DESCRIPTION

METHOD FOR SOLUBILIZING INSOLUBLE PROTEIN AND/OR PEPTIDE

Technical Field

[0001]

The present invention relates to a method for solubilizing an insoluble protein and/or peptide. It also relates to a method for transducing an insoluble protein and/or peptide into a cell. It further relates to a production method for an antigen presenting cell that presents an insoluble antigen, an antigen presenting cell produced by the production method, a cytotoxic T lymphocyte inducing method and a cytotoxic T lymphocyte inducing agent using the antigen presenting cell, as well as a method and an agent for preventing or treating cancer and/or infectious diseases using the antigen presenting cell.

Background Art

[0002]

As a new treatment method for intractable diseases including cancer, recently, much attention has been paid to an immuno-cell therapy. The immuno-cell therapy is a treating method for artificially activating the immunity by ex vivo culture of immune cells, particularly leukocyte, of a patient, activating the cell, and then returning the activated cell to the patient himself/herself again. Because the immuno-cell therapy uses cells of the patient himself/herself, it is particularly advantageous in that side effect is especially reduced over conventional treatment using anti-cancer drugs.

[0003]

As the immuno-cell therapy, currently, an activated auto-lymphocyte therapy is being prevalent, in which lymphocyte is activated by lymphokine in an antigen-nonspecific manner ex vivo, and the activated lymphocyte is returned to the body. Furthermore, treatment methods using cytotoxic T lymphocyte (hereinafter, also referred to as "CTL") having a strong cytotoxic activity and specifically recognizing and injuring a lesion is being expected to be prevail.

[0004]

As the currently attempted treatment method for inducing CTL, a method for directly administering a cancer antigen peptide or the like to a patient has been employed. This method has problems that the peptide is degraded by peptidase in the tissue, the suppression of

the immune response of a patient, and the like, and therefore CTL inducing ability is not expected so much. Another method is a dendritic cell vaccine therapy or the like (for example, Non Patent Literature 1) in which an antigen presenting cell such as a dendritic cell (hereinafter, also referred to as "DC") is induced from the cell derived from a patient ex vivo, and is pulsed with an antigen so as to strongly present an antigen, and the DC is returned to the body of the patient.

[0005]

Specifically, the dendritic cell vaccine therapy is a therapy in which DCs incorporate a disease antigenic protein or the like (for example, cancer antigenic protein, or antigenic protein related to infectious diseases) into the cells, carries out processing in the cells, and then a part thereof is presented to the major histocompatibility complex antigen (MHC antigen, major histocompatibility antigen, histocompatibility antigen, in the case of human, human leukocyte antigen: HLA), the DCs are administered as a vaccine so as to induce a disease antigen specific CTL which selectively attacks abnormal cells expressing a disease antigen in the body (see, for example, Non Patent Literature 1). In this way, because the DC vaccine can induce disease-specific CTL, it is one of the most promising therapeutic effects among the immuno-cell therapies.

[0006]

The MHC antigen is classified into the MHC class I and the class II, the class I induces an antigen-specific CTL, and the class II induces helper T (Th) cells. Furthermore, in the case of a human, the class I is further classified into subtypes such as HLA-A02 and HLA-A24, and even when antigenic proteins are the same, presented peptides are different.

[0007]

In the DC vaccine therapy, an important thing is to present the antigen or the like to DC. As the presenting method, two methods are employed currently. First one is a method of directly bonding antigen peptide derived from an antigenic protein to a MHC antigen (HLA) on the surface of DC, and second one is a method of allowing the protein to be incorporated into the DC by endocytosis, or the like.

[0008]

Among them, when the method of directly binding an MHC class I-restrictive antigen peptide derived from an antigenic protein to the MHC class I antigen of the antigen presenting cell is used, the antigen presenting cell is cultured and sufficiently matured, and then a peptide dissolved in a physiological solvent is added to the culture solution so that the concentration is about 1 μg/mL, and the solution is incubated for 2 to 24 hours, and then allowed

to be bound to the MHC class I molecule.

[0009]

However, in the method of directly binding an antigen peptide derived from an antigenic protein to the MHC antigen of the antigen presenting cell, CTL can be induced by presenting the antigen by the class I. In the case of, for example, human, specific HLA, for example, antigen peptide bound to the core-class I, for example, A02 and A24 are being identified, but other HLA types have not been identified. That is to say, since the peptide is restrictive to specific HLA, it can be used only for patients having particular HLA compatibility. Furthermore, in this case, since the class II cannot be used either, CD4 positive (helper) T cell function cannot be used.

[0010]

On the other hand, in the method of incorporating the antigenic protein by endocytosis, tumor tissue is made into homogenates (pulverized) in a physiological buffer solution such as a phosphate-buffered saline (hereinafter, referred to as "PBS"), and protein or peptide in the lysate (supernatant) is simply added to the culture solution of DC. In this case, the incorporated protein is processed in lysosome, loaded on a pathway mainly presented by a class II histocompatibility antigen (class II), and mainly stimulates CD4 positive (helper) T cells so as to induce its activation. Furthermore, as a system for incorporating an extracellular substance, a mechanism for processing waste matter, which incorporates a substance bound to a receptor, is present, for example, a mannose receptor is known to incorporate sugar protein or the like bound to the receptor into a cell. This also proceeds to a pathway presented by class II via lysosome. On the contrary, the protein in the cytoplasm is processed with proteasome, and loaded on a pathway that is presented mainly by class I histocompatibility antigen (class I), and stimulates mainly CD8 positive (killer) T cells (CTL) so as to induce its activity.

[0011]

When tumor tissue is made into homogenates in a physiological buffer solution, the soluble fraction is added to DC as supernatant, but the insoluble fraction is removed as precipitate. Therefore, antigenic protein contained in the insoluble fraction cannot be used. Even if a protein is produced as a recombinant protein, it is recovered into the insoluble fraction, so that an antigen cannot be presented by DC.

[0012]

Furthermore, when dendritic cells are allowed to incorporate an antigen by endocytosis, the antigen is transported to an antigen-presenting pathway toward class II in essence, and therefore antigen cannot be sufficiently presented to CD8 positive (killer) T cells

and CTL from the class I. In order to solve the problem, recently, a method for transducing a protein into cells, electroporation or the like have been able to be applied. However, in order to transduce insoluble proteins, the proteins need to have been solubilized in a physiological solvent.

[0013]

In order to solubilize an insoluble protein, generally, a technique of dissolving the protein once in a solvent such as urea or guanidine hydrochloride having a denaturing effect, and adding a reducing agent such as dithiothreitol (DTT) when the protein is not easily dissolved was used.

[0014]

Thereafter, for the purpose of suppressing random intermolecular disulfide bond, glutathione was added. According to circumstances, for the purpose of suppressing the aggregation between molecules, arginine was also added and then an oxidation-reduction reaction was caused (converted into glutathione). After the oxidation-reduction reaction, dialysis or the like was carried out with a buffer solution of PBS or the like (according to circumstances, PBS to which about 10% glycerol has been added), thereby replacing the solvent with a physiological solvent to carry out solubilization and purification (Non Patent Literature 2). However, with such methods, many proteins cannot be solubilized, and almost all proteins, even proteins that can be solubilized, may be precipitated when they are dialyzed with a physiological solvent. Thus, sufficient effects could not be obtained.

[0015]

As described above, in order to give a sufficient anti-tumor effect, a target antigenic protein is allowed to be incorporated into dendritic cells in a state in which it is solubilized and to be presented to the MHC class I, and therefore, it has been thought that a technique for dissolving an insoluble protein in a physiological solvent is essential.

Citation List

Non Patent Literature

[0016]

Non Patent Literature 1: Jonathan M. Weiss et al, J. Immunother, VOL. 28: 542-550 (2005).

Non Patent Literature 2: Method in Enzymology 1999; 309, 217-236

Summary of Invention

Technical Problem

[0017]

The present invention has been made in view of the above-mentioned circumstances, and provides a method for dissolving an insoluble protein and/or peptide (hereinafter, also referred to as an "insoluble protein") in a physiological solvent, which has not been able to be solubilized in a conventional method sufficiently. Furthermore, the present invention also provides a method for efficiently transducing an insoluble protein into a cell. Furthermore, the present invention provides a production method for an antigen presenting cell that presents an insoluble antigen, an antigen presenting cell produced by the production method, a cytotoxic T lymphocyte inducing method and a cytotoxic T lymphocyte inducing agent using the antigen presenting cell, as well as a method or an agent for preventing and treating cancer and/or infectious diseases using the antigen presenting cell.

Solution to Problem

[0018]

The present inventors have carried out various studies in order to solve the above-mentioned problems, and have found that solubilization can be efficiently carried out with the addition of nucleotide when a solution in which a protein is dissolved by using a denaturing agent is replaced with a physiological solvent. Furthermore, it is demonstrated that a protein solubilized by the solubilization method of the present invention can be transduced into a cell by electroporation. Therefore, the present inventors have found that when a disease antigenic protein of cancer, infectious disease, or the like, is solubilized and transduced into an antigen presenting cell, CTL specific to a disease antigen can be induced. Thus, the present inventors completed the present invention.

That is to say, the present invention provides the following (1) to (42).

[0019]

(1) A method for solubilizing an insoluble protein and/or peptide, the method comprising:

a step of mixing an insoluble protein and/or peptide and a denaturing agent in one solution, and dissolving the insoluble protein and/or peptide, thereby forming a solution of the insoluble protein and/or peptide;

a step of adding a nucleotide to the solution; and

a step of removing the denaturing agent from the solution.

(2) The method for solubilizing an insoluble protein and/or peptide according to (1), wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

(3) The method for solubilizing an insoluble protein and/or peptide according to (1) or (2), wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil, and a combination of one or more of the nucleotides is added.

(4) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (3), wherein the number of phosphate groups constituting the nucleotide is one to three.

(5) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (4), wherein the nucleotide is added after or concurrently with mixing the insoluble protein and/or peptide with the denaturing agent.

(6) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (5), wherein in the step of removing the denaturing agent from the solution, the denaturing agent is removed while the solution is replaced with a physiological solvent, and preferably the physiological solvent includes nucleotide.

(7) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (6), wherein the insoluble protein and/or peptide is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

(8) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (7), wherein the insoluble protein and/or peptide is an antigenic protein.

(9) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (8), wherein the denaturing agent is urea and/or guanidine hydrochloride.

(10) The method for solubilizing an insoluble protein and/or peptide according to any one of claims (1) to (9), wherein in the step of mixing the insoluble protein and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

(11) The method for solubilizing an insoluble protein and/or peptide according to any one of (1) to (10), wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

[0020]

(12) A method for transducing an insoluble protein and/or peptide into a cell, the method comprising:

a step of mixing an insoluble protein and/or peptide and a denaturing agent in one solution, and dissolving the insoluble protein and/or peptide, thereby forming a solution of the insoluble protein and/or peptide;

a step of adding a nucleotide to the solution;

a step of removing the denaturing agent while the solution is replaced with a

physiological solvent, thereby forming a physiological solution of the insoluble protein and/or peptide; and

transducing the physiological solution into cytoplasm or inside of a desired cell.

(13) The method for transducing an insoluble protein and/or peptide into a cell according to (12), wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

(14) The method for transducing an insoluble protein and/or peptide into a cell according to (12) or (13), wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil; and a combination of one or more of the nucleotides is added.

(15) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (14), wherein the number of phosphate groups constituting the nucleotide is one to three.

(16) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (15), wherein the nucleotide is added after or concurrently with mixing the insoluble protein and/or peptide with the denaturing agent.

(17) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (16), wherein the physiological solvent to be used for replacing the solution of the protein and/or peptide includes nucleotide.

(18) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (17), wherein the cell is a cell having an antigen presenting function.

(19) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (18), wherein the cell having the antigen presenting function is a dendritic cell.

(20) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (19), wherein the insoluble protein and/or peptide is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

(21) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (20), wherein the insoluble protein and/or peptide is an antigenic protein or peptide.

(22) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (21), wherein the denaturing agent is urea and/or guanidine hydrochloride.

(23) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (22), wherein in the step of mixing the insoluble protein and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

(24) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (23), wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

(25) The method for transducing an insoluble protein and/or peptide into a cell according to any one of (12) to (24), wherein the solubilized insoluble protein and/or peptide is transduced into cytoplasm and/or inside a cell by electroporation.

[0021]

(26) A method for producing an antigen presenting cell that presents an insoluble antigen, the method comprising:

a step of mixing the insoluble antigen and a denaturing agent in one solution, and dissolving the insoluble antigen, thereby forming a solution containing the insoluble antigen;

a step of adding a nucleotide to the solution;

a step of removing the denaturing agent while the solution is replaced with a physiological solvent, thereby forming a physiological solution containing the insoluble antigen; and

transducing the physiological solution containing the insoluble antigen into cytoplasm or inside of the antigen presenting cell.

(27) The method for producing an antigen presenting cell according to (26), wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

(28) The method for producing an antigen presenting cell according to (26) or (27), wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil; and a combination of one or more of the nucleotides is added.

(29) The method for producing an antigen presenting cell according to any one of (26) to (28), wherein the number of phosphate groups constituting the nucleotide is one to three.

(30) The method for producing an antigen presenting cell according to any one of (26) to (29), wherein the physiological solvent to be used for replacing the solution of the antigen includes nucleotide.

(31) The method for producing an antigen presenting cell according to any one of (26) to (30), wherein the antigen is an insoluble fraction obtained from a fractured product of a tumor cell, a tumor tissue, an infectious disease cell or a pathogenic cell.

(32) The method for producing an antigen presenting cell according to any one of

(26) to (31), wherein the antigen is a protein or a peptide.

(33) The method for producing an antigen presenting cell according to any one of (26) to (32), wherein the antigen is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

(34) The method for producing an antigen presenting cell according to any one of (26) to (33), wherein the antigen presenting cell is a dendritic cell.

(35) The method for producing an antigen presenting cell according to any one of (26) to (34), wherein the denaturing agent is urea and/or guanidine hydrochloride.

(36) The method for producing an antigen presenting cell according to any one of (26) to (35), wherein in the step of mixing the insoluble protein and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

(37) The method for producing an antigen presenting cell according to any one of (26) to (36), wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

(38) An antigen presenting cell produced by the producing method according to any one of (26) to (37).

(39) A method for inducing a cytotoxic T lymphocyte, the method comprising a step of administering an antigen presenting cell produced by a producing method according to any one of (26) to (37) to a mammalian including a human.

(40) A method for treating or preventing cancer and/or an infectious disease, the method comprising a step of administering an antigen presenting cell produced by a producing method according to any one of (26) to (37) to a mammalian including a human.

(41) A composition for inducing a cytotoxic T lymphocyte, the composition comprising, as an active ingredient, an antigen presenting cell produced by a producing method according to any one of (26) to (37).

(42) A composition for treating or preventing cancer and/or an infectious disease, the composition comprising, as an active ingredient, an antigen presenting cell produced by a producing method according to any one of (26) to (37).

Advantageous Effects of Invention

[0022]

With the solubilization method of the present invention, insoluble proteins such as naturally occurring proteins and recombinant proteins can be solubilized. Furthermore, when the solubilized proteins are used, they can be directly transduced into the cytoplasm by

electroporation. Specific methods of use of the present invention include a production method for an antigen presenting cell that presents an insoluble antigen, application for production of immune response drugs and preparation of reagents, and medicine such as a protein vaccine, a peptide vaccine or a dendritic cell vaccine containing a solubilized protein antigen.

Brief Description of Drawings

[0023]

[Fig. 1] Fig. 1 shows results of solubilization of TRP-2 fused protein with the addition of arginine, nucleotide, and both of them, and detection of TRP-2 fused protein contained in supernatant and precipitate by SDS-PAGE.

[Fig. 2] Fig. 2 shows results of solubilization of TRP-2 by adding four types of nucleotides respectively or in combination thereof, and detection of TRP-2 contained in supernatant and precipitate by SDS-PAGE.

[Fig. 3] Fig. 3 shows results of solubilization of TRP-2 by adding ribonucleotide (GTP) or deoxyribonucleotide (dGTP), and detection of TRP-2 contained in supernatant and precipitate by SDS-PAGE.

[Fig. 4] Fig. 4 shows results of solubilization of TRP-2 by adding GMP or GTP, and detection of TRP-2 contained in supernatant and precipitate by SDS-PAGE.

[Fig. 5] Fig. 5 shows results of solubilization of TRP-2 by adding UMP or UTP, and detection of TRP-2 contained in supernatant and precipitate by SDS-PAGE.

[Fig. 6] Fig. 6 shows results of solubilization of TRP-2 by adding UMP, dialysis with the use of solutions having different pHs, and detection of TRP-2 contained in supernatant and precipitate by SDS-PAGE.

[Fig. 7] Fig. 7a shows results of CBB staining of protein remaining in a gel without being transferred when a soluble fraction (S) and an insoluble fraction (P) of human melanoma cell line homogenates are analyzed by Western Blotting. Fig. 7b shows results of the Western Blotting with the use of an anti-MART-1 antibody.

[Fig. 8] Fig. 8 shows results when a Mewo insoluble fraction was solubilized by the solubilization method of the present invention, and subjected to Western Blotting with the use of an anti-MART-1 antibody.

[Fig. 9] Fig. 9 shows results when a precipitate fraction of mouse melanoma (B16) homogenates was solubilized with the use of UMP, and the amount of protein contained in the supernatant or the precipitate was detected by SDS-PAGE.

[Fig. 10] Fig. 10 shows results when a precipitate fraction of human breast cancer (MDA-MB-

231) homogenates was solubilized with the use of UMP, and the amount of protein contained in the supernatant or the precipitate was detected by SDS-PAGE.

[Fig. 11] Fig. 11 shows results when a precipitate fraction of human prostate cancer (DU145) homogenates was solubilized with the use of UMP, and the amount of protein contained in the supernatant or the precipitate was detected by SDS-PAGE.

[Fig. 12] Fig. 12 shows analysis of a phenotype after inducing a dendritic cell with CD11b/CD11c and CD80/CD86 antibodies.

[Fig. 13] Fig. 13 shows confirmation of a CTL inducing effect specific to TRP-2 by tetramer analysis.

[Fig. 14] Fig. 14a is a graph showing confirmation of expression of H-2K^b and H-2D^b of B16. Fig. 14b is a graph showing confirmation of induction of CTL that specifically recognizes a cancer antigen of B16 in a body of a mouse immunized with DCs by measuring the cytotoxic activity using Terascan VP system when lysate (soluble fraction: sup) of B16 or a solubilized insoluble fraction (ppt) was transduced into the DCs by electroporation.

Description of Embodiments

[0024]

Hereinafter, embodiments for carrying out the present invention are described. However, needless to say, the present invention is not limited to these embodiments, and the present invention can be carried out in various embodiments in a scope that is not beyond the summary.

[0025]

Method for solubilizing insoluble protein and/or peptide

Firstly, a method for solubilizing an insoluble protein and/or peptide according to the present invention (hereinafter, also referred to as "a method for solubilizing of the present invention") is described.

The "insoluble" to be used in the specification refers to a property of a protein, and a property that a protein cannot be dissolved or less dissolved in a liquid, in particular, in water or a physiological solvent, so as to form a homogeneous mixed solution at a room temperature. In the case where the protein is present in water, water and salt, as well as water and a physiological solvent that does not denature the protein, after the solution of the protein is centrifuged (subjected to a centrifugation) at 15,100×g for one hour, when the protein is not substantially present in supernatant, or at least 50%, desirably 75% or more, and more desirably 100% of the protein is not present in the supernatant, natural insoluble proteins of the kind

described in the specification is referred to as insoluble.

[0026]

The "protein" used herein includes peptide, polypeptide, and the like, and it is not limited to naturally occurring proteins that present in nature, but it includes a recombinant protein derived from cells transformed by gene transfer or the like, a protein expressed in vitro by using a cell-free protein expression system, and a synthesized protein produced in an organic synthetic chemical manner. Furthermore, a protein in which a part or all of amino acids constituting the protein is provided with functional groups for acetylation, phosphorylation, methylation, or the like, or modified with sugar chain, protein, lipid, or the like, may be included. Note here that in the specification, claims, and abstract, in order to clarify that an insoluble peptide is also encompassed in the present invention, in some cases, both protein and peptide may be described as in "insoluble protein and/or peptide."

[0027]

The "insoluble protein" to be used herein refers to a protein that is not dissolved or less dissolved in water or a physiological solvent even when it is stirred at room temperature, and a protein that is dissolved with the use of a denaturing agent but produces precipitate when the solvent is replaced with a physiological solvent. Furthermore, even if a natural form is originally a soluble protein, if it is expressed by the use of different types of organs as a recombinant protein (for example, in a case where an expression system Escherichia coli is constructed in gene recombination technology), a target protein recovered as an inclusion body (inclusion body) is also referred to as an insoluble protein.

[0028]

Examples of the insoluble protein contained in a fractured product of tumor tissue or a tumor cell may include a membrane protein having a transmembrane structure. Since the membrane protein includes a hydrophilic moiety and a hydrophobic moiety, it is generally known that it is recovered in the insoluble fraction. The membrane proteins include a molecule that is known as a cancer antigen, it is extremely important that these proteins are solubilized and used for immuno-cell therapy. Examples of the insoluble protein contained in a fractured product of tumor tissue or a tumor cell may include MART-1, PSMA, and TRP-2. Furthermore, it is also thought that when originally soluble antigenic proteins are mutated, the composition of the amino acid is changed and the proteins cannot have a normal structure and thereby become insoluble. The above-mentioned phenomenon has been observed in proteins such as prion (J. Mol. Neurosci. 2007; 32: 90-96), superoxide dismutase (J. Biol. Chem. 2008; 283: 866-874), and alpha A-crystallin (Biochemistry 2008; 47: 9697-9706).

[0029]

The term "solubilization" in this specification refers to dissolving an insoluble protein in a physiological solvent with the amino acid sequence thereof maintained. It refers to increasing the amount of insoluble proteins to be recovered in supernatant after centrifugation when a solution dissolving the insoluble protein with the use of a denaturing agent is replaced with a physiological solvent, and centrifuged.

[0030]

Nucleotide to be used in the solubilization method of the present invention is a compound in which base, sugar, and phosphoric acid are bonded. Nucleotide whose sugar moiety is D-2-deoxyribose is referred to as deoxyribonucleotide, which is a moiety constituting DNA. Furthermore, nucleotide whose sugar moiety is ribose is referred to as ribonucleotide, which is a moiety constituting RNA. To the phosphoric acid moiety, one to three phosphoric acids are bonded. To the base moiety is adenine, cytosine, guanine, thymine, and uracil are bonded.

[0031]

In the solubilization method of the present invention, any of deoxyribonucleotide and ribonucleotide can be used. Furthermore, the number of phosphoric acids bound to nucleotide may be any of one to three. Nucleotide to which any base of adenine, cytosine, guanine, thymine, and uracil, as the base constituting nucleotide, is bound can be used. However, since the efficiency of the solubilization is different depending upon the constituting base and solubilized protein, it is desirable to use a combination of any one or more bases. Examples include adenosine monophosphate (AMP), uridine monophosphate (UMP), guanosine monophosphate (GMP), cytidine monophosphate (CMP), adenosine diphosphate (ADP), uridine diphosphate (UDP), guanosine diphosphate (GDP), cytidine diphosphate (CDP), adenosine triphosphate (ATP), guanosine triphosphate (GTP), uridine triphosphate (UTP), cytidine triphosphate (CTP), deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxyadenosine diphosphate (dADP), deoxythymidine diphosphate (dTDP), deoxyguanosine diphosphate (dGDP), deoxycytidine diphosphate (dCDP), deoxyadenosine monophosphate (dAMP), deoxythymidine monophosphate (dTMP), deoxyguanosine monophosphate (dGMP), deoxycytidine monophosphate (dCMP), deoxynucleotide triphosphate (dNTP; mixture of dATP, dTTP, dGTP, and dCTP).

[0032]

The denaturing agent used in the solubilization method of the present invention is

generally urea, guanidine hydrochloride, surfactant, or the like, and refers to an agent having property of cleaving an ion bond, a hydrogen bond, hydrophobic interaction, or the like, which form an insoluble three-dimensional structure. Such denaturing agents are used in a state in which they are dissolved in an aqueous solution. For example, a buffer solution such as a sodium phosphate aqueous solution whose pH can be adjusted is preferable.

[0033]

The reducing agent used in the solubilization method of the present invention is dithiothreitol (DTT), 2-mercaptoethanol (2ME), or the like, and refers to an agent having a property that cleaves a disulfide bond that forms an insoluble three-dimensional structure. Such reducing agents may be used in a state in which they are mixed with the denaturing agent in advance or dissolved in different solvents.

[0034]

In the solubilization method of the present invention, examples of the method of removing the denaturing agent from the protein solution containing the denaturing agent include a method of replacing the solvent by dialysis or gel filtration. In particular, from the viewpoint of the ease in operation, dialysis is preferable. The solvent to be used for substitution may be any solvents that do not contain the denaturing agent, and, for example, water and a physiological solvent can be used. In particular, when the protein solvent is added to a culture solution of cells, or when the protein solvent is administered to a living body, it is preferable that solution is replaced with a physiological solvent. For example, as the physiological solvent, PBS (phosphate buffered saline), physiological saline, and the like are preferable. Furthermore, when nucleotide is added to a physiological solvent, insoluble protein can be solubilized more efficiently. The concentration of the nucleotide to be added to the physiological solvent is preferably about 10 μM.

[0035]

Embodiments of the solubilization method of the present invention are different depending upon the origins of proteins.

For example, a) proteins obtained from tumor tissue and cell, as a naturally occurring protein, are solubilized as follows.

a-1) A tissue is subdivided, then a cell is destroyed by freezing and thawing repeatedly, or a cell is pulverized by ultrasonic wave or the like, and fractionated into supernatant (soluble fraction) and precipitates (insoluble fraction) by centrifugation.

a-2) The recovered precipitates are dissolved with the use of a denaturing agent, for example, 4 to 8 M urea. When the precipitates are not easily dissolved, a reducing agent,

for example, DTT, 2ME, or the like, may be added.

a-3) When the reducing agent is added, oxidized glutathione or the like is preferably added.

a-4) Nucleotide is added in a state in which an insoluble protein is solubilized with denaturing agent. It is preferable that nucleotides are added in an amount so that the final concentration is 0.5 mM to 10 mM.

a-5) The denaturing agent and the like are removed by dialysis with a physiological solvent containing about 10 μM nucleotide.

[0036]

For example, b) insoluble recombinant proteins expressed by using eukaryotic cells, fungus bodies, or the like, are solubilized as follows.

b-1) A cell or a fungus is pulverized by, for example, ultrasonic wave and centrifuged to separate an insoluble fraction and recover insoluble proteins.

b-2) The recovered insoluble proteins are dissolved with a denaturing agent, for example, 4 to 8 M urea. When the precipitates are not easily dissolved, a reducing agent, for example, DTT, 2ME, or the like, is added.

b-3) Purification with a chromatography system is carried out to obtain a target protein. Any chromatography systems may be used as long as the target protein can be extracted regardless with a solvent, for example, affinity chromatography with a protein-specific antibody is preferable, or when a protein include a histidine tag, or the like, nickel affinity chromatography or the like is preferable.

b-4) Further, when purification is carried out, proteins are purified by using a purification method according to the properties or solvents of the target protein to be used, for example, molecular exclusion chromatography, gel filtration chromatography, gel permeation chromatography, ion exchange chromatography, or the like (such chromatography may be combined with high-performance liquid chromatography).

b-5) A reducing agent is added to the protein solution before purification, during purification, or after purification. In the case where the reducing agent is added after purification, after addition of the reduction, the solution is stood still for about one hour at room temperature to cause a reduction reaction. As the reducing agent, for example, DTT is preferable.

b-6) An excessive reducing agent is removed by dialysis with the use of a denaturing agent without containing a reducing agent.

b-7) It is preferable that oxidized glutathione (reduced glutathione, if necessary)

or the like is added.

b-8) Nucleotide is added and stood still for one hour at room temperature. It is preferable that nucleotide is added in an amount so that the final concentration is 0.5 mM to 10 mM.

b-9) The denaturing agent is removed by dialysis with the use of physiological solvent containing about 10 μM nucleotide.

[0037]

For example, c) insoluble synthesized proteins are solubilized is as follows.

c-1)Target insoluble proteins are recovered from a synthesized protein solution containing amino acid and randomly bonded peptide fragments by, for example, high-performance liquid chromatography.

c-2) The recovered insoluble proteins are dissolved with a denaturing agent, for example, 4 to 8 M urea. When the insoluble proteins are not easily dissolved, a reducing agent, for example, DTT, 2ME, or the like, may be added.

c-3) When the reducing agent is added, oxidized glutathione or the like is preferably added.

c-4) Nucleotide is added in a state in which an insoluble protein is solubilized with denaturing agent. It is preferable that nucleotides are added in an amount so that the final concentration is 0.5 mM to 10 mM.

c-5) The denaturing agent and the like are removed by dialysis with a physiological solvent containing about 10 μM nucleotide.

[0038]

The protein and/or peptide dissolved in a physiological solvent in this way is not different from an insoluble state and can be subjected to sterilization using a filter, it can be administered to a patient as a vaccine.

[0039]

As to a method for transducing an insoluble protein into a cell:

Next, a method for transducing an insoluble protein into a cell according to the present invention is described. The insoluble protein that has been solubilized by the above-mentioned solubilization method of the present invention is transduced into cytoplasm and/or inside the cell by, for example, electroporation, an antigen presenting cell can be produced.

[0040]

According to the above-mentioned solubilization method of the present invention, an insoluble protein is dissolved in a physiological solvent. When the insoluble protein is

dissolved in a physiological solvent, problems that cells are killed by the toxicity of a solvent during or after the transduction of the protein do not easily occur.

In the method for transducing into a cell of the present invention, as a method for transducing the insoluble protein dissolved in a physiological solvent, generally used various techniques can be used. For example, electroporation can be employed.

[0041]

In the transduction method of the present invention, when a solubilized insoluble protein is transduced into an antigen presenting cell (for example, dendritic cell) by electroporation, the transduced protein and/or peptide is presented to the MHC class I, and CTL can be induced. Furthermore, a part of peptide is presented to the MHC class II by cross presentation, and it activates Th cells. The Th1 cells activated by an antigen presented to the MHC class II produce cytokine necessary for cellular immunization and promote the induction of CTL.

[0042]

The electroporation is a transduction method that applies the physical principle in which when the cells are exposed to an electric pulse, a membrane of prokaryotic/eukaryotic cell is transitorily ruptured. For example, when an electric pulse is given in a state in which an antigenic protein or the like is added to a cell culture solution, a solubilized protein can be directly transduced into the cytoplasm from an extended pore of the cell membrane.

[0043]

The culture solution of the cultured cells is replaced with a serum-free medium, PBS, or the like, and prepared at about 1×10^6 cells/mL. The protein solution is added to the cell suspension, the cell suspension is placed into a cuvette, and set to an electrode connected to an electroporation machine main body. Electric pulse is carried out rapidly, and the protein is recovered in a 5-fold volume or more of culture medium. Thereafter, it is cultured for at least one hour, and used for the intended application.

[0044]

When a dendritic cell into which the solubilized protein and/or peptide has been transduced is suspended in a medically acceptable solvent (for example, physiological saline), it can be administered to a patient as a dendritic cell vaccine.

[0045]

Next, a production method for an antigen presenting cell according to the present invention is described.

[0046]

In the production method for an antigen presenting cell of the present invention, examples of the antigen insoluble with respect to a physiological solvent to be used include an insoluble fraction obtained from a fractured product of tumor tissue or a tumor cell, an insoluble fraction obtained from a fractured product of infectious disease cells infected with virus, an insoluble fraction obtained from a fractured product of pathogenic bacteria, artificially synthesized insoluble protein or peptide, or the like. In particular, the insoluble fraction obtained from a fractured product of tumor tissue or a tumor cell, the insoluble fraction obtained from a fractured product of infectious disease cells infected with virus, and the insoluble fraction obtained from a fractured product of pathogenic bacteria contain an insoluble protein antigen specifically expressing in tumor tissue or a tumor cell, in infectious disease cell, and pathogenic bacteria. When such insoluble fractions are solubilized and allowed to present antigen, higher anti-tumor activity, anti-(virus) infectious disease activity, anti-pathogenic bacteria activity are expected to be induced as compared with a conventional method of inducing CTL with only supernatant.

[0047]

The insoluble antigen and the denaturing agent (for example, 4 to 8 M urea) are mixed in one solution to produce a solution containing the insoluble antigen. If necessary, DTT or 2ME is added as a reducing agent. Next, nucleotide is added in the solution containing the insoluble antigen.

[0048]

The solution containing the insoluble antigen is replaced to a physiological solvent, thereby removing the denaturing agent. Thus, a physiological solution containing the insoluble antigen can be obtained.

[0049]

The physiological solution containing the insoluble antigen is transduced into an antigen presenting cell (for example, a dendritic cell). As an transducing method, a method for transduced the insoluble antigen directly into the cytoplasm is preferable, and for example, electroporation can be employed.

[0050]

Since in the thus produced antigen presenting cells, the transduced antigen is degraded in the cell and presented to the MHC class I, CTL specific to the antigen can be induced. In particular, when the protein is transduced, since a plurality of epitopes contained in the sequence are presented, a plurality of types of CTLs specific to the antigen can be activated.

Furthermore, since an epitope of the MHC class II is also presented, Th1 cells are activated, and the activation of CTL can be further promoted.

Therefore, the thus produced antigen presenting cell can be used for a cytotoxic T lymphocyte inducing method, a cytotoxic T lymphocyte inducing agent, a method for preventing or treating cancer and/or infectious diseases, and an agent for preventing or treating cancer and/or infectious diseases. More specifically, for example, an insoluble fraction is obtained from a fractured product of tumor tissue or a tumor cell collected from a cancer patient, or an insoluble fraction is obtained from a fractured product that is the same virus as that from a patient with virus infectious disease or an insoluble fraction is obtained from a fractured product that is the same pathogenic bacterium as that from a patient with pathogenic bacterial infectious disease is obtained, and such insoluble fractions are subjected to the solubilization method of the present invention and solubilized, and the solubilized fractions are added to antigen presenting cells such as the lymphocyte or the dendritic cells collected from the same patient and cultured so as to induce an antigen presenting cell, and the induced antigen presenting cell is administered to the same patient. Thereby, a cytotoxic T lymphocyte specific to a tumor cell, virus or pathogenic bacteria can be induced, thus enabling diseases such as cancer or virus infectious diseases to be prevented or treated. Furthermore, solubilized proteins obtained by solubilizing a recombinant protein or synthesized protein by the solubilization method of the present invention are added to antigen presenting cells such as the lymphocyte or the dendritic cell collected from the patient and cultured to induce an antigen presenting cell, and the induced antigen presenting cell can be administered to the same patient. The induced antigen presenting cell may be administered to a patient by a usual method, for example, it may be administered to a patient as it is, or may be administered to a patient in a usual form such as injection, and may be administered as a usual vaccine formulation. Furthermore, an amount of the antigen presenting cell to be administered can be arbitrarily determined according to symptoms or types of diseases of patients and, for prevention, according to types of diseases and subjects. Furthermore, by using the produced antigen presenting cell, the lymphocyte collected from a patient and the antigen presenting cell are mixed and cultured so as to induce CTL in vitro.

Example 1

[0051]

<Examination of Effectiveness of Nucleotide>

A recombinant mouse Tyrosinase-Related Protein-2 (hereinafter, also referred to as "TRP-2") was used as an insoluble protein, and a solubilization method using a nucleotide was examined.

Firstly, a recombinant fused protein (hereinafter, referred to as "UT") consisting of an ubiquitin fragment (hereinafter, referred to as "Ubi") and TRP-2 in which a histidine tag (His-tag) had been added to N-terminal was prepared. Ubi was isolated from cDNA encoding the amino acid sequence set forth in SEQ ID NO: 1, and TRP-2 was isolated from cDNA encoding glutamic acid (E) that is the 56th residue from the N-terminal to serine (S) that is the 472nd residue from the N-terminal based on the amino acid sequence described in AC No. P29812 of SWISS-PROT database. Ubi and TRP-2 were inserted into a pET19b vector (Novagen) to form an expression plasmid (pET19b/UT). When Escherichia coli (Rosetta-gami2 (DE3) pLysS: Novagen, #71352-3) was transformed with pET19b/UT to express a recombinant protein, a fused protein (UT protein) having a sequence: NH_2-His-Ubi-TRP-2-CO_2H (SEQ ID NO: 2) was produced, but it formed an inclusion body (inclusion body) and was insoluble.

[0052]

Escherichia coli that had expressed the UT protein was suspended in PBS containing a protease inhibitor (Complete EDTA-free, Roche, #04 693 132 001), destroyed by using an ultrasonic wave device, subjected to centrifugation at 15,100×g for one hour at 4°C by using an M-X100 centrifuge, TMP-11 rotor (TOMY).

[0053]

An insoluble fraction (precipitate) after centrifugation was suspended in 4M urea/PBS (pH7.4), the suspension was stirred for one hour at room temperature, and centrifuged at 15,100×g for one hour at 4°C.

The insoluble fraction (precipitate) after centrifugation

1) was dissolved in 8 M urea /150 mM NaCl/5 mM DTT/20 mM sodium phosphate (pH8.5), and the solution was subjected to

2) nickel affinity (His Trap HP column, Amersham Pharmasia Biotech, #17-5247-01) with the use of an AKTA explorer liquid chromatography system (Amersham Pharmasia Biotech), and

3) gel filtration chromatography (Superose12 HR column, Amersham Pharmasia Biotech, #17-0538-01) with 6M urea /150 mM NaCl/5 mM DTT/20 mM sodium phosphate (pH8.5) so as to partly purify a recombinant protein.

[0054]

DTT was added to a sample that had undergone gel filtration purification so that the concentration was 5 mM, and the mixture was reacted for one hour, followed by carrying out dialysis with 6 M urea/150 mM NaCl/20 mM sodium phosphate (pH8.5) to remove unreacted

DTT.

[0055]

With the above-mentioned process, a protein sample was obtained. However, the protein sample obtained in the above-mentioned process was in a state in which a denaturing agent was contained. Accordingly, this sample was not able to be used as it is for being transduced into a cell, and therefore, the solvent was necessary to be exchanged with a physiological solvent. Then, by the processes 4 to 10, the protein was attempted to be dissolved in a physiological solvent (hereinafter, dissolving a protein in a physiological solvent is referred to as "solubilization"). A method of adding arginine is a well-known technique, and this was made to be a control.

[0056]

4) Glutathione (GSSG) was added to the protein sample obtained in 3) to carry out an oxidation-reduction reaction.

5) An additive agent (arginine or dNTP) was added and the mixture was reacted for one hour at room temperature.

6) A glycerol was added (10%).

7) Dialysis was carried out with a physiological solvent (for example, PBS).

8) A sample was recovered and centrifuged at 15,100×g for one hour at 4°C.

9) The sample was separated into supernatant (a soluble fraction) and precipitate (an insoluble fraction) and they were recovered.

10) Solubilization efficiency was analyzed by, for example, SDS-PAGE.

[0057]

The processes 4 to 10 are described in detail. To the UT sample dissolved in 6 M urea/150 mM NaCl/20 mM sodium phosphate (pH8.5), 2 mM DTT was added, and the solution was stood still for one hour at room temperature, followed by carrying out dialysis with 6 M urea/150 mM NaCl/20 mM sodium phosphate (pH8.5) to remove excessive DTT. The sample was dispensed into three sample tubes (1), (2), and (3), and 2.5 mM oxidized glutathione (GSSG) was added to each tube (each concentration is the final concentration). Furthermore, arginine (L(+) Arginine, Wako, #015-04617) was added only to the tube (3) so that the final concentration was 125 mM. All the tubes were stood still for one hour. After that, to the tube (3) to which arginine had been added and to the tube (2), 10% glycerol (final concentration) was added. These three types of reaction solutions were individually dialyzed with 10% glycerol/150 mM NaCl/20 mM Tris(hydroxymethyl)aminomethane (pH8.4), the solvent was replaced. Since this dialyzing operation produced an insoluble protein, the insoluble protein

was centrifuged at 15,100×g for one hour at 10°C and fractionated into a supernatant and a precipitate (protein that had not been solubilized was recovered in the precipitate, and protein that had been solubilized was recovered in the supernatant).

[0058]

To the supernatant, a 2×SDS-PAGE sample buffer solution (tris-SDS-2ME sample treatment solution: Daiichi Pure Chemicals Co., Ltd., #423437) was added. The precipitate fraction was dissolved in a 1×SDS-PAGE sample buffer solution in an amount that is the same as that of the supernatant, and an equal amount each was subjected to SDS-PAGE. After electrophoresis, the solution was stained with Coomassie brilliant blue G250 stain solution and the amount of protein recovered in each of the supernatant and the precipitate was estimated. As shown in Fig. 1 and (1), (2), and (3) of Table 1, protein was not recovered in any soluble fractions. The rate of solubilization shown in Table 1 was calculated from the following formula in which a gel stained with Coomassie brilliant blue was taken as an image by using a CCD camera of Gel Doc 2000 (BIO RAD), and an area of each band "a" and "b" was multiplied by the concentration so as to be represented numerically (v). The band "b" was the degradation product of "a."

Rate of solubilization (%) = S(v) × 100/S(v) + P(v)

S (v): area of band "a" of supernatant × concentration + area of band "b" of supernatant × concentration

P (v): area of band "a" of precipitate × concentration + area of band "b" of precipitate × concentration

[0059]

The same protein dissolved in 6 M urea/150 mM NaCl/20 mM sodium phosphate (pH8.5) was used, the solubilization in a case where dNTP (illustra dNTP Set, 100 mM Solutions, GE Healthcare, 27-2035-01) instead of arginine was added so that the final concentration was 12.5 mM, and the solubilization in a case where 12.5 mM dNTP and 125 mM arginine were added were examined. The other operations were the same as those in (1) to (3). Respective results are shown in Fig. 1 and (4) and (5) of Table 1. In the case where dNTP was added, a clear band was observed in the supernatant, and the rate was 50.8%. Also in the case where dNTP and arginine (Arg) were added, a clear band was observed in the supernatant, but the rate was 20.7%.

[0060]

[Table 1]

Rate of solubilization of UT fused protein when arginine or dNTP was added

	(1)	(2)	(3)	(4)	(5)
Rate of solubilization (%)	0	0	0	50.8	20.7

(1) not added
(2) 10% Glycerol
(3) (2)+Arg
(4) (2)+dNTP
(5) (2)+Arg+dNTP

[0061]

From the above-mentioned results, it was found that the protein that was not be able to be solubilized by a conventional method can be solubilized with the addition of dNTP. Furthermore, it was considered that when dNTP was added, the addition of arginine was not necessary.

Example 2

[0062]

<Examination of Types of Nucleotides (Bases)>

As a model of an insoluble protein, a recombinant mouse TRP-2 (rTRP-2; SEQ ID NO: 3) was used. rTRP-2 was isolated from cDNA encoding glutamic acid (E) that is the 56th residue from the N-terminal to serine (S) that is the 472nd residue from the N-terminal based on the amino acid sequence described in AC No. P29812 of SWISS-PROT database, and inserted into a pET19b vector (Novagen) to form an expression plasmid (pET19b/mdT). When Escherichia coli (Rosetta-gami2 (DE3) pLysS: Novagen, #71352-3) was transformed with pET19b/mdT and was expressed, a recombinant protein having a histidine tag (His-tag) at the N-terminal was obtained. The recombinant protein formed an inclusion body, and was insoluble. The insoluble fraction was dissolved in 8 M urea/PBS/5 mM DTT (pH8.5) or 8 M urea/PBS/10 mM DTT (pH8.0), and the solution partially purified by nickel affinity (His Trap HP column, Amersham Pharmasia Biotech, #17-5247-01) and gel filtration chromatography (HiPrep 26/60Sephacryl S300 HR column, Amersham Pharmasia Biotech, #17-1196-01) by using an AKTA explorer liquid chromatography system (Amersham Pharmasia Biotech). In order to replace the solvent of the purified sample with a physiological buffer solution, dialysis with PBS was carried out, but almost a whole amount was precipitated.

[0063]

rTRP-2 dissolved in 6 M urea/PBS/5 mM DTT (pH8.5) was prepared in five sample tubes. To all the tubes, 2.5 mM reduced glutathione (GSH) and 2.5 mM GSSG were

added. To respective tubes, dATP (dA, Sigma, #D6500), dTTP (dT, Sigma, #D5288), dCTP (dC, Sigma, #D4635), dGTP (dG, Sigma, #D4010) or dNTP was added so that the final concentration was 10 mM, and all the tubes were stood still for one hour. After that, to each tube, 10% glycerol was added. These solutions were individually dialyzed with 10% glycerol/PBS (pH8.0), and the solvent was replaced. Then, the solution was centrifuged at 15,100×g for one hour at 10°C and fractionated into supernatant and precipitate, and the precipitate fraction was dissolved in 8M urea/PBS (pH8.0). Hereinafter, analysis was carried out with SDS-PAGE similarly to Example 1.

[0064]

As shown in Fig. 2, when any bases were used, it was confirmed that the rTRP-2 was recovered in the soluble fraction. Among them, when dT and dG were added, almost whole amounts of the protein were able to be solubilized.

Example 3

[0065]

<Examination of Types of Nucleotides (Ribonucleotide and Deoxyribonucleotide)>

rTRP-2 dissolved in 6 M urea/PBS/5 mM DTT (pH8.5) was prepared in six sample tubes, and 2.5 mM GSH and 2.5 mM GSSG were added to all the tubes. GTP (Sigma, #G8877) or dGTP was added to the respective tubes so that the concentration was 2 mM, 10 mM or 50 mM, and all the tubes were stood still for one hour. After that, 10% glycerol was added to the respective tubes. Dialysis was carried out with 10% glycerol/PBS (pH8.0), and the solvent was replaced. After that, the solution was centrifuged at 15,100×g for one hour at 10°C, and fractionated into supernatant and precipitate, and then analysis by SDS-PAGE was carried out similarly to Example 1.

[0066]

As shown in Fig. 3, about 50% of 2 to 10 mM GTP and 2 to 10 mM dGTP were solubilized and recovered in the supernatant. However, when the concentrations of the GTP and the dGTP were made to be 50 mM, almost whole amounts were insoluble, and recovered in the precipitate fraction. From the above mention, it is considered to be appropriate that nucleotide to be added is 10 mM or less. Thus, it was revealed that nucleotide constituting ribonucleic acid and nucleotide constituting deoxyribonucleic acid had the same effect.

Example 4

[0067]

<Examination of Types of Nucleotides (Monophosphate and Triphosphate) (Example of Guanine)>

rTRP-2 dissolved in 6 M urea/PBS/5 mM DTT (pH8.5) (the same as in Example 3) was prepared in six sample tubes, and 2.5 mM GSH and 2.5 mM GSSG were added to all the tubes. 10 mM GMP (Sigma, #G8377), 2 mM GMP, 0.5 mM GMP, 10 mM GTP, 2 mM GTP or 0.5 mM GTP was added to the respective tubes, and all the tubes were stood still for one hour. After that, 10% glycerol was added to the respective tubes. Dialysis was carried out with 10% glycerol/PBS (pH8.0), and the solvent was replaced. After that, the solution was centrifuged at 15,100×g for one hour at 10°C, fractionated into supernatant and precipitate, and thus analysis by SDS-PAGE was carried out similarly to Example 1.

[0068]

As shown in Fig. 4, in the case where 0.5 to 10 mM GMP and 0.5 to 2 mM GTP were added, almost whole amounts of TRP-2 were solubilized and recovered in the supernatant. In the case where 10 mM GTP was added, it was recovered in a partially insoluble state but 50% or more was recovered in a soluble state. From the above mention, it was revealed that both of GMP and GTP had the equal effect although they had a different appropriate range of addition concentration.

Example 5

[0069]

<Examination of Types of Nucleotides (Monophosphate and Triphosphate) (Example of Uracil)>

This Example exampled an effect of uracil that is a base constituting ribonucleic acid corresponding to dTTP whose solubilization efficiency was high in Example 2. Similar to the example of guanine, the same examination as in Example 4 except that UMP (Sigma, #U6375) was used instead of GMP, and UTP (Sigma, #U6625) was used instead of GTP.

[0070]

As shown in Fig. 5, in the case where 0.5 to 10 mM UMP and 0.5 to 2 mM UTP were added, almost whole amounts of rTRP-2 were solubilized and recovered in the supernatant. In the case where 10 mM UTP was added, rTRP-2 was recovered in a partially insoluble state but 50% or more was recovered in a soluble state. From the above mention, it was revealed that both of UMP and UTP had the equal solubilization effect although they had a different appropriate range of addition concentration. From the examination in Example 2 and the examination of this Example, it was revealed that both of deoxyribonucleotide and ribonucleotide, in which the number of phosphoric acids was different, of nucleotide were able to be used.

Example 6

[0071]

<Examination of pH>

rTRP-2 dissolved in 6 M urea/PBS/5 mM DTT (pH8.5) to which 10 mM UMP had been added as in Example 5 and the rTRP-2 to which 10 mM UMP had not been added were prepared, and they were reacted for one hour. Then, the rTRP-2 to which UMP had been added was divided into two parts, and dialyzed with 10 μM UMP /10% glycerol/PBS (pH7.4) or (pH8.0). The rTRP-2 to which UMP had not been added was dialyzed with 10% glycerol/PBS (pH7.4). The recovered samples were centrifuged at 15,100×g for one hour at 10°C, and fractionated into supernatant and precipitate. The precipitate fraction was dissolved in 8M urea-PBS. Hereinafter, analysis by SDS-PAGE was carried out similarly to Example 1.

[0072]

As a result, as shown in Fig. 6, it was revealed that in the case where dialysis was carried out at pH7.4, when UMP had not been added, the protein was not detected in the soluble fraction and almost all of the protein was insoluble and precipitated, but that when UMP had been added, protein was recovered also in the soluble fraction. Furthermore, it was revealed that when pH is made to 8.0, the solubilization efficiency was increased.

Example 7

[0073]

<Quantification of Natural Insoluble Protein Derived from Cell>

75 cm^2 each of Malme-3M, Mewo and G-361 as human melanoma cell lines was cultured in a culture flask. When the cells were spread over the bottom surface, the cultured cells were washed with PBS three times. After washing, cells were scraped off, and centrifuged at 1,000×g for 5 minutes at 4°C and recovered as precipitate. The wet weight thereof was measured.

[0074]

From each wet weight, the cells were suspended again in PBS so that the cells were 400 μL/100 mg. After that, freezing (-80°C) and thawing (30°C) were carried out six times, and centrifugation (hereinafter, this centrifugation operation is referred to as simply "centrifugation") at 15,100×g for one hour at 4°C, fractionated into supernatant (soluble fraction: S) and precipitate (insoluble fraction: P). The quantification of protein in the supernatant was measured by a BCA method, and the quantification of the protein in precipitate was measured by a Bradford method after it was dissolved in 8M urea-PBS whose volume was the same as the supernatant.

[0075]

The results are shown in Table 2. In Malme-3M and G-361, 50% or more of protein was recovered in the precipitate, in Mewo, 47% of protein was recovered in the precipitate. Therefore, it was confirmed that a soluble protein that had been used in a conventional processing method of dendritic cells (DCs) for preparing the lysate-pulse DCs was about 50% in the total amount of proteins contained in the cells.

[0076]

[Table 2]

Amount of protein contained in supernatant (sup) or precipitate (ppt) after freezing and thawing of melanoma cell line

cell lines	Malme-3M	Mewo	G-361
wet weight (mg)	37	52	57
sup (μg)	611±0	853±21	671±16
ppt (μg)	720±36	768±30	964±44

± SE (n=2)

Example 8

[0077]

<Detection of Cancer Antigen in Natural Insoluble Protein Derived from Cell>

In order to estimate how many cancer antigens were contained in the precipitate (insoluble) fraction, Western Blotting using an antibody that specifically recognizes a MART-1 protein known as a cancer antigen of melanoma was carried out. The supernatant and precipitate derived from each melanoma cell line, which had been obtained above, were separated into equal volumes by SDS-PAGE. They were transferred to an Immun-Blot PVDF Membrane (BIO-RAD, #162-0176), and then blocked with Super Block Blocking Buffer in TBS (Thermo SCIENTIFIC, #37535). After that, anti-human MART-1 monoclonal antibody (MART-1 Mouse Mab: SPRING, #E6704), Biotinylated Anti-Mouse Ig (Whole Ab: GE Healthcare, #RPN1001-2ML), and Amersham Streptavidin-alkaline phosphatase conjugate (GE Healthcare, #RPN1234) were reacted, and colored by BCIP/NBT Solution (Wako, #547-01941) as a substrate. Before respective reactions, a PVDF membrane was washed with 0.1% Tween20-PBS.

[0078]

As a result, a band of MART-1 was not detected in the supernatant of Malme-3M, and was slightly detected in the supernatant of Mewo and G-361. Furthermore, a clear band of MART-1 was observed in precipitate derived from each cell (Fig. 7). From the above-

mentioned results, it was assumed that a large amount of cancer antigenic proteins were contained in the insoluble fraction of the cancer cell.

[0079]

It was thought that if the protein contained in the insoluble fraction can be solubilized in a physiological solvent, a larger amount of (and different) cancer antigenic proteins were able to be pulsed to the dendritic cells as compared with the case of using only the protein in the lysate that had been used in a conventional method.

[0080]

Then, in order to examine what degree of the protein contained in the insoluble fraction can be solubilized by the solubilization method of the present invention, the following experiments were carried out.

Example 9

[0081]

<Solubilization of Insoluble Recombinant Protein>

From Examples 4 and 5, since the solubilization effect of, in particular, GMP and UMP among nucleotides was strong and stable, the nucleotide to be added in the following experiments were limited to these two nucleotides.

[0082]

rTRP-2 dissolved in 8 M urea-PBS was divided into three test tubes in an equal volume each (0.367 mg each was contained in one test tube). GSSG with the final concentration of 2.5 mM was added to these test tubes. Furthermore, GMP with the final concentration of 10 mM was added to the first test tube; UMP with the final concentration of 10 mM was added to the second test tube; and 6 M urea-PBS was added to the third test tube, in an equal amount each. They were reacted for one hour at room temperature. After that, to the reaction solutions, 1/10 volume of 50% glycerol and 6 M urea-PBS were added, and the solutions were dialyzed with 10% glycerol-PBS (pH8.0) containing corresponding 10 μM nucleotide (GMP, UMP or none). The recovered sample was divided into the supernatant and the precipitate by centrifugation, and the amount of the protein (solubilized protein) dissolved in the supernatant was quantified by a BCA method.

[0083]

The results are shown in Table 3. In the case where no nucleotide was added, rTRP-2 that was recovered in a soluble state was 82.8%, while in the case where GMP was added, it was 97.2%, and in the case where UMP was added, it was 100%. The effectiveness of the solubilization method of the present invention was confirmed.

[0084]

[Table 3]

Effect of Nucleotide in Solubilization of rTRP-2

Nucleotide	GMP	UMP	none
Amount of Solubilized Protein (μg)	357±31.1	372±51.6	304±4.24
Recovery Rate (%)	97.2	100	82.8

± SD (n=2)

Example 10

[0085]

<Solubilization of Natural Insoluble Protein Derived from Cell>

An insoluble fraction of Mewo cell was obtained as in Example 7. This was dissolved in 8M urea-PBS, and the resultant solution was divided into three test tubes in equal volume each (each test tube contained 182 μg of insoluble protein, respectively). GSSG with the final concentration of 10 mM was added to these test tubes. Furthermore, GMP with the final concentration of 10 mM was added to the first test tube; UMP with the final concentration of 10 mM was added to the second test tube; and 6 M urea-PBS was added to the third test tube, in an equal amount each. They were reacted for one hour at room temperature. After that, to the reaction solution, 1/10 volume of 50% glycerol and 6 M urea-PBS were added, and the solution was dialyzed with 10% glycerol PBS (pH7.4) containing 10 μM corresponding nucleotide (GMP, UMP or none). The recovered sample was divided into supernatant and precipitate by centrifugation, and the amount of protein dissolved in the supernatant was quantified by a BCA method.

[0086]

The results are shown in Table 4. In the case where no nucleotide was added, protein recovered in a soluble state was 35.9%, while in the case where GMP was added, it was 45.8% ($p<0.02$ in comparison with the case where no nucleotide was added), and in the case where UMP was added, it was 59.9% ($p<0.02$ in comparison with the case where no nucleotide was added). It was confirmed that the effectiveness of the solubilization method of the present invention was significantly increased.

[0087]

[Table 4]

Effect of Nucleotide in Solubilization of Memo Insoluble Fraction

Nucleotide	GMP	UMP	none
Amount of Solubilized Protein (μg)	83.3±2.97*	109±8.62*	65.0±11.2
Recovery Rate (%)	45.8	59.9	35.9

± SD (n=4) *P<0.02

[0088]

Furthermore, whether or not MART-1 is contained in the thus obtained solubilized protein was estimated by Western Blotting as in Example 8. The supernatant (S) after solubilization and the precipitate (P) dissolved in a 2×SDS-PAGE sample treatment solution having the same volume as that of the supernatant were separated by SDS-PAGE in equal volume each. Hereinafter, MART-1 was detected by the same operation as in Example 8 (Fig. 8). Furthermore, this image was taken by using Gel Doc 2000 (BIO RAD), and the solubilized rate was calculated from the produce of the concentration and the area of the resultant band. As a result, it was revealed that when solubilization was carried out with the addition of GMP, 46.6% was recovered in the supernatant (soluble fraction), and when solubilization was carried out with the addition of UMP, 47.3% was recovered in the supernatant (soluble fraction).

Example 11

[0089]

From the examination in a recombinant protein, the same level of effect was confirmed in both the case in which the nucleotide to be added was monophosphate and the case in which the nucleotide was triphosphate. The replicating property in naturally occurring protein was confirmed. Example 7 carried out an examination by using a protein derived from a human cell. This Example carried out verification by using an experiment system in which insoluble protein derived from a mouse cell to which UMP as an example of the monophosphate had been added.

[0090]

B16 melanoma had been cultured in vitro, and the attached cells were scraped off and recovered by using a scraper in PBS. The solution was centrifuged at 700×g for 5 min at 4°C, and supernatant was discarded and precipitate of the cells was frozen at -80°C. After that, PBS was added again, and the cells were pulverized by freezing and thawing (six times) and ultrasonic wave. Hereinafter, the same operations as in Example 7 were carried out, and the insoluble fraction of B16 melanoma was dissolved by using a denaturing agent to form a

solution. To the solution, 10 mM UMP as nucleotide was added. One hour later, the solution was dialyzed with 10 μM UMP/10% glycerol/PBS (pH7.4), and centrifuged. After the centrifugation, the supernatant and precipitate were recovered, and analyzed by SDS-PAGE and by protein quantification. The results are shown in Fig. 9 and Table 5. In the SDS-PAGE, proteins were observed in the supernatant both in the case where nucleotide after dialysis had not been added (PBS added: "-" in figure) and the case where UMP had been added ("+" in figure). In the protein quantification, however, the sup recovery rate was 27.6% in (-) while it was 41.2% in (+). It was determined that the solubilization efficiency was increased with the addition of UMP. That is to say, it was confirmed that the monophosphate nucleotide was effective in the solubilization of natural insoluble protein similar to the triphosphate nucleotide.

[0091]

[Table 5]

Sup recovery rate

UMP	(-)	(+)
sup recovery rate (%)	27.6	41.2

Example 12

[0092]

Furthermore, by using UMP, solubilization in an insoluble protein derived from a human cell was verified. By using a breast cancer cell line (MDA-MB-231) and a prostate cancer cell line (DU145), experiment was carried out similar to Example 7. The results are shown in Figs. 10 and 11 and Table 6. In MDA-MB-231, the Sup recovery rate was 40.0% when UMP had not been added, 45.0% when UMP had been added. Meanwhile, in DU145, the Sup recovery rate was 18.5% when UMP had not been added, 22.6% when UMP had been added. In both cases, with the addition of UMP, the solubilization efficiency was increased.

From the above-mentioned result, it was confirmed that the addition of nucleotide was effective in solubilization of an insoluble protein.

[0093]

[Table 6]

Sup recovery rate of insoluble protein of two types of cell lines

UMP		(-)	(+)
sup recovery rate (%)	MDA-MB-231	40.0	45.0
	DU-145	18.5	22.6

Example 13

[0094]

<Induction of Mouse DC>

In order to confirm that a protein solubilized according to the method of the present invention was useful for research and development of medicines, application to an antigen presenting method using mouse dendritic cells was attempted. According to the Non Patent Literature 1 (Jonathan M. Weiss et al, J. Immunother, VOL. 28: 542-550 (2005)), a soluble protein derived from a cancer cell dissolved in a physiological solvent was transduced into the cytoplasm of the dendritic cell by electroporation, the transduced protein was fragmented in the cell, and presented to the MHC class I as an antigen peptide, and immune response was able to be induced in an individual to which the DC had been administered.

[0095]

Verification was carried out whether or not antigen presentation was actually made when the insoluble rTRP-2 produced in Example 2 was used, solubilized, and then transduced into DC by electroporation.

Male C57BL/6, which had been purchased at 8 weeks old, was bred in an SPF environment, and the bone marrow cells were collected at 9 weeks or later. The collected bone marrow cells were seeded in an AIM-V (Invitrogen, 087-0112DK) medium in a culture flask, incubated for 4 to 6 hours at 37°C in 5% CO_2. Then, floating cells were removed. The attached cells were cultured for one day in AIM-V to which 10 ng/ml mouse IL-4 (Peprotech, #214-14) and 20 ng/ml mouse GM-CSF (Peprotech, #315-03) had been added, and the medium was replaced with a fresh medium on the following day. Furthermore, the medium was replaced with a new one every other day. Cells that naturally float until the seventh day after the start of culture of the attached cells were used as mature DCs.

[0096]

Fig. 12 shows an example in which the cell surface antigen of the mature DC was confirmed. Analysis of each CD was carried out by using a flow cytometer (CYTOMICS FC 500, BECKMAN COULTER) by labeling with PE anti-CD11c, Clone N418 (BioLegend, #117307) and PE Armenian Hamster IgG Isotype control, Clone HTK888 (BioLegend, #400907) in the analysis of CD11c; by labeling with FITC anti-mouse CD11b, Clone M1/70 (BioLegend, #101205), and FITC Rat IgG2b, Isotype control, Clone RTK4530 (BioLegend, #400605) in the analysis of CD11b; by labeling with FITC anti-mouse CD80, Clone 16-10A1 (BioLegend, #104705), and FITC Armenian Hamster IgG Isotype control, Clone HTK888 (BioLegend, #400905) in the analysis of CD80; as well as by labeling with Biotin anti-mouse CD86, Clone

GL-1 (BioLegend, #105003), and Biotin Rat IgG2a, Isotype control, Clone RTK2758 (BioLegend, #400503) and then labeling with Streptavidin-PE/Cy5 (BioLegend, #405205)in the analysis of CD86.

[0097]

Fig. 12 a) is a graph showing development with CD11c and CD11b, showing that 82.3% of the cells was CD11c positive. Meanwhile, Fig. 12 b) is a graph showing development with CD80 and CD86, showing that 81.6% of the cells was CD80 positive or CD86 positive. From the above mention, it was confirmed that cells to be used were the mature DCs.

[0098]

<Electroporation>

The medium of DCs recovered on Day 7 of culture was exchanged with X-VIVO 20 (Takara Bio Inc., #04-448Q), and the number of cells was counted. rTRP-2 solubilized with the addition of UMP or GMP with respect to the number of living cells, 2×10^5 cells, was added so that the concentration was 150 pmole, was prepared with a buffer solution so that the total volume was 0.4 mL, and the total number of cells was about 1×10^6 cells. At this time, the rate of the medium was at least 75%. This cell suspension was placed in NEPA cuvette electrode 2 mm gap (NEPA GENE, #EC-002S), and pulsed at 500 v for 500 μsec by using Electro square Porator (BTX, ECM830). Immediately after being pulsed, cells were recovered with 5-fold volume of X-VIVO 20, and incubated for one hour at 37°C in 5% CO_2. The rate of living cells after the cells were incubated was approximately 20%. FITC-dextran (Sigma, #FD 500S) was pulsed by this technique, it was confirmed in the previous examination that FITC-dextran was transduced in almost all the cells. After the cells were incubated for one hour, 10 ng/ml IL-4, 20 ng/ml GM-CSF and 10 ng/ml mouse IFN-γ (Pepratech, #315-05) were added, and the cells were cultured for about 15 hours. After the cells were cultured, the cells were recovered, the medium was replaced with PBS containing 0.5% normal C57BL/6 serum, and prepared so that the number of living cells was about 2×10^5 cells/200 μl.

[0099]

<Immunization>

A part of DCs used for electroporation were cultured as it is, 1 μg/ml of TRP-2 peptide (SVYDFFVWL: SEQ ID NO: 4) was added thereto on the day of immunization, cultured for six hours, and recovered and similarly prepared so that the number of living cells was about 2×10^5 cells/200 μl (peptide-pulsed DCs). Peptide-pulsed DC was used as a positive control (peptide), DC (T(U)) into which rTRP-2 solubilized with the addition of UMP had been

transduced by electroporation or DC (T(G)) into which rTRP-2 solubilized with the addition of GMP had been transduced by electroporation were intraperitoneally administered to the C57BL/6 mice in the number of living cells of about 2×10^5 cells/200 μl/mouse, thus immunization was carried out. The immunization was carried out three times every other week. One week after the final immunization, splenocytes were recovered.

[0100]

<Confirmation of Induction of CTL>

The recovered splenocytes were labeled with H-2K^b TRP-2 tetramer–SVYDFFVWL (SEQ ID NO: 4)–(MBL, #TS-5004-1) or H-2K^b OVA tetramer–SIINFEKL (SEQ ID NO: 5)–(BCI, #TS-5001-1) as a negative control, and at the same time, analysis of TRP-2 specific CTL was carried out by a flow cytometer according to the technique presented by MBL by using an anit-CD8a antibody–FITC, Clone 53-6.7 (BD Bioscience, #553030) or Rat IgG2a, k isotype control, Clone R35-95 (BD Bioscience, #553929).

[0101]

The results are shown in Table 13. The CD8 positive cells and the OVA tetramer positive ($CD8^+OVA^+$) cells were not detected in any mouse-derived splenocytes. In untreated mouse-derived splenocytes marked with a negative control group, the CD8 positive cells and TRP-2 tetramer positive ($CD8^+TRP2^+$) cells were 0.0%. In mouse-derived splenocytes which had been immunized with peptide-pulsed DC marked with peptide, the $CD8^+TRP2^+$ cells were 2.4%. At this time, in the mouse-derived splenocytes which had been immunized with T(U), 2.3% $CD8^+TRP2^+$ cells were induced; and in the mouse-derived splenocytes which had been immunized with T(G), 1.5% $CD8^+TRP2^+$ cells were induced. From the above-mentioned results, it was confirmed that the solubilized rTRP-2 was transduced into DCs and immunized, TRP-2-specific CTL was induced.

[0102]

In the result of the comparison of induction efficiency of CTL with respect to a single epitope (SVYDFFVWL: SEQ ID NO: 4), the method of the present invention for transducing insoluble protein and/or peptide into the cells showed the CTL induction effect substantially equal to that in the case where a peptide was pulsed. In the case where a peptide is pulsed, although only CTL specific to the pulsed peptide can be induced, in the case where protein solubilized by the transducing method of the present invention is transduced into the DCs, since a plurality of epitopes contained in the protein can be presented to a various types of MHC including not only the class I but also the class II, it was assumed that the effect was able to be enhanced as compared with the peptide-pulsed DC vaccine.

Example 14

[0103]

<Induction of CTL Using Insoluble Fraction of Cancer Cell Homogenates>

As shown in Example 13, when an insoluble recombinant protein (rTRP-2) was solubilized, rTRP-2 was able to be incorporated into DCs by electroporation. It was revealed by a tetramer assay that when the DCs incorporating a protein were administered to a mouse, the CTL was able to be induced in a mouse body. This time, the insoluble fraction of B16 mouse melanoma cell homogenates that had been solubilized by the method of the present invention was used to prepare DCs, and an effect on the anti-tumor immunization in a mouse body was examined.

[0104]

<Preparation of B16 Lysate and Solubilized Insoluble Fraction>

B16 was cultured in 75 cm^2-culture flask. When the cells were spread all over the bottom surface, the cultured cells were washed with PBS three times. After washing, the cells were scraped off, and centrifuged at 1,000×g for 5 minutes at 4°C and recovered as precipitate. The wet weight of the precipitate was measured.

[0105]

From each wet weight, the precipitate was suspended again in PBS so that the concentration was 400 μL/100 mg. After that, the suspension was subjected to freezing (-80°C) and thawing (30°C) six times, centrifuged, and fractionated into supernatant and precipitate. The quantification of protein in the supernatant (lysate: sup) was measured by a BCA method. The precipitate was dissolved in 8 M urea-PBS, GSSG with the final concentration of 10 mM and UMP with the final concentration of 10 mM were added to the solution, and they were reacted for one hour at room temperature. After that, 1/5 volume of 50% glycerol and 6 M urea-PBS were added into a reaction solution, and the reaction solution was dialyzed with 10% glycerol-PBS containing 10 μM UMP. The recovered samples were centrifuged and the amount of protein dissolved in the supernatant (solubilized insoluble fraction: ppt) was quantified by a BCA method.

[0106]

<Preparation and Immunization of DC>

Similar to Example 10, mature DCs were induced from the bone marrow cells of C57BL/6 mouse. The mature DCs were suspended in an X-VIVO20 medium so that the concentration was 1×10^7 cells /mL, and the suspension was dispensed into 0.2 mL each. To this suspension, 0.2 mL of 10% glycerol-PBS containing 10 μM UMP, 0.2 mL of B16 lysate (final

concentration: 2 mg/mL) that had been previously prepared or 0.2 mL of solubilized insoluble fraction of B16 solubilization (final concentration: 2 mg/mL) was added, and electroporation was carried out. Cells were recovered immediately after electric pulse, and they were suspended in an AIM-V medium containing 5-fold volume of 0.2% normal C57BL/6 mouse serum, and the suspension was incubated for one hour at 37°C in 5% CO_2. Thereafter, GM-CSF with the final concentration of 20 ng/mL, IL-4 with the final concentration of 10 ng/mL, and IFN-γ with the final concentration of 10 ng/mL were added, and the mixed suspension was similarly cultured overnight. Then, respective DCs were recovered and washed. The DCs were suspended in PBS containing 0.2% normal C57BL/6 mouse serum, 5×10^5 cells/mouse were intraperitoneally administered to C57BL/6 mice. As the negative control, DCs that had not been subjected to electroporation and co-cultured with lysate or the solubilized insoluble fraction were similarly administered. The immunization was carried out again one week later, and further one week later, splenocytes were collected from each mouse.

[0107]

<Cytotoxicity test>

$CD8a^+$ cells were isolated and separated from the splenocytes by using $CD8a^+$ T Cell isolation kit (Miltenyi Biotec, #130-090-859), and then suspended in a RPMI1640 (complete) medium containing 10% FBS, 25 mM HEPES (Invitrogen, #15630-080), 5×10^{-5} M 2-ME (Invitrogen, #21985-023), 2 mM L-glutamine (Invitrogen, #25030-081), 1×10^{-5} M sodium pyruvate (Invitrogen, #11360-070), 1% non-essential amino acid (Invitrogen, #11140-050), and 100 μg/mL streptmycin, and 100 u/mL penicillin (Invitrogen, #15070-063) so that the concentration was 1×10^6 cells /mL. 10 U/mL IL-2 (Chairon) was added to the suspension, and the suspension was cultured for three days to give effector cells.

[0108]

For target cells, sub-cultured B16 cells to which IFN-γ with the final concentration of 1 ng/mL had been added and which had been cultured for 24 hours was used. The expression of the MHC class I molecule that had been treated with IFN-γ was analyzed by a flow cytometer by using an anti-H-$2K^b$ antibody (BD Pharmingen PE, #553570) and an anti-H-$2D^b$ antibody (BD Pharmingen FITC, #553573). As shown in Fig. 14a, the positive cell rate was 37.4%. This means that the maximum cytotoxicity due to antigen-specific recognition was 37.4%. B16 cells that had been treated with IFN-γ were suspended in a RPMI1640 medium containing 5% FBS so that the concentration was 1×10^6 cells /mL, and Calcein-AM (Doujin Kagaku Kenkyusho, #349-07201) was added and stirred so that final concentration was 2

μg/mL, and stirred every 10 min at 37°C in the presence of 5% CO_2, and incubated for 30 min. With this operation, B16 cells labeled with Calcein-AM were washed with a RPMI1640 medium containing 5% FBS three times to give the target cells. The effector cells and the target cells were mixed at the ratio of the number of effector cells (E) and the number of target cells (T) (E/T ratio) of 5, 10 and 20, and the cells were incubated for two hours at 37°C in the presence of 5% CO_2. Thus, the cytotoxicity test was carried out. The measurement of the cytotoxicity was carried out by using Terascan VP (MINERVA TECH) system.

[0109]

The results are shown in Fig. 14b. The axis of ordinates shows a mean value of the cytotoxicity by the effector cells derived from a group consisting of two, and an error bar means the SD thereof. Mouse-derived effector cells immunized with DC (Co sup) that had been co-cultured with lysate and DC (Co ppt) that had been co-cultured with the solubilized insoluble fraction have the same level of cytotoxicity as that of the mouse-derived effector cells to which DC (Ep(-)) electroporated without an antigen had been administered. That is to say, only a non-specific cytotoxicity was recognized. Furthermore, also in mouse-derived effector cells immunized with TRP-2 peptide-pulsed DC (Pep), specific cytotoxicity was not recognized. On the other hand, in the mouse-derived effector cells immunized with DC (Ep sup) in which the lysate had been incorporated by electroporation, E/T was 20 and specific cytotoxicity was recognized. Furthermore, in the mouse-derived effector cells immunized with DC (Ep ppt) in which the solubilized insoluble fraction had been incorporated by electroporation, E/T was 10 and 20, and stronger antigen-specific cytotoxicity was exhibited.

[0110]

From the above-mentioned results, when a protein solubilized by the solubilization method described in this specification was used as an antigen, CTL having a higher effect than that by a conventional method can be induced, it was suggested that anti-cancer immunization stronger than that by a single epitope peptide can be induced. Furthermore, it is shown that an insoluble fraction of tumor tissue homogenates, which had been conventionally discarded, can be used in the present solubilization method.

Industrial Applicability

[0111]

As described above, use of a method of the present invention enables an insoluble protein to be solubilized efficiently. Furthermore, a protein solubilized by the method of the present invention can be easily transduced into a cell. Accordingly, it is effective to induce

CTL by an antigen presenting cell. With the use of techniques of the present invention, antigen presenting cells induced by solubilizing, for example, insoluble antigenic proteins of cancer and infectious diseases and transducing it into dendritic cells or the like can be used as CTL inducing agents specific to the antigenic protein, and can be used for treatment or the like for patients with cancer and infectious diseases having the antigenic protein.

CLAIMS

[Claim 1]

A method for solubilizing an insoluble protein and/or peptide, the method comprising:

a step of mixing an insoluble protein and/or peptide and a denaturing agent in one solution, and dissolving the insoluble protein and/or peptide, thereby forming a solution of the insoluble protein and/or peptide;

a step of adding a nucleotide to the solution; and

a step of removing the denaturing agent from the solution.

[Claim 2]

The method for solubilizing an insoluble protein and/or peptide according claim 1, wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

[Claim 3]

The method for solubilizing an insoluble protein and/or peptide according claim 1 or 2, wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil; and a combination of one or more of the nucleotides is added.

[Claim 4]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 3, wherein the number of phosphate groups constituting the nucleotide is one to three.

[Claim 5]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 4, wherein the nucleotide is added after or concurrently with mixing the insoluble protein and/or peptide with the denaturing agent.

[Claim 6]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 5, wherein in the step of removing the denaturing agent from the solution, the denaturing agent is removed while the solution is replaced with a physiological solvent, and preferably the physiological solvent includes nucleotide.

[Claim 7]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 6, wherein the insoluble protein and/or peptide is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

[Claim 8]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 7, wherein the insoluble protein and/or peptide is an antigenic protein.

[Claim 9]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 8, wherein the denaturing agent is urea and/or guanidine hydrochloride.

[Claim 10]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 9, wherein in the step of mixing the insoluble protein and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

[Claim 11]

The method for solubilizing an insoluble protein and/or peptide according to any one of claims 1 to 10, wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

[Claim 12]

A method for transducing an insoluble protein and/or peptide into a cell, the method comprising:

a step of mixing an insoluble protein and/or peptide and a denaturing agent in one solution, and dissolving the insoluble protein and/or peptide, thereby forming a solution of the insoluble protein and/or peptide;

a step of adding a nucleotide to the solution;

a step of removing the denaturing agent while the solution is replaced with a physiological solvent, thereby forming a physiological solution of the insoluble protein and/or peptide; and

transducing the physiological solution into cytoplasm or inside of a desired cell.

[Claim 13]

The method for transducing an insoluble protein and/or peptide into a cell according claim 12, wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

[Claim 14]

The method for transducing an insoluble protein and/or peptide into a cell according claim 12 or 13, wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil; and a combination of one or more of the nucleotides is added.

[Claim 15]

The method for transducing an insoluble protein and/or peptide into a cell

according to any one of claims 12 to 14, wherein the number of phosphate groups constituting the nucleotide is one to three.

[Claim 16]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 15, wherein the nucleotide is added after or concurrently with mixing the insoluble protein and/or peptide with the denaturing agent.

[Claim 17]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 16, wherein the physiological solvent to be used for replacing the solution of the protein and/or peptide includes nucleotide.

[Claim 18]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 17, wherein the cell is a cell having an antigen presenting function.

[Claim 19]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 18, wherein the cell having the antigen presenting function is a dendritic cell.

[Claim 20]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 19, wherein the insoluble protein and/or peptide is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

[Claim 21]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 20, wherein the insoluble protein and/or peptide is an antigenic protein or peptide.

[Claim 22]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 21, wherein the denaturing agent is urea and/or guanidine hydrochloride.

[Claim 23]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 22, wherein in the step of mixing the insoluble protein

and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

[Claim 24]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 23, wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

[Claim 25]

The method for transducing an insoluble protein and/or peptide into a cell according to any one of claims 12 to 24, wherein the solubilized insoluble protein and/or peptide is transduced into cytoplasm and/or inside a cell by electroporation.

[Claim 26]

A method for producing an antigen presenting cell that presents an insoluble antigen, the method comprising:

a step of mixing the insoluble antigen and a denaturing agent in one solution, and dissolving the insoluble antigen, thereby forming a solution containing the insoluble antigen;

a step of adding a nucleotide to the solution;

a step of removing the denaturing agent while the solution is replaced with a physiological solvent, thereby forming a physiological solution containing the insoluble antigen; and

transducing the physiological solution containing the insoluble antigen into cytoplasm or inside of the antigen presenting cell.

[Claim 27]

The method for producing an antigen presenting cell according to claim 26, wherein the nucleotide is deoxyribonucleotide or ribonucleotide.

[Claim 28]

The method for producing an antigen presenting cell according to claim 26 or 27, wherein a base constituting the nucleotide is any one of adenine, cytosine, guanine, thymine, and uracil; and a combination of one or more of the nucleotides is added.

[Claim 29]

The method for producing an antigen presenting cell according to any one of claims 26 to 28, wherein the number of phosphate groups constituting the nucleotide is one to three.

[Claim 30]

The method for producing an antigen presenting cell according to any one of claims 26 to 29, wherein the physiological solvent to be used for replacing the solution of the

antigen includes nucleotide.

[Claim 31]

The method for producing an antigen presenting cell according to any one of claims 26 to 30, wherein the antigen is an insoluble fraction obtained from a fractured product of a tumor cell, a tumor tissue, an infectious disease cell or a pathogenic cell.

[Claim 32]

The method for producing an antigen presenting cell according to any one of claims 26 to 31, wherein the antigen is a protein or a peptide.

[Claim 33]

The method for producing an antigen presenting cell according to any one of claims 26 to 32, wherein the antigen is a naturally occurring protein and/or peptide, a protein and/or peptide produced by a gene recombination technology, or chemically synthesized protein and/or peptide.

[Claim 34]

The method for producing an antigen presenting cell according to any one of claims 26 to 33, wherein the antigen presenting cell is a dendritic cell.

[Claim 35]

The method for producing an antigen presenting cell according to any one of claims 26 to 34, wherein the denaturing agent is urea and/or guanidine hydrochloride.

[Claim 36]

The method for producing an antigen presenting cell according to any one of claims 26 to 35, wherein in the step of mixing the insoluble protein and/or peptide and the denaturing agent in one solution, a reducing agent is further added.

[Claim 37]

The method for producing an antigen presenting cell according to any one of claims 26 to 36, wherein the reducing agent is dithiothreitol or 2-mercaptoethanol.

[Claim 38]

An antigen presenting cell produced by the producing method according to any one of claims 26 to 37.

[Claim 39]

A method for inducing a cytotoxic T lymphocyte, the method comprising a step of administering an antigen presenting cell produced by a producing method according to any one of claims 26 to 37 to a mammalian including a human.

[Claim 40]

A method for treating or preventing cancer and/or an infectious disease, the method comprising a step of administering an antigen presenting cell produced by a producing method according to any one of claims 26 to 37 to a mammalian including a human.

[Claim 41]

A composition for inducing a cytotoxic T lymphocyte, the composition comprising, as an active ingredient, an antigen presenting cell produced by a producing method according to any one of claims 26 to 37.

[Claim 42]

A composition for treating or preventing cancer and/or an infectious disease, the composition comprising, as an active ingredient, an antigen presenting cell produced by a producing method according to any one of claims 26 to 37.

SEQUENCE LISTING

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Leu Ala Lys Lys Ser Ile His Pro Asp Tyr Val Ile Thr Thr Gln His
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Trp Leu Gly Leu Leu Gly Pro Asn Gly Thr Gln Pro Gln Ile Ala Asn
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Cys Ser Val Tyr Asp Phe Phe Val Trp Leu His Tyr Tyr Ser Val Arg
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Asp Thr Leu Leu Gly Pro Gly Arg Pro Tyr Lys Ala Ile Asp Phe Ser
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Asp Asp Trp Leu Gly Ala Ala Arg Gln Asp Asp Pro Thr Leu Ile Ser
305 310 315 320

Arg Asn Ser Arg Phe Ser Thr Trp Glu Ile Val Cys Asp Ser Leu Asp
325 330 335

Asp Tyr Asn Arg Arg Val Thr Leu Cys Asn Gly Thr Tyr Glu Gly Leu
340 345 350

Leu Arg Arg Asn Lys Val Gly Arg Asn Asn Glu Lys Leu Pro Thr Leu
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Phe Phe Gln Asn Ser Thr Phe Ser Phe Arg Asn Ala Leu Glu Gly Phe
385 390 395 400

Asp Lys Ala Asp Gly Thr Leu Asp Ser Gln Val Met Asn Leu His Asn
405 410 415

Leu Ala His Ser Phe Leu Asn Gly Thr Asn Ala Leu Pro His Ser Ala
420 425 430

Ala Asn Asp Pro Val Phe Val Val Leu His Ser Phe Thr Asp Ala Ile
435 440 445

Phe Asp Glu Trp Leu Lys Arg Asn Asn Pro Ser Thr Asp Ala Trp Pro
450 455 460

Gln Glu Leu Ala Pro Ile Gly His Asn Arg Met Tyr Asn Met Val Pro
465 470 475 480

Phe Phe Pro Pro Val Thr Asn Glu Glu Leu Phe Leu Thr Ala Glu Gln
485 490 495

Leu Gly Tyr Asn Tyr Ala Val Asp Leu Ser Glu Glu Glu Ala Pro Val
500 505 510

Trp Ser Thr Thr Leu Ser Phe
515

<210> 3
<211> 438
<212> PRT
<213> Mus musculus

<400> 3

Met Ser Gly Ser His His His His His His Ser Ser Gly Ile Glu Gly
1 5 10 15

Arg Val Asp Arg Glu Gly Arg Gly Gln Cys Ala Glu Val Gln Thr Asp
20 25 30

Thr Arg Pro Trp Ser Gly Pro Tyr Ile Leu Arg Asn Gln Asp Asp Arg
35 40 45

Glu Gln Trp Pro Arg Lys Phe Phe Asn Arg Thr Cys Lys Cys Thr Gly
50 55 60

Asn Phe Ala Gly Tyr Asn Cys Gly Gly Cys Lys Phe Gly Trp Thr Gly
65 70 75 80

Pro Asp Cys Asn Arg Lys Lys Pro Ala Ile Leu Arg Arg Asn Ile His
85 90 95

Ser Leu Thr Ala Gln Glu Arg Glu Gln Phe Leu Gly Ala Leu Asp Leu
100 105 110

Ala Lys Lys Ser Ile His Pro Asp Tyr Val Ile Thr Thr Gln His Trp
115 120 125

Leu Gly Leu Leu Gly Pro Asn Gly Thr Gln Pro Gln Ile Ala Asn Cys
130 135 140

Ser Val Tyr Asp Phe Phe Val Trp Leu His Tyr Tyr Ser Val Arg Asp
145 150 155 160

Thr Leu Leu Gly Pro Gly Arg Pro Tyr Lys Ala Ile Asp Phe Ser His
165 170 175

Gln Gly Pro Ala Phe Val Thr Trp His Arg Tyr His Leu Leu Trp Leu
180 185 190

Glu Arg Glu Leu Gln Arg Leu Thr Gly Asn Glu Ser Phe Ala Leu Pro
195 200 205

Tyr Trp Asn Phe Ala Thr Gly Lys Asn Glu Cys Asp Val Cys Thr Asp
210 215 220

Asp Trp Leu Gly Ala Ala Arg Gln Asp Asp Pro Thr Leu Ile Ser Arg
225 230 235 240

Asn Ser Arg Phe Ser Thr Trp Glu Ile Val Cys Asp Ser Leu Asp Asp
245 250 255

Tyr Asn Arg Arg Val Thr Leu Cys Asn Gly Thr Tyr Glu Gly Leu Leu
260 265 270

Arg Arg Asn Lys Val Gly Arg Asn Asn Glu Lys Leu Pro Thr Leu Lys
275 280 285

Asn Val Gln Asp Cys Leu Ser Leu Gln Lys Phe Asp Ser Pro Pro Phe
290 295 300

Phe Gln Asn Ser Thr Phe Ser Phe Arg Asn Ala Leu Glu Gly Phe Asp
305 310 315 320

Lys Ala Asp Gly Thr Leu Asp Ser Gln Val Met Asn Leu His Asn Leu
325 330 335

Ala His Ser Phe Leu Asn Gly Thr Asn Ala Leu Pro His Ser Ala Ala
340 345 350

Asn Asp Pro Val Phe Val Val Leu His Ser Phe Thr Asp Ala Ile Phe
355 360 365

Asp Glu Trp Leu Lys Arg Asn Asn Pro Ser Thr Asp Ala Trp Pro Gln
370 375 380

Glu Leu Ala Pro Ile Gly His Asn Arg Met Tyr Asn Met Val Pro Phe
385 390 395 400

Phe Pro Pro Val Thr Asn Glu Glu Leu Phe Leu Thr Ala Glu Gln Leu
405 410 415

Gly Tyr Asn Tyr Ala Val Asp Leu Ser Glu Glu Glu Ala Pro Val Trp
420 425 430

Ser Thr Thr Leu Ser Phe
435

<210> 4
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<212> PRT
<213> Mus musculus

<400> 4

Ser Val Tyr Asp Phe Phe Val Trp Leu
1 5

<210> 5
<211> 8
<212> PRT
<213> Gallus gallus

<400> 5

Ser Ile Ile Asn Phe Glu Lys Leu
1 5