was then heated at 95°C for 3 minutes. This reaction mixture was defined as a sample for SDS-PAGE. The above described sample was electrophoresed in 1 × SDS buffer, using Mini-PROTEAN TGX GEL (4%-20%, manufactured by BIO-RAD). After completion of the electrophoresis, the gel was washed with water, was then stained with a staining solution (SimplyBlueTM SafeStain), and was then destained with water. The results are shown in Figure 7. In SP27L0 having no linkers, a band was not detected around the size of Trastuzumab scFv (approximately 25 kDa) (the arrow in the upper case in the figure), but in SP27L6 and SP3L22 into which a linker has been incorporated, a band with the estimated size of Trastuzumab scFv was detected (Figure 7).

[0047]

13. Analysis of purified protein by LC-MS/MS

Recombinant bifidobacteria, B. longum 105A/pHuSP27L6-scFv, was cultured, and a culture supernatant was then purified using a histidine tag-fused protein purification kit (TALON Metal Affinity Resin, manufactured by TAKARA BIO INC.). The purified protein was electrophoresed on 4%-20% polyacrylamide gel (Mini-PROTEAN TGX GEL, manufactured by Bio-Rad) and was then stained with Simply Blue Stain (manufactured by Invitrogen), and a band was cut out. A band was cut out

of the stained gel to make a gel cube with a size of 1 × 1 mm^3, and the gel was then distained using destaining solution (an aqueous solution containing 30% acetonitrile (manufactured by Wako Pure Chemical Industries, Ltd.) and 25 mM ammonium bicarbonate (manufactured by SIGMA)), and was then reduced by 10 mM DTT ((±)-dithiothreitol, manufactured by Wako Pure Chemical Industries, Ltd.) (56°C, 45 min). Thereafter, it was subjected to air cooling to a room temperature, and was then alkylated with 55 mM ICH_2CONH_2 (iodoacetoamide, manufactured by Wako Pure Chemical Industries, Ltd.) (at room temperature under light-shielding conditions for 30 min). After completion of the alkylation, in-gel digestion (37°C, 16 h) was carried out using 12.5 ng/mL trypsin (manufactured by Promega), and the digested peptide fragment was extracted from the gel and was then concentrated (Reference: Shevchenko A. et al. Anal. Chem. 68, 850-858, 1996.). The obtained peptide fragment was analyzed by LC-MS/MS (Waters nano ACQUITY UPLC, manufactured by Xevo QTOF), and the identified peptide was compared with the amino acid sequence of Trastuzumab scFv (Figure 8). As a result of the LC-MS/MS analysis, peptide fragments matched with the amino acid sequence of Trastuzumab scFv are shown in Figure 9.

[0048]

14. Construction of recombinant vector (pUC119 plasmid) and production of Trastuzumab scFv

DNA, which had been artificially synthesized based on the Trastuzumab cDNA sequence obtained from the PDB database as in the aforementioned case, was incorporated

into a pUC119 plasmid to produce a vector, and the thus produced vector was used as a Trastuzumab scFv expression vector. A Trastuzumab-scFv-His plasmid was introduced into Rosetta2 E. coli (Merck Millipore, Darmstadt, Germany), and colonies were then selected using agar containing ampicillin and chloramphenicol. The colonies were allowed to grow at 37°C in 10 mL of ampicillin-containing LB medium that comprised 2% glucose. Thereafter, the colonies were transferred into 200 mL of LB medium containing IPTG, and the culture was continuously carried out at 25°C for 20 hours. Thereafter, E. coli was recovered, and a cell lysate was then extracted with BugBuster Protein Extraction Reagent (manufactured by Novagen) containing benzonuclease. After that, the cell lysate was centrifuged at 10000 × g for 5 minutes, so that a supernatant containing an antibody protein was recovered. The His tag-attached antibody protein was purified using HisTrap HP Ni Sepharose column and Sephadex G25 gel filtration. After completion of the purification, SDS-PAGE was carried out using Tris-Glycine gel, and as a result, a band of Trastuzumab scFv that was matched with 30 kDa was detected (Figure 10). The purified scFv antibody was used in flow cytometry or a surface plasmon resonance (SPR) assay using Biacore.

[0049]

15. Measurement of affinity of Trastuzumab scFv with HER2 extracellular domain by Biacore X100 using surface plasmon resonance (SPR) method

Trastuzumab scFv produced using E. coli Rosetta2 strain was used, and also, a HER2 extracellular domain

protein (HER2. ex.) and a Trastuzumab full-body antibody, which had been produced using an insect-derived HF cell line, were used. An SPR analysis was carried out using Biacore X100. All of reagents and sensor chips used in the measurement were purchased from GE Healthcare. For immobilization of HER2. ex. on a CM5 sensor chip, a 10 mM acetate solution with pH 5.0 was used, and for immobilization of a Trastuzumab full-body antibody thereon, the solution with pH 5.5 was used. The measurement was carried out using a 0.05% or 0.005% Tween-PBS buffer (pH 7.4) at a flow rate of 30 μL/mL at 25°C. The binding affinity of Trastuzumab scFv with an antigen was obtained by two methods, namely, a multi-cycle method, which comprises observing the interaction of the added Trastuzumab scFv with HER2. ex. immobilized on the sensor chip, then completely removing the bound Trastuzumab scFv with a regeneration solution, then repeating the same measurement as described above even on Trastuzumab scFv with a different concentration, and comprehensively analyzing the obtained results; and a single cycle method capable of obtaining information necessary for analysis only by successively adding Trastuzumab scFv with 5 different concentrations during a single measurement cycle, wherein the removal of Trastuzumab scFv binding to HER2. ex. is not required. Dynamic constants regarding binding and dissociation were calculated using Biacore X100 evaluation software. The results of analyses are shown in Figure 11.

[0050]

16. Measurement of affinity of Anti-HER2/neu antibody with

HER2 extracellular domain by Biacore X100 using a surface plasmon resonance (SPR) method

As described above, using Trastuzumab full-body antibody on an immobilized side and HER2. ex. on a flow channel side, the binding affinity of a Trastuzumab full-body antibody with a HER2 antigen was analyzed according to a single cycle method employing Biacore X100. The obtained results were compared with the binding affinity of the produced Trastuzumab scFv. For the sake of convenience, since HER2. ex. was used on an immobilization side and Trastuzumab scFv was used on a flow channel side in the analysis of Trastuzumab scFv, the relationship between the flow channel and the immobilization became opposite to the case of the analysis of a Trastuzumab full-body antibody (Figure 12). As a result, it was found that the produced Trastuzumab scFv has binding affinity equivalent to that of the Trastuzumab full-body antibody. Moreover, from the ka value (the larger the ka value, the higher the affinity with an antigen) and the kd value (the smaller the kd value, the stronger the binding ability with an antigen) used as reaction rate constants, it was demonstrated that the produced Trastuzumab scFv has higher affinity with an antigen than the Trastuzumab full-body antibody, but that the binding ability thereof with an antigen is low.

[0051]

17. FACS analysis of binding ability of Trastuzumab scFv and Trastuzumab full-body antibody to human breast cancer cell lines

As human breast cancer cell lines, a HER2-positive line (SKBR-3) and a HER2-negative line (MDA-MD231, 468) were used. As an anti-His antibody used for FACS, a PE-labeled anti-His antibody (CAT. 130-092-691, manufactured by Miltenyi) was used. In addition, as a control of Trastuzumab scFv, human CMVpp65 scFv was used. Regarding reagents, PBS(-) + 0.1% BSA + 0.1% sodium azide was used as an Ab buffer, PBS(-) + 1% FBS + 0.1% sodium azide + 2 mM EDTA was used as a FACS buffer, and FACS buffer + 0.5% PFA (paraform aldehyde) was used as a fixation buffer. Trastuzumab scFv and human CMVpp65 scFv were each adjusted to give a concentration of 10 μg/mL. As a secondary antibody, a PE-anti-His antibody was used.

1 to 2 × 10^5/well cells were suspended in the FACS buffer, and the obtained suspension was then seeded on a 96-well round-bottom plate. The plate was centrifuged at 1400 rpm for 2 minutes, and a supernatant was then aspirated. 10 μL of the Ab buffer was added to and suspended in the reaction solution, and thereafter, 10 μL of Trastuzumab scFv or human CMVpp65 scFv (10 μg/mL) used as a control was added thereto. The obtained mixture was incubated at 4°C for 15 minutes, and the reaction solution was then washed with 150 μL of the FACS buffer twice. The reaction solution was centrifuged at 1400 rpm for 4 minutes, and a supernatant was then removed by aspiration. 10 μL of the Ab buffer was added to the resulting solution, 10 μL of the PE-anti-His antibody (not diluted) was further added thereto, and the obtained mixture was incubated at 4°C for 15 minutes. The reaction solution was washed with 150 μL of the FACS buffer twice, and was

then centrifuged at 1400 rpm for 2 minutes, and thereafter, a supernatant was aspirated. 400 µL of the fixation buffer was added to the resulting solution, and the obtained solution was transferred into a FACS tube by pipetting. Thereafter, the measurement was carried out using Flow Cytometry (FACSCanto, manufactured by BD Biosciences) within 24 hours. The results are shown in Figure 13. It was demonstrated that Trastuzumab scFv is an antibody that is sufficiently separable from negative control cells, although it is slightly inferior to a full-body antibody thereof (Figure 13, center and right). Moreover, it was also demonstrated that Trastuzumab scFv specifically binds to an antigen, from the comparison with anti-cytomegalovirus antigen scFv as a control, in which a HER2 antigen expressed by SKBR-3 cells cannot be detected (Figure 13, left).

[0052]

18. In vivo dynamic imaging of Cy5.5-labeled Trastuzumab scFv using in vivo tumor models

A human mammary tumor cell line MDA-MB-361 (5×10^6 cells/mouse [containing Matrigel]), which highly expresses a HER2 antigen, was transplanted into the mammary epithelium of a BALB/cA-nu/nu mouse (female, 7-week-old, manufactured by CLEA Japan, Inc.) that immunologically deleted T cell function. To the MDA-MB-361 cancer-bearing mouse (single mouse) whose tumor volume had reached approximately 290 mm^3, fluorochrome Cy5.5-labeled Trastuzumab scFv (corresponding to 3.75 mg/kg) was intratumorally administered once (100 µL). In order to confirm the retention of the Cy5.5-labeled Trastuzumab

scFv in the tumor according to intratumoral administration, the mouse was measured under isoflurane inhalation anesthesia for 1 week, using in vivo imaging device eXplore Optix (manufactured by GE Healthcare) (Figure 14). On the first week, the mouse was sacrificed by euthanasia, the tumor, liver, spleen, lung and small intestine were then excised from the mouse, and imaging ability was then measured. As a result, it was demonstrated that the Cy5.5-labeled Trastuzumab scFv can be detected even on the 7th day after intratumoral administration thereof.

[0053]

19. In vivo dynamic imaging of Cy5.5-labeled Trastuzumab full-body antibody using in vivo tumor models

As described above, using a cancer-bearing nude mouse system, into the mammary epithelium of which 4×10^6 cells/mouse MDA-MB-361 was transplanted, in order to confirm the retention of the Cy5.5-labeled Trastuzumab full-body antibody in the tumor according to intratumoral administration, a Cy5.5-labeled Trastuzumab full-body antibody (corresponding to 3.75 mg/kg) was intratumorally administered to a cancer-bearing mouse (400 mm^3) on the 56th day after the transplantation. Intratumoral retention was measured until 5th day after the administration, under isoflurane inhalation anesthesia, using eXplore Optix (Figure 15). Even 5 days after the intratumoral administration, the fluorescence-labeled antibody could be observed in the living body of the mouse. Thus, it was demonstrated that the Cy5.5-labeled Trastuzumab full-body antibody can be detected for a long

period of time, as in the case of the Cy5.5-labeled Trastuzumab scFv.

[0054]

20. Antitumor effect of Trastuzumab scFv on orthotropic grafted tumor of human breast cancer MDA-MB-361 cells

A human mammary tumor cell line MDA-MB-361 (5×10^6 cells/mouse [containing Matrigel]) highly expressing a HER2 antigen was transplanted into the mammary epithelium of the aforementioned BALB/cA-nu/nu mouse (n = 4 to 5/group). To the MDA-MB-361 cancer-bearing mice whose mean tumor volume had reached 250 mm^3, Trastuzumab scFv (corresponding to 3.75 mg/kg) was intratumorally administered (50 μL) five times every other day. The tumor volume was measured over time, after initiation of the administration of Trastuzumab scFv, and the antitumor activity thereof was compared with the antitumor activity in a non-treated group (control group). From the results obtained using the tumor volume measured every 3 or 4 days as an indicator, it was demonstrated that Trastuzumab scFv has a distinct antiproliferative effect on the tumor cells (Figure 16).

Example 2

[0055]

21. Production of secretion plasmid (pHuSP7L20-opt-Trastuzumab scFv) optimized to codon of bifidobacteria

The nucleotide sequence of a Trastuzumab scFv gene was optimized to the codon of bifidobacteria (opt-Trastuzumab scFv gene: SEQ ID NO: 92). Moreover, a secretion plasmid (pHuSP7L20-opt-Trastuzumab scFv: SEQ ID

NO: 94), into which the opt-Trastuzumab scFv gene was incorporated, was produced.

The opt-Trastuzumab scFv gene was artificially synthesized as a plasmid pUC57-opt-Trastuzumab scFv subcloned into a plasmid pUC57 for E. coli (manufactured by GenScript Japan Inc.). As a first stage, a Trastuzumab scFv gene of pHuSP27L0-Trastuzumab scFv (SEQ ID NO: 93) that was an existing Trastuzumab scFv expression-secretion plasmid was replaced by an opt-Trastuzumab scFv gene, so as to produce a plasmid pHuSP27L0-opt-Trastuzumab scFv. As a second stage, SP27L0 that was a signal peptide + a linker sequence was replaced by SP7L20, so as to produce pHuSP7L20-opt-Trastuzumab scFv (SEQ ID NO: 94). As a third stage, the Hu promoter of pHuSP7L20-opt-Trastuzumab scFv was replaced by a P30 promoter, so as to produce pP30SP7L20-opt-Trastuzumab scFv (SEQ ID NO: 95). The details are as follows.

[0056]

22. Preparation of insert in production of pHuSP27L0-opt-Trastuzumab scFv

The procedures for replacing the Trastuzumab scFv gene portion of pHuSP27L0-Trastuzumab scFv with an opt-Trastuzumab scFv gene will be described below. First, an opt-Trastuzumab scFv insert was prepared as follows. Using a plasmid pUC57-opt-Trastuzumab scFv having an opt-Trastuzumab scFv-coding sequence (containing a His-tag sequence at the terminus thereof) as a template, PCR was carried out. Using, as primers, opt-Trastuzumab scFv_ins_INF_F1 primer (SEQ ID NO: 96) and opt-Trastuzumab scFv_ins_INF_R1 primer (SEQ ID NO: 97) (wherein 15

nucleotides on the 5'-terminal side of each primer have a sequence homologous to a vector as shown below), an opt-Trastuzumab scFv-coding region was amplified to obtain a 777-bp insert PCR product. Using 2.0% agarose gel (1 × TEB buffer, containing ethidium bromide), the insert PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0057]

23. Preparation of vector in production of pHuSP27L0-opt-Trastuzumab scFv

A vector was prepared as follows. Using, as a template, pHuSP27L0-Trastuzumab scFv (comprising a Trastuzumab scFv gene, a replication origin of E. coli, a replication origin of bifidobacteria, and a spectinomycin resistance gene), PCR was carried out. Using, as primers, SP1_Vec_F1 primer (SEQ ID NO: 98) and d0018_0aa_Vec_R3 primer (SEQ ID NO: 99), a region from which the Trastuzumab scFv gene was excluded was amplified, so as to obtain an approximately 4-kb vector PCR product (PrimeSTAR; registered trademark: HS Premix, manufactured by TAKARA BIO INC.). Using 0.8% agarose gel (1 × TEB buffer, containing ethidium bromide), the vector PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0058]

24. Fusing of insert to vector by In-Fusion reaction in production of pHuSP27L0-opt-Trastuzumab scFv

Using In-Fusion (registered trademark) HD Cloning Kit with Cloning Enhancer (manufactured by Clontech), the insert PCR product was fused to the vector PCR product. First, referring to the website of Clontech, In-Fusion (registered trademark) Molar Ratio Calculator (http://bioinfo.clontech.com/infusion /molarRatio.do), necessary amounts of the insert and the vector were calculated. 2 μL of 5 × In-Fusion HD Enzymes premix, 1 μL of Cloning Enhancer, and necessary amounts of the insert and the vector were mixed with one another, and sterilized water was then added to the obtained mixture, so that a total amount of the reaction system was adjusted to 10 μL. After completion of a reaction at 37°C for 15 minutes, the reaction product was treated at 50°C for 15 minutes and was then left at rest at 4°C.

[0059]

25. Transformation of E. coli, plasmid extraction and sequencing in production of pHuSP27L0-opt-Trastuzumab scFv

Using 2 μL of In-Fusion reaction solution, E. coli HST16CR Competent Cells (manufactured by TAKARA BIO INC.) were transformed, and the resultant was then transferred onto an LB (75 μg/mL, containing spectinomycin) plate, followed by performing a culture at 37°C overnight. Conditions for the transformation were as described in the product instruction. The transformed E. coli colonies were cultured in an LB (75 μg/mL, containing spectinomycin) liquid medium at 37°C overnight, and a plasmid was then extracted from the culture (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). It was confirmed that the whole nucleotide sequence of this plasmid was as

designed, and the plasmid was designated as a plasmid pHuSP27L0-opt-Trastuzumab scFv.

[0060]

26. Preparation of insert in production of pHuSP7L20-opt-Trastuzumab scFv

The procedures for replacing the signal peptide + linker sequence (SP27L0) of pHuSP27L0-opt-Trastuzumab scFv with SP7L20 will be described below. First, using the genomic DNA of Bifidobacterium longum 105A as a template, PCR was carried out, and a signal peptide insert was prepared as follows. Using SP7L20-opt-Trastuzumab scFv_ins_INF_F1 primer (SEQ ID NO: 100) and SP7L20-opt-Trastuzumab scFv_ins_INF_R1 primer (SEQ ID NO: 101) (wherein 15 nucleotides on the 5'-terminal side of each primer have a sequence homologous to a vector as shown below), PCR was carried out, so as to obtain a 189-bp insert PCR product (SP7L20). Using 2.0% agarose gel, the insert PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0061]

27. Preparation of vector in production of pHuSP7L20-opt-Trastuzumab scFv

Using the plasmid pHuSP27L0-opt-Trastuzumab scFv as a template, PCR was carried out to prepare a vector. Using, as primers, Hu-opt-Trastuzumab_vec_F1 primer (SEQ ID NO: 102) and Hu-Vec_R1 primer (SEQ ID NO: 18), a region from which the signal peptide + linker portion was excluded was amplified, so as to obtain an approximately 4.5-kb vector PCR product. Using 0.8% agarose gel, the

vector PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0062]

28. Fusing of insert to vector by In-Fusion reaction in production of pHuSP7L20-opt-Trastuzumab scFv

As described above, using In-Fusion (registered trademark) HD Cloning Kit with Cloning Enhancer (manufactured by Clontech), an insert PCR product was fused to a vector PCR product.

[0063]

29. Transformation of E. coli, plasmid extraction and sequencing in production of pHuSP7L20-opt-Trastuzumab scFv

As described above, using In-Fusion reaction solution, transformation of E. coli HST16CR Competent Cells, culture, and plasmid extraction were carried out. Thereafter, it was confirmed that the nucleotide sequence of an opt-Trastuzumab scFv expression cassette (from the Hu promoter to the terminator) of this plasmid was as designed.

[0064]

30. Preparation of insert in production of pP30SP7L20-opt-Trastuzumab scFv

The promoter of pHuSP7L20-opt-Trastuzumab scFv was replaced by the P30 promoter of a gene existing in the genome of B. longum 105A.

First, using the genomic DNA of B. longum 105A as a template, PCR was carried out, and a signal peptide insert was prepared as follows. Using P30_ins_F1 primer (SEQ ID NO: 103) and P30_SP7_ins_R1 primer (SEQ ID NO: 104)

(wherein 15 nucleotides on the 5'-terminal side of each primer have a sequence homologous to a vector as shown below), PCR was carried out to obtain a 265-bp insert PCR product. Using 2.0% agarose gel, the insert PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0065]

31. Preparation of vector in production of pP30SP7L20-opt-Trastuzumab scFv

Using the plasmid pHuSP7L20-opt-Trastuzumab scFv as a template, PCR was carried out to prepare a vector. Using, as primers, SP7_Vec_F1 primer (SEQ ID NO: 105) and pUC_ori_Vec_R2 primer (SEQ ID NO: 106), the region of the opt-Trastuzumab scFv expression unit, from which a promoter was excluded, was amplified to obtain an approximately 4.4-kb vector PCR product. Using 0.8% agarose gel, the vector PCR product, together with a DNA concentration marker, was subjected to electrophoresis, so that the concentration was estimated.

[0066]

32. Fusing of insert to vector by In-Fusion reaction in production of pP30SP7L20-opt-Trastuzumab scFv

As described above, using In-Fusion (registered trademark) HD Cloning Kit with Cloning Enhancer (manufactured by Clontech), an insert PCR product was fused to a vector PCR product.

[0067]

33. Transformation of E. coli, plasmid extraction and sequencing in production of pP30SP7L20-opt-Trastuzumab scFv

By the same operations as those described above, transformation of E. coli HST16CR Competent Cells, culture, and plasmid extraction were carried out using In-Fusion reaction solution. Thereafter, it was confirmed that the nucleotide sequence of an opt-Trastuzumab scFv expression cassette (from the P30 promoter to the terminator) of this plasmid was as designed.

[0068]

34. Transformation of bifidobacteria

Using the plasmids pHuSP7L20-opt-Trastuzumab scFv and pP30SP7L20-opt-Trastuzumab scFv, bifidobacteria were transformed as follows. Using the plasmid DNA, bifidobacteria, B. longum 105A, was transformed according to an electroporation system (Gene Pulser II, manufactured by Bio-Rad Laboratories). After completion of the electric shock, a mixed solution of 800 µL of IMR liquid medium and 50 µL of vitamin C-added solution was immediately added to a cuvette, and it was then recovered into a 2-mL sterilized microtube. The same operations as described above were carried out on each tube, and the cap of each 2-mL tube was then loosened. The resulting solution, together with AneroPack, was placed in a hermetically sealed vessel, it was then placed in an incubator that had been set at 37°C, and it was then incubated for 3 hours. After completion of the incubation, each suspension was fully mixed, and 100 µL of the mixed suspension was taken and was then each applied to one IMR agar medium (containing 75 µg/mL SPCM). Such an agar medium, together with a deoxidant/carbon dioxide generator (AneroPack-Kenki, manufactured by Mitsubishi Gas Chemical

Company), was placed in a hermetically sealed vessel, and was then cultured in an incubator that had been set at 37°C for 2 to 3 days. The colonies growing on the plate were picked up with a disposable stick, and were then streaked on a BL-bS agar medium (containing 75 μg/mL SPCM). The medium, together with a deoxidant/carbon dioxide generator (AneroPack-Kenki, manufactured by Mitsubishi Gas Chemical Company), was placed in a hermetically sealed vessel, and was then cultured in an incubator that had been set at 37°C for 1 day, so as to obtain a streaked culture, thereby obtaining recombinant bifidobacteria, B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv and B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv.

[0069]

35. Purification of Trastuzumab scFv from bifidobacteria

Trastuzumab scFv was purified using the above-produced Trastuzumab scFv secretion bifidobacteria, B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv.

B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv was inoculated into APS-2S-2.5SE medium (containing 75 μg/mL spectinomycin), and it was then subjected to an anaerobic culture at 37°C for 24 hours. Subsequently, 0.5% of this culture solution was added to a medium prepared by adding spectinomycin to DMEM : APS-2S-2.5SE (= 9 : 1) to a concentration of 75 μg/mL. The thus obtained mixture was subjected to an anaerobic culture at 37°C for 18 hours.

While a culture supernatant obtained by centrifugation of the above described culture solution was stirred, ammonium sulfate was slowly added to the culture supernatant to 80% saturation. The obtained mixture was

stirred at 4°C overnight so as to conduct salting-out. The resultant was centrifuged, and thereafter, a precipitate was recovered and was then purified by employing a histidine tag-added protein purification kit (TALON resin, manufactured by TAKARA BIO INC.), using a histidine tag as an indicator. The thus purified solution was concentrated by ultrafiltration (Amicon Ultra-0.5, manufactured by Merck Millipore).

SDS-PAGE was performed on a part of the above-purified single-chain antibody, and thereafter, Coomassie Blue staining (manufactured by Life Technologies, SimplyBlueTM SafeStain) was carried out thereon, so that it was confirmed that Trastuzumab scFv was purified at a purity of approximately 90%. The concentration of the purified protein was measured by a Bradford method (Coomassie Plus Protein Assay, manufactured by Thermo Scientific).

The results of the SDS-PAGE analysis are shown in Figure 17. A band was detected around the size of Trastuzumab scFv (approximately 25 kDa).

[0070]

36. Confirmation of binding of Trastuzumab scFv to human breast cancer cell lines by fluorescent antibody technique

Using Trastuzumab scFv purified from B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv, the binding of the Trastuzumab scFv to human breast cancer cell lines was confirmed.

As human breast cancer cell lines, HER2-positive lines (SK-BR-3 and BT-474) and a HER2-negative line (SK-MEL-28) were used (all of which were purchased from

American Type Culture Collection, ATCC). As an anti-His antibody for immunostaining, an Alexa Fluor 488-labeled anti-His antibody (Cat. D291-A48, manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.) was used. In addition, an anti-HER2 full-body antibody (Cat. 427041, manufactured by NICHIREI BIOSCIENCE INC.) was added, so that the expression of HER2 in the cells was confirmed. Regarding reagents, PBS(-) was used as a wash buffer, PBS(-) + 1.5% BSA was used as an Ab buffer, and 4% PFA (paraformaldehyde) + phosphate buffer (manufactured by Wako Pure Chemical Industries, Ltd.) was used as a fixation buffer. Trastuzumab scFv and an anti-HER2 full-body antibody were prepared to a concentration of 5 μg/mL by addition of the Ab buffer. As a secondary antibody against Trastuzumab scFv, Anti-His-tag mAb-Alexa Fluor 488 was used, and as a secondary antibody against an anti-HER2 full-body antibody, DyLight 594 goat anti-mouse IgG (Cat. 405311, manufactured by BioLegend) was used. As a mounting agent, VECTASHIELD Mounting Medium with DAPI (Cat. H-1200, manufactured by VECTOR Laboratories) was used.

A cover glass with a size of 18 mm × 18 mm (manufactured by Matsunami Glass Ind., Ltd.) was placed on a 6-well plate (manufactured by NEST), and it was then coated with Poly-L-lysine (manufactured by Sigma-Aldrich). Thereafter, the cells were cultured thereon at a density of 1 to 2 × 10^4 cells/glass. One day later, the plate was left at rest on ice, and the medium was aspirated and cells were then washed with a wash buffer three times. Thereafter, 100 μL of the Ab buffer was added to the cells, and the obtained mixture was then incubated on ice for 30

minutes. Thereafter, 100 μL of Trastuzumab scFv, or an Ab buffer used as a negative control, was added, and the obtained mixture was incubated on ice for 1 hour. After completion of a scFv reaction, the resultant was washed with a wash buffer three times. After completion of the washing, 100 μL of Anti-His-tag mAb-Alexa Fluor 488 (400-fold diluted) was added to the resultant, and the obtained mixture was then incubated on ice for 30 minutes. After completion of the incubation, the reaction product was washed with a wash buffer three times, 500 μL of the fixation buffer was then added to the resultant, and the obtained mixture was then incubated on ice for 10 minutes. Thereafter, the reaction product was washed with a wash buffer three times, and was then mounted with a DAPI-containing mounting agent, followed by observation under a fluorescence microscope (DM5000B, manufactured by Leica MICROSYSTEMS).

The results are shown in Figure 18. It was confirmed that HER2 was expressed in HER2-positive cells, and that HER2 was not expressed in HER2-negative cells. Furthermore, it was also demonstrated that Trastuzumab scFv specifically binds to HER2-positive cells and is co-localized with HER2 on the cell surface.

[0071]

37. Confirmation of binding of Trastuzumab scFv to human breast cancer cell lines by flow cytometry

Using Trastuzumab scFv purified from B. longum 105A/pHuSP7L20-opt-Trastuzumab scFv, the binding of the Trastuzumab scFv to human breast cancer cell lines was confirmed.

BT-474 cells and SK-BR-3 cells, in which human HER2 was positive, and SK-MEL-28 cells in which human HER2 was negative (all of which were purchased from American Type Culture Collection, ATCC), were used. These cells were cultured in a 100-mm petri dish (manufactured by NIPPON Genetics Co, Ltd.). BT-474 cells were cultured in a Hybri-care medium, SK-BR-3 cells were cultured in a McCoy's 5A medium, and SK-MEL-28 cells were cultured in an E-MEM medium. Into all of these media, inactivated 10% fetal bovine serum (manufactured by EQUITECH-BIO) and penicillin/streptomycin solution (manufactured by COSMO BIO Co., Ltd.) were mixed.

At the stage in which the above described cells were gathered to a certain extent, the medium was removed, and the cells were washed with PBS(-) (manufactured by Wako Pure Chemical Industries, Ltd.). Thereafter, the cells were detached from the petri dish, using Trypsin-EDTA (manufactured by Life Technologies), and were then transferred into a 15-mL conical tube (manufactured by SANPLATEC CORP.). The cells were centrifuged at 1000 rpm for 5 minutes, using a desktop small centrifuge (manufactured by KUBOTA CO., LTD.), a supernatant was then removed, and 5 mL of medium was then added thereto. The number of cells contained in this cell suspension was counted using a hemocytometer, and the cells were then dispensed in a 1.5-mL tube (manufactured by Eppendorf) to a cell density of 3×10^4 cells/tube. The cells dispensed in the 1.5-mL tube were centrifuged at 5000 rpm at 4°C for 1 minute, using a trace-amount refrigerated centrifuge (manufactured by TOMY), and after completion of the

centrifugation, a supernatant was removed. Cell pellets remaining in the tube were washed with 1 mL of PBS(-) twice, and 100 µL of the purified anti-HER2 scFv was added in a concentration of 10 µg/mL thereto. It was then left at rest on ice for 30 minutes. To the reaction product, 20 µL of a fluorescence-labeled anti-His-tag antibody (Anti-His-tag Alexa Fluor 488, manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.) was added, and the obtained mixture was fully mixed by pipetting. The reaction mixture was then left at rest on ice for 20 minutes. After leaving at rest, 500 µL of FACS buffer (PBS containing 1% BSA and 0.1% NaN_3) was added to the tube, and the cells were then suspended therein. The obtained mixture was centrifuged at 5000 rpm at 4°C for 1 minute, using a trace-amount refrigerated centrifuge, and a supernatant was then removed. This washing operation was carried out again, and 500 µL of FACS buffer was added to the tube to suspend the cells therein. Thereafter, the cells were transferred into a 5-mL polystyrene round-bottom tube (manufactured by Becton, Dickinson and Company).

Immediately before performing an analysis, 5 µL of a propidium iodide solution (5 µg/mL) was added to the tube, and thereafter, an analysis was carried out using a BD FACS cantoII flow cytometer (manufactured by Becton, Dickinson and Company) and flow cytometric analysis software Kaluza ver 1.2 (manufactured by BECKMAN COULTER).

The results are shown in Figure 19. In the upper case of Figure 19, it was confirmed that Trastuzumab scFv binds to BT-474 cells and SK-BR-3 cells, which were both

HER2-positive cells. On the other hand, in the lower case of Figure 19, the binding of Trastuzumab scFv to SK-MEL-28 cells, which were HER2-negative cells, was not observed.

[0072]

38. Antiproliferative activity of Trastuzumab scFv against cancer cells

With regard to the physiological activity of Trastuzumab scFv, Trastuzumab scFv obtained by His tag purification of a culture supernatant of B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv (PBS replacement) was added to HER2-positive cells (BT474 (breast cancer) cells), and the antiproliferative activity was then measured.

BT474 cells were cultured in a McCoy's 5A medium (containing 10% (v/v) FBS) at 37°C in 5% CO_2. Thereafter, the cells were seeded on a 96-well plate at a cell density of 1×10^4 cells/well, and were then cultured at 37°C in 5% CO_2 for 24 hours. After completion of the culture, the medium was removed by aspiration, and 98 µL each of fresh McCoy's 5A medium (containing 10% (v/v) FBS) was added. Subsequently, 2 µL each of anti-Trastuzumab scFv in PBS(-) that was adjusted to 244 ng/mL to 1 mg/mL was added as a measurement sample. This plate was cultured at 37°C in 5% CO_2 for 5 days.

After completion of the culture for 5 days, the medium was removed by aspiration, and 100 µL each of 1 mL of Cell Counting Kit-8 added to 9 mL of fresh McCoy's 5A medium (containing 10% (v/v) FBS) was added thereto. The obtained mixture was further incubated at 37°C in 5% CO_2 for 3 hours, and the absorbance was then measured at wavelengths of 450 nm and 630 nm (reference wavelengths),

so that the antiproliferative activity of Trastuzumab scFv against the above described HER2-positive cells was measured.

The results are shown in Figure 20. It was confirmed that Trastuzumab scFv purified from B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv exhibits a dose-dependent antiproliferative activity on BT474 breast cancer cells, and that Trastuzumab scFv has physiological activity.

[0073]

39. Confirmation of antitumor effect of Trastuzumab scFv secretion bifidobacteria, B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv

Using cancer-bearing nude mice with the human stomach cancer cell line NCI-N87, the antitumor effect of B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv was confirmed as follows.

The human stomach cancer cell line NCI-N87 (purchased from ATCC) was cultured in a RPMI1640 medium (manufactured by Wako Pure Chemical Industries, Ltd.), to which 10% FBS (manufactured by EQUITECH-BIO, INC.) had been added, and the cells were then transplanted into nude mice (manufactured by Japan SLC, Inc.) to produce cancer-bearing mice. For the experiment, cancer-bearing mice, the tumor volume of which had reached approximately 200 mm^3, were used. Constitution of groups is as follows: Group 1: a non-treated group (control group); Group 2: a B. longum 105A/pBEshuttle strain (Trastuzumab scFv-not-expressing bacteria) administration group; and Group 3: a B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv

(Trastuzumab scFv-expressing bacteria) administration group. Eight cancer-bearing mice were used for each group.

Bifidobacteria were administered to the mice through the caudal vein, at a dose of 6×10^8 cfu, twice a week. In addition, to the bifidobacteria administration groups (Group 2 and Group 3), 1 mL each of 10% maltose solution was administered to the mice at a frequency of twice a day, five times a week (administration for 5 days, then suspension for 2 days). The test period was set at 3 weeks, and tumor volume was measured over time. On the 22nd day, the tumor was excised, and was then used for Gram staining and immunohistostaining.

A change over time in tumor volume and the results regarding antitumor effect are shown in Figure 21. The tumor volume in the B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv administration group transited at a level lower than other groups throughout the test period. At the time of termination of the test (Day 22), the tumor volume in the B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv administration group was significantly reduced in comparison to that in the B. longum 105A/pBEshuttle administration group, and B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv was confirmed to have an antitumor effect.

[0074]

40. Confirmation of the presence of B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv in tumor, and detection of secreted Trastuzumab scFv

Using the tumor excised in the above section 39, localization of the bacteria of genus Bifidobacterium in the tumor was confirmed by Gram staining, and localization

of Trastuzumab scFv therein was confirmed by immunohistostaining using an anti-His-tag antibody.

The excised tumor was subjected to frozen embedding using O.C.T. compound (manufactured by Sakura Finetek Japan Co., Ltd.), and then, thin slide specimens were produced using freezing microtome Leica CM1900 (manufactured by Leica) and were then subjected to each histostaining.

[0075]

The procedures for Gram staining will be described below. The above described thin slide specimen was air-dried, and was then immersed in 4% PFA (manufactured by Wako Pure Chemical Industries, Ltd.) for 10 minutes, so that it was fixed. After completion of the fixation, the specimen was pre-stained with Barmi M Crystal Violet Solution (manufactured by Muto Pure Chemicals Co., Ltd.) for 2 minutes, and was then treated with Barmi M Iodine-Sodium Hydroxide Solution (manufactured by Muto Pure Chemicals Co., Ltd.) for 1 minute. Thereafter, the specimen was discolored with Barmi M acetone-ethyl alcohol mixed solution (manufactured by Muto Pure Chemicals Co., Ltd.), and was then stained with Barmi M 0.1% fuchsin solution (manufactured by Muto Pure Chemicals Co., Ltd.) for 1 minute. After completion of the staining, the specimen was washed with purified water, was then dehydrated with 99.5% ethanol (manufactured by Wako Pure Chemical Industries, Ltd.), and was then dialyzed with Lemosol (manufactured by Wako Pure Chemical Industries, Ltd.). Thereafter, the specimen was mounted with Entellan new (manufactured by MERCK KGaA).

The results are shown in Figure 22. As a result of the Gram staining, the presence of B. longum 105A/pBEshuttle and B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv was confirmed in the tumor tissues (the arrow portion of Figure 22).

[0076]

The procedures for immunohistostaining using an anti-histidine tag antibody will be described below. The above described thin slide specimen was air-dried, and was then immersed in 4% PFA (manufactured by Wako Pure Chemical Industries, Ltd.) for approximately 4 hours, so that it was fixed. After completion of the fixation, the specimen was washed with purified water for 1 minute, and was then washed with 1 × PBS(-) for 5 minutes three times. Water around the tissues was wiped off, and the tissues were then enclosed with Dako pen (manufactured by Dako). Thereafter, 3% BSA-PBS was added dropwise to the tissues, and the reaction was carried out for 60 minutes, so that non-specific bindings were inhibited. Anti-His-tag mAb-Alexa Fluor (registered trademark) 594 (manufactured by MBL) was mixed and diluted with Can Get Signal (registered trademark) immunostain (manufactured by TOYOBO), and the mixed solution was used as an antibody reaction solution and was added dropwise to the tissues. The reaction was carried out at 4°C overnight. After completion of the antibody reaction, the tissues were washed with 1 × PBS(-) for 5 minutes three times, and was then mounted with VECTASHIELD (registered trademark) Mounting Medium with DAPI. The stained section was subjected to microscopic examination under a microscope DM5000B (manufactured by

Leica), and the image thereof was then photographed.

The results are shown in Figure 23. As a result of the immunohistostaining for a histidine tag, a histidine tag-positive image (Trastuzumab scFv) was observed (the arrow portion of Figure 23).

[0077]

As a result of the Gram staining and the immunohistostaining, it was confirmed that, when B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv is intravenously administered to human stomach cancer NCI-N87-bearing mice, it is engrafted in the tumor, and also that Trastuzumab scFv secreted from B. longum 105A/pP30SP7L20-opt-Trastuzumab scFv is present in the tumor.

Industrial Applicability

[0078]

The vector of the present invention or intestinal bacteria transformed with the vector can efficiently supply a therapeutic agent to the diseased site in anaerobic diseased tissues, when compared with conventional ones. Accordingly, the vector of the present invention or intestinal bacteria transformed with the vector is useful in the field of pharmaceutical products or therapeutic field.

CLAIMS

1. A DNA encoding a signal peptide consisting of an amino acid sequence described in the following a) or b):
a) an amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107; or
b) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids, with respect to the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107, wherein a peptide consisting of the amino acid sequence functions as a signal peptide in Bifidobacterium longum.

2. The DNA according to claim 1, consisting of a nucleotide sequence shown in SEQ ID NO: 2 or SEQ ID NO: 108.

3. A DNA encoding a signal peptide-linker conjugate having a linker consisting of an amino acid sequence linked to the C-terminus of a signal peptide consisting of an amino acid sequence described in the following a) or b):
a) an amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107; or
b) an amino acid sequence comprising a deletion, substitution or addition of one or two amino acids, with respect to the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 107, wherein a peptide consisting of the amino acid sequence functions as a signal peptide in Bifidobacterium longum.

4. The DNA according to claim 3, wherein the signal peptide-linker conjugate consists of an amino acid sequence shown in SEQ ID NO: 3 or SEQ ID NO: 109.

5. The DNA according to claim 4, consisting of a nucleotide sequence shown in SEQ ID NO: 4 or SEQ ID NO: 110.

6. A DNA insert, wherein the 5'-terminus of an antibody gene is linked to the 3'-terminus of the DNA according to any one of claims 1 to 5.

7. The DNA insert according to claim 6, wherein the antibody gene is the gene of an antibody having an anticancer activity.

8. The DNA insert according to claim 7, wherein the antibody having an anticancer activity is Trastuzumab.

9. The DNA insert according to claim 8, wherein the Trastuzumab is a Trastuzumab single-chain antibody.

10. A vector having the DNA insert according to any one of claims 6 to 9 inserted thereinto.

11. An intestinal bacterium transformed with the vector according to claim 10.

12. The intestinal bacterium according to claim 11, wherein the bacterium is a microorganism belonging to genus Bifidobacterium.

13. The intestinal bacterium according to claim 12, wherein the microorganism belonging to genus Bifidobacterium is Bifidobacterium longum.

14. An antibody drug composition comprising, as an active ingredient, the intestinal bacterium according to any one of claims 10 to 13.

15. The antibody drug composition according to claim 14, which is an anticancer agent composition.

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<223> Inventor: TANIGUCHI, Shun-ichiro; AKIYAMA, Yasuto; MASAKI Takeshi ; SHIMIZU Hitomi

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