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Protein which induces interferon-gamma production by immunocompetent cell

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Abstract of the Disclosure

A protein of human cell origin, which induces the IFN-γ production by immunocompetent cells and has the amino acid sequence of SEQ ID NO:1 near or at the N-terminus. It can be produced from human cells such as lymphoblasts, lymphocytes, monoblasts, monocytes, myeloblasts, myelocytes, granulocytes and macrophages, and used for preventing and/or treating IFN-γ susceptive diseases.

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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

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Invention Title: "PROTEIN WHICH INDUCES INTERFERON-GAMMA PRODUCTION BY IMMUNOCOMPETENT CELL"

The following statement is a full description of this invention, including the best method of performing it known to me:-

PROTEIN WHICH INDUCES INTERFERON-γ PRODUCTION

BY IMMUNOCOMPETENT CELL

Background of the Invention

Field of the Invention

The present invention relates to a novel protein which induces interferon-γ (hereinafter abbreviated as "IFN-γ") production by immunocompetent cells.

Description of the Prior Art

It is known that IFN-γ is a protein which has antiviral-, antioncotic- and immunoregulatory-activities which is produced by immunocompetent cells that are stimulated with antigens or mitogens. Because of these biological activities, it has been expected, since its discovery, that IFN-γ would be useful as an antitumor agent, and it has been studied energetically in clinical trials as a therapeutic agent for malignant tumors, in general, including brain tumors. IFN-γ preparations commercially available, now, are roughly classified into two groups, i.e. one group of natural IFN-γs produced by immunocompetent cells and another group of recombinant IFN-γs produced by transformants obtained by introducing DNAs which encode natural IFN-γs into microorganisms of the species *Escherichia coli.* In the above clinical trials, one of these two groups of IFN-γs was administered to patients as an "exogenous IFN-γ".

Among these IFN-γs, natural IFN-γs are usually produced by culturing established immunocompetent cell lines in nutrient culture media admixed with IFN-γ inducers to produce

AUSTRALIAN PATENT OFFICE

IFN-γs, and purifying the produced IFN-γs from the resulting cultures. It is known that IFN-γ inducers greatly influence IFN-γ yield, the ease of IFN-γ purification, and the safety of final IFN-γ preparations. Generally, mitogens such as concanavalin A (Con A), lentil lectin, pokeweed lectin, endotoxin and lipopolysaccharides can be used as IFN-γ inducers. However, these mitogens have the following problems: (i) their molecular structure and quality varies and changes depending on their origin and purification method, and (ii) preparations with a consistent IFN-γ inducibility are not readily prepared in a satisfactory yield. In addition, most of these mitogens might induce unfavorable side effects when administered to living bodies, and some of them might cause toxicity, so that it is reasonably difficult to induce IFN-γ production by directly administering IFN-γ inducers to living bodies.

Summary of the Invention

The present invention was made based on a novel protein which induces interferon-γ production by immunocompetent cells. During the study of cytokines produced by mammalian cells, the present inventors noticed the existence of a substance which induces IFN-γ production in mouse liver cells which had been treated with a lipopolysaccharide and inactivated whole cells of *Corynebacterium*. They isolated the substance by many purification methods, using column chromatography as a main technique, and studied its properties and features, and have found that the substance is a protein

AUSTRALIAN PATENT OFFICE

having the following physicochemical properties:

(1) Molecular weight:
19,000±5,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE);

(2) Isoelectric point (pI)
pI of 4.8±1.0 on chromatofocusing;

(3) Partial amino acid sequence:
Having the partial amino acid sequences of SEQ ID NOs:8 and 9; and

(4) Biological activity:
Inducing IFN-γ production by immunocompetent cells.

The data established that the substance is novel because no protein with these physicochemical properties is known. The present inventors continued studying mouse liver cells and have succeeded in isolating DNA which encodes the protein. The inventors decoded the DNA and have found that it consists of 471 base pairs and encodes the amino acid sequence of SEQ ID NO:10 (where the symbol "Xaa" means "methionine" or "threonine").

Based on these findings, the present inventors further studied human liver cells to obtain a DNA which encodes another novel substance that induces IFN-γ production by immunocompetent cells. They determined that the substance is a polypeptide, then decoded the DNA and found that it has the amino acid sequence of SEQ ID NO:6 (where the symbol "Xaa" is "isoleucine" or "threonine"). They introduced the DNA into *Escherichia coli* to express the polypeptide and to produce it

AUSTRALIAN PATENT OFFICE

in the resulting culture in a satisfactorily high yield. These findings were disclosed in Japanese Patent Laid-Open Nos.27,189/96 and 193,098/96, applied for by the present applicant. In Japanese Patent Application No.78,357/95 applied for by the present applicant, the polypeptide is disclosed as an agent for susceptive diseases. Although biologically active proteins which are administered to humans after mixing with pharmaceuticals should generally be of human cell origin, no human cell which produces such a polypeptide has been reported.

In view of the foregoing, the present invention provides, in a first aspect, a protein of human cell origin, which induces IFN-γ production by immunocompetent cells.

The present invention also provides, in a second aspect, a process for producing the protein.

The present invention further provides, in a third aspect, the use of the protein as an agent for susceptive diseases.

The first aspect of the present invention provides a protein of human cell origin which induces IFN-γ production by immunocompetent cells and has the amino acid sequence of SEQ ID NO:1 near or at its N-terminus.

The second aspect of the present invention provides a process for producing the protein by propagating human cells which produce the protein, and collecting the protein from the propagated cells.

The third aspect of the present invention provides an agent for susceptive diseases, which contains the protein as an effective ingredient.

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Brief Explanation of the Accompanying Drawing

FIG.1 is a peptide map of the protein of Experiment 1.

Detailed Description of the Invention

The protein according to the present invention induces IFN-γ production by immunocompetent cells when allowed to act on the cells alone or together with an appropriate cofactor.

The protein is derived from human cells, and it can be readily prepared by the process of the present invention, using human cells.

The agent for susceptive diseases, according to the present invention, induces IFN-γ production by immunocompetent cells in the human body when administered to humans, and exerts positive effects in the treatment and prevention of IFN-γ susceptive diseases. When the protein augments the cytotoxicity of killer cells or induces the formation of killer cells, it exerts positive effects on inveterate diseases including malignant tumors.

The preferred embodiments according to the present invention will be described hereinafter. The term "protein" as referred to in the present invention means polypeptides and glycoproteins in general which induce IFN-γ production by immunocompetent cells and have the amino acid sequence of SEQ ID NO:1 near or at their N-termini. Depending on the types and propagation conditions of human cells, the protein may have the amino acid sequence of SEQ ID NO: 6 which comprises the amino acid sequences of SEQ ID NOs:1 and 3 near or at the N- and C-termini, respectively and can

AUSTRALIAN PATENT OFFICE

include the amino acid sequences of SEQ ID NOs:4 and 5 as internal fragments (where the symbol "Xaa" means "isoleucine" or "threonine"). The protein is detected as a protein band at a position corresponding to a molecular weight of 14,000-24,000 daltons, usually, 18,000-19,500 daltons when determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent. Depending on the types and propagating conditions of the human cells, one or more amino acids may be added to the above N- and/or C-termini of SEQ ID NOs:1 and 3 or one or more amino acids in the N- and/or C-termini may be deleted. Any protein can be used in the present invention as long as it is derived from a human cell, as well as having either of these amino acid sequences and inducing IFN-γ production when acting on immunocompetent cells alone or together with an appropriate cofactor.

These proteins can be produced by the process of the present invention using human cells. Usually, the human cells used in the present invention include cell lines derived from human hematopoietic cells such as lymphoblasts, lymphocytes, monoblasts, monocytes, myeloblasts, myelocytes, granulocytes and macrophages. Examples of these cell lines are lymphomas and leukemias such as myelocytic leukemia, promyelocytic leukemia, adult T-cell leukemia, and hairy cell leukemia, specifically, HBL-38 cells, HL-60 cells (ATCC CCL240), K-562 (ATCC CCL243), KG-1 cells (ATCC CCL246), Mo cells (ATCC CRL8066), THP-1 cells (ATCC TIB202), and U-937 cells (ATCC CRL1593) as reported by Jun MINOWADA in "*Cancer Review*", Vol.10, pp.1-18 (1988), and A-253 cells (ATCC HTB41),

AUSTRALIAN PATENT OFFICE

an epidermoid carcinoma from the submaxillary gland of a human. Mutants of these cell lines can also be used in the present invention. Because these cell lines readily proliferate and, moreover, produce the protein of the present invention, they can be advantageously used in the present invention. In particular, epidermoid carcinoma cell lines such as A-253 cells, and human myelomonocytic cell lines such as HBL-38 cells, HL-60 cells, KG-1 cells, THP-1 cells, and U-937 cells have an extremely high productivity with respect to the protein of the present invention and are most satisfactorily used in the present invention.

In the process of the present invention, the above human cells are first allowed to propagate, then the protein of the present invention is collected from the propagated cells. The method used to propagate these human cells in the present invention is not specifically restricted, and any conventional *in vivo* or *in vitro* propagation method can be used. The *in vitro* propagation method is a method of propagating cells using nutrient culture medium, which comprises, for instance, suspending human cells in RPMI 1640 medium, MEM medium or DEM medium, which are conventionally used to propagate animal cells in this field, supplemented with 0.3-30 w/v % of fetal bovine serum to give a cell density of about $1x10^4$-$1x10^7$ cells/ml, preferably, about $1x10^5$-$1x10^6$ cells/ml, and culturing these cells at a temperature of 36-38°C, preferably, a temperature of about 37°C and at a pH of 7-8, preferably, a pH of 7.2-7.4, for about 1-7 days while replacing the medium with fresh medium. Thereafter, the propagated cells were separated from the cultures to obtain the protein. Depending on the types and culture conditions of the human cells, some cells excrete the protein of the present invention extracellularly during

AUSTRALIAN PATENT OFFICE

culturing. When together in culture media with inducers such as mitogens and/or IFN-γs which induce the production of the protein of the present invention by human cells, most of, or all of, the protein may be produced extracellularly. In this case, the protein can be collected from the culture supernatants.

The *in vivo* propagation method for human cells using warm-blooded animals, excluding humans, comprises, in order to suppress immunoreaction by the animals, injecting antilymphocyte antibodies derived from rabbits into the animals which are preferably rodents such as new born mice, nude mice, rats, nude rats, guinea pigs, and hamsters, injecting subcutaneously or intraperitoneally about $1x10^5$-$1x10^8$ cells/animal of the human cells into the animals or placing the human cells in diffusion chambers embedded in or out of the animal's body while allowing the animal's body fluid to circulate in the chamber, and feeding the animals by conventional methods for about 2-10 weeks. During the feeding, the human cells propagate while receiving the animal's body fluid. The propagated human cells are collected in the form of a tumor mass, ascites or cell suspension. If necessary, the protein of the invention is collected after suspending and washing these human cells in and with an appropriate solvent. The *in vivo* propagation method has merit in that as compared with the *in vitro* propagation method it yields the protein of the present invention at a lower labor cost and in less time and in a satisfactorily high yield. The *in vivo* propagation method is disclosed, for example, in Japanese Patent Publication No.54,158/81.

To collect the protein of the present invention from the propagated cells, these cells are disrupted by ultrasonication before or after

AUSTRALIAN PATENT OFFICE

separating the protein of the invention from the cultures, homogenizing, freezing and thawing, or by soaking these cells in considerably low osmotic solvents, then the protein is collected from the resulting cell debris or from a mixture of cell debris and culture supernatant. To collect the protein from the cell debris or the mixture, the cell debris or the mixture can be subjected directly or after incubation at about 37°C for 1-24 hours to the following conventional methods for purifying biologically active substances in this field: salting out, dialysis, filtration, concentration, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, adsorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and/or isoelectrophoresis. Two or more of these conventional methods can be selectively used in combination. The collected protein can be concentrated and/or lyophilized into a liquid or solid form to suit its final use.

The monoclonal antibody described in Japanese Patent Application No.58,240/95 applied for by the present applicant (EP 0712 931 published 22 May 1996) is advantageously used to purify the present protein.

Immunoaffinity chromatography using the monoclonal antibody yields the highest possible purity of the protein at the lowest cost and labor.

As described above, the protein according to the present invention has the property of inducing IFN-γ production by immunocompetent cells. Thus it can be satisfactorily used as an inducer for IFN-γ production by cell culture methods and used in the treatment and prevention of IFN-γ susceptive diseases including viral diseases such as AIDS and

AUSTRALIAN PATENT OFFICE

condyloma acuminatum; malignant tumors such as malignant nephroma, granuloma, mycosis fungoides, and brain tumor; and immunopathies such as articular rheumatism and allergosis.

The protein of the present invention is usually added to nutrient culture media for IFN-γ production by culturing immunocompetent cells or administered to humans to treat and/or prevent IFN-γ susceptive diseases. In the former case, leukocytes separated from mammalian peripheral blood and established cell lines of immunocompetent cells such as HBL-38 cells, Mo cells (ATCC CRL8066), Jurkat cells (ATCC CRL8163), HuT78 cells (ATCC TIB161), EL4 cells (ATCC TIB39), L12-R4 cells, and mutants thereof are suspended in culture media containing about 0.1-1,000 ng/ml of the protein of the present invention, and preferably, about 1-100 ng/ml of the protein. If necessary, these cells are cultured in nutrient culture media supplemented with T-cell stimulants such as mitogen, interleukin 2, and anti-CD3 antibody for about 1-100 hours in a conventional manner while replacing the culture media with fresh media. From the resulting cultures, the protein of the present invention can be collected by one or more conventional methods used to purify IFN-γ such as salting out, dialysis, filtration, concentration, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, adsorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and isoelectrophoresis.

Because the protein of the present invention induces IFN-γ production by human immunocompetent cells, agents for susceptive diseases containing the protein as an effective ingredient

AUSTRALIAN PATENT OFFICE

stimulate human immunocompetent cells to produce IFN-γ when administered to humans, and exert positive effects on the treatment and/or the prevention of IFN-γ susceptive diseases. Killer cells participate in the treatment and/or prevention of susceptive diseases when the protein of the present invention induces IFN-γ production by immunocompetent cells, accelerates the cytotoxicity of killer cells such as cytotoxic T-cells and lymphokine activating killer cells including NK- and LAK-cells, and induces the formation of killer cells similarly to the proteins in the later described Experiments and Examples. The term "susceptive diseases" as referred to in the present invention means diseases in general including IFN-γ susceptive diseases, which can be treated and/or prevented by IFN-γs and/or killer cells: For example, viral diseases such as hepatitis, herpes, condyloma acuminatum, and AIDS; infectious diseases such as candidiasis, malaria, cryptococcosis, and *Yersinia;* malignant solid tumors such as malignant tumor, mycosis fungoides, and chronic granulomatous disease; hematopoietic malignant tumors such as adult T-cell leukemia, chronic myelocytic leukemia, and malignant tumor; and immunopathies such as allergosis and rheumatism. When used with interleukin 3, the protein of the present invention positively effects the complete cure or the remission of leukopenia and thrombocytopenia induced by radio- and chemo-therapies used to treat leukemia, myeloma, and malignant tumors.

The present agent for susceptive diseases is widely used in the treatment and/or the prevention of the above susceptive diseases as an antitumor agent, antiviral agent, antiseptic, immunotherapeutic agent, platelet-increasing agent,

AUSTRALIAN PATENT OFFICE

or leukocyte-increasing agent. Depending on the type of agent and the symptoms of the susceptive diseases to be treated, the present agent is generally processed into a liquid, paste or solid form which contains 0.000001-100 w/w %, preferably, 0.0001-0.1 w/w % of the protein, on a dry solid basis (d.s.b.).

The present agent can be used as it is or processed into compositions by mixing with physiologically-acceptable carriers, adjuvants, excipients, diluents and/or stabilizers, and, if necessary, further mixing with one or more other biologically-active substances such as interferon-α, interferon-β, interleukin 2, interleukin 3, interleukin 12, TNF-α, TNF-β, carboquone, cyclophosphamide, aclarubicin, thiotepa, busulfan, ancitabine, cytarabine, 5-fluorouracil, 5-fluoro-1-(tetrahydro-2-furyl)uracil, methotrexate, actinomycin D, chromomycin A_3, daunorubicin, doxorubicin, bleomycin, mitomycin C, vincristine, vinblastine, L-asparaginase, radio gold colloidal, Krestin®, picibanil, lentinan, and Maruyama vaccine. Among these combinations, a combination of the protein of the present invention and interleukin 2 is especially useful because interleukin 2 acts as a cofactor for the protein when the protein induces IFN-γ production by immunocompetent cells. A combination of the protein and a natural or recombinant human interleukin 2 induces a relatively high level of IFN-γ production using only a small amount of the protein which does not substantially induce IFN-γ production by immunocompetent cells, while a combination of the protein and interleukin 12 induces a greater level of IFN-γ production which could not be readily attained by each agent on its own. Because the protein of the present invention increases the activity of

AUSTRALIAN PATENT OFFICE

interleukin 12 in inhibiting the production of immunoglobulin E antibody in the human body, the protein is advantageously used as an agent for immunopathies such as atopic diseases including atopic asthma, atopic bronchial asthma, hay fever, allergic rhinitis, atopic dermatitis, angioedema, and atopic digestive system disorder. Occasionally a relatively small amount of interleukin 12 exists in humans. In this case, administration of the protein alone to humans can attain the desired effect.

The form of the present agent for susceptive diseases includes those in a unit dose form which means a physically formulated medicament suitable for administration and contains the protein in an amount from 1/40 to several folds, i.e. up to 4 folds of a dosage. Examples of these are injections, liquids, powders, granules, tablets, capsules, sublinguals, ophthalmic solutions, nasal drops, and suppositories.

The present agent can be orally or parenterally administered to patients, and as described below it can be used to activate antitumor cells *in vitro*. In both administrations, the agent exerts a satisfactory effect in the treatment and/or prevention of susceptive diseases. Depending on the types of susceptive diseases and the symptoms of patients before and after administration, the agent is orally administered to them or parenterally administered to their intradermal- and subcutaneous-tissues, muscles, or veins at a dose of about 0.1μg to 50 mg per shot, and preferably, about one μg to one mg per shot, 1-4 times/day or 1-5 times/week, for one day to one year.

The present agent can be also used in so called

AUSTRALIAN PATENT OFFICE

"antitumor immunotherapy" using interleukin 2. Generally, the antitumor immunotherapy is roughly classified into (i) a method for directly administering interleukin 2 to patients with malignant tumors, and (ii) a method for introducing antitumor cells which have been previously activated *in vitro* by interleukin 2, i.e. an adoptive immunotherapy. The protein of the present invention significantly enhances the above immunotherapeutic effect of interleukin 2 when used in combination with it. In method (i), the protein is administered to patients in an amount of about 0.1 μg/shot/adult to one mg/shot/adult at 1-10 times before the administration of interleukin 2 or at the same time. The dose of interleukin 2 is generally about 10,000-1,000,000 units/shot/adult, though it varies depending on the types of malignant tumors, patient's symptoms, and the dose of the protein of the present invention. In method (ii), mononuclear cells and lymphocytes, collected from patients with malignant tumors, are cultured in the presence of interleukin 2 and about 0.1 ng to one μg of the protein per $1x10^6$ cells of the blood cells. After culturing for a prescribed period of time, NK cells or LAK cells are collected from the culture and introduced into the same patient. Diseases which can be treated by the present antitumor immunotherapy are, for example, hematopoietic malignant tumors such as leukemia and malignant lymphoma, and solid malignant tumors such as colonic cancer, rectal cancer, large intestinal cancer, gastric cancer, thyroid carcinoma, cancer of the tongue, bladder carcinoma, choriocarcinoma, hepatoma, prostatic cancer, carcinoma uteri, laryngeal, lung cancer, breast cancer, malignant melanoma, Kaposi's sarcoma,

AUSTRALIAN PATENT OFFICE

cerebral tumor, neuroblastoma, tumor of the ovary, testicular tumor, osteosarcoma, cancer of the pancreas, renal cancer, hypernephroma and hemangioendothelioma.

The following experiments explain the protein of the present invention:

Experiment 1

Preparation of protein

New born hamsters immunosuppressed in a conventional manner by injecting a rabbit antiserum to hamster thymus into the hamsters, had about $5x10^5$ cells/hamster of THP-1 cells (ATCC TIB202), a myelomonocytic cell line of a human acute monocytic leukemia transplanted to their dorsal subcutaneous tissues and were fed for 3 weeks in a conventional manner. Tumor masses formed in their subcutaneous tissues, of about 15 g weight per hamster, were extracted, dispersed in a conventional manner in physiological saline, and washed with phosphate buffered saline (hereinafter abbreviated as "PBS").

The propagated cells thus obtained were washed with 10-fold volumes of cold 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, and 0.1 mM disodium ethylenediaminetetraacetate, allowed to stand in 3-fold volumes of a fresh preparation of the same buffer under ice-chilled conditions, frozen at -80°C, and thawed to disrupt the cells. The disrupted cells were centrifuged to obtain a supernatant which was then fed to a column packed with "DEAE-SEPHAROSE", a gel for ion-exchange column chromatography commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been previously equilibrated with 10 mM phosphate buffer (pH 6.6), followed by washing the column with

AUSTRALIAN PATENT OFFICE

10 mM phosphate buffer (pH 6.6). The supernatant was fed to the column with a gradient buffer of sodium chloride which increased stepwise from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.6), and a fraction eluting at about 0.2 M sodium chloride was collected.

The fraction was dialyzed against 10 mM phosphate buffer (pH 6.8) and fed to a column packed with "DEAE 5PW", a gel for ion-exchange chromatography commercialized by Tosoh Corporation, Tokyo, Japan, followed by feeding the column with a gradient buffer of sodium chloride which increased stepwise from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.8), and collecting fractions eluted at about 0.2-0.3 M sodium chloride.

The resulting fractions were pooled, then dialyzed against PBS, fed to a plastic cylindrical column packed with a gel for immunoaffinity chromatography using a monoclonal antibody which had been prepared according to the method disclosed in Japanese Patent Application No.58,240/95 applied for by the present applicant (EP 0712 931 published 22 May 1996), and washed with PBS. The column was fed with 100 mM glycine-HCl buffer (pH 2.5) in order to collect from the eluate, fractions containing a protein which induces IFN-γ production by immunocompetent cells. These fractions were pooled, dialyzed against sterile, distilled water, concentrated with a membrane filter, and lyophilized to obtain a purified, solid protein in a yield of about 50 ng per hamster.

Experiment 2

Molecular weight

In accordance with the method reported by U. K. Laemmli in *Nature*, Vol.227, pp.680-685 (1970), a purified protein prepared by the method of Experiment 1 was

AUSTRALIAN PATENT OFFICE

electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) in the presence of 2 w/v % dithiothreitol, resulting in a main protein band with IFN-γ inducibility at a position corresponding to about 18,000-19,500 daltons. The marker proteins used in this experiment were bovine serum albumin (MW=67,000 daltons), ovalbumin (MW=45,000 daltons), carbonic anhydrase (MW=30,000 daltons), soy bean trypsin inhibitor (MW=20,100 daltons), and α-lactalbumin (MW=14,400 daltons).

Experiment 3

Amino acid sequence and peptide mapping of the N-terminus

Experiment 3-1

Amino acid sequence of the N-terminus

The purified protein of Experiment 1 was analyzed on a "MODEL 473A" protein sequencer commercialized by Perkin-Elmer Corp., Instrument Div., Norwalk, USA, which revealed that it has the amino acid sequence of SEQ ID NO:l, particularly, SEQ ID NO:2 at or near the N-terminus.

Experiment 3-2

Peptide mapping

A purified protein obtained by the method of Experiment 1 was dissolved in an adequate amount of sterile, distilled water, and the solution was fed to a column packed with "ASAHIPAK® C4P-50 4E", a gel for high-performance liquid chromatography (HPLC) commercialized by Showa Denko, K.K., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid solution, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid solution and feeding a linear gradient solution of

AUSTRALIAN PATENT OFFICE

acetonitrile increasing from 0 v/v % to 90 v/v % in a mixture of trifluoroacetic acid and acetonitrile at a flow rate of 60 ml/hour, to the column. Fractions containing a protein which induces IFN-γ production by immunocompetent cells were collected from the eluted fractions, pooled, neutralized with 1 M aqueous Tris solution (pH 11.2), and concentrated in a conventional manner. The protein was added to 50 mM Tris-HCl buffer (pH 8.5), dissolving an adequate amount of clostripain commercialized by Sigma Chemical Company, St. Louis, Missouri, USA, in an amount of about 50 folds of the clostripain by molar ratio while removing acetonitrile, and the resulting mixture was allowed to react at a pH of 8-9 and at 37°C for 12 hours to obtain a reaction mixture containing fragments of the protein.

The reaction mixture was fed to a column packed with "ODS-120T", a gel for HPLC commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid solution, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid solution and feeding a linear gradient solution of acetonitrile increasing from 0 v/v % to 70 v/v % in a mixture of trifluoroacetic acid, acetonitrile and water (where the concentration of trifluoroacetic acid was 0.09 v/v %) at a flow rate of 30 ml/hour to the column while monitoring the absorption level of the peptide, i.e. the concentration of the peptide, at a wave length of 214 nm. FIG.1 is the resulting peptide map.

In FIG.l, peptide fragments eluted at about 59, 62 and 68 min after initiating the elution are respectively named peptide fragments 1, 2 and 3. These peptide fragments were

AUSTRALIAN PATENT OFFICE

collected separately and analyzed for amino acid sequence on a "MODEL 473A" protein sequencer commercialized by Perkin-Elmer Corp., Instrument Div., Norwalk, USA, in a conventional manner. As a result, it was revealed that peptide fragments 1 and 2 have the amino acid sequences of SEQ ID NOs:3 and 7, respectively, while peptide fragment 3 has those of SEQ ID NOs:4 and 5. The comparison of these amino acid sequences with that of SEQ ID NO:6 revealed that peptide fragments 1 to 3 correspond to positions 148-157, 1-13 and 45-58 or 80-96 in the amino acid sequence of SEQ ID NO:6, respectively. These results confirmed that peptide fragments 1 and 2 correspond to the C- and N-terminal fragments of the protein analysed, and peptide fragment 3 corresponds to an internal fragment of the protein.

It is concluded that: the purified protein obtained by the method of Experiment 1 contains the amino acid sequence of SEQ ID NO:6 when totally evaluating these results; as revealed in Experiment 2 the purified protein has a main protein band at a position corresponding to a molecular weight of about 18,000-19,500 daltons on SDS-PAGE; and the purified protein is calculated to have a molecular weight of 18,199 daltons from the amino acid sequence of SEQ ID NO:6.

Experiment 4

Biological activity

Experiment 4-1

IFN-γ production by immunocompetent cells

Blood was sampled from a healthy volunteer using a heparinized syringe and diluted 2-fold with serum free RPMI

AUSTRALIAN

1640 medium (pH 7.4). The diluted blood was overlaid on a Ficoll commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by centrifugation to collect lymphocytes. These lymphocytes were washed with RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal bovine serum and suspended in a fresh preparation of the same medium to give a cell density of $5x10^6$ cells/ml. The cell suspension was distributed to a 96-well microplate in a volume of 0.15 ml/well.

A purified protein obtained by the method of Experiment 1 was diluted with RPMI 1640 (pH 7.4) supplemented with 10 v/v % fetal bovine serum, and the dilution was distributed to the microplate in a volume of 0.05 ml/well. A fresh preparation of the same buffer either with or without 2.5 μg/ml Con A or 50 units/ml of a recombinant human interleukin 2 in a volume of 0.05 ml/well was added to the microplate, and the microplate was incubated at 37°C for 24 hours in a 5 v/v % CO_2 incubator. After completion of the culture, 0.1 ml of culture supernatant was sampled from each well and assayed for IFN-γ activity by conventional enzyme immunosorbent assay (EIA). As a control, a system free of the purified protein was provided and treated in a similar manner to that described above. The results are shown in Table 1 where the IFN-γ content was assayed and expressed in international units (IU) with respect to "Gg23-901-530", an international standard for IFN-γ obtained from the National Institute for Health, Bethesda, MD, USA.

AUSTRALIAN PATENT OFFICE

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Table 1

Protein concentration (ng/ml)	IFN-γ yield (IU/ml)		
	Protein	Protein plus Con A	Protein plus interleukin 2
0	<0.5	<2	<0.5
0.32	<0.5	6±2	2±1
1.6	10±2	70±20	60±20
8	140±10	490±80	570±30
40	180±20	620±10	880±50
200	260±20	800±20	1500±400

Note : In the table, the term "protein" means the present protein.

The results in Table 1 show that lymphocytes as immunocompetent cells produce IFN-γ by action of the protein of the present invention. As is evident from the results, IFN-γ production is increased in the presence of interleukin 2 or Con A as a cofactor.

Experiment 4-2

Increase of cytotoxicity by NK cells

Blood was sampled from a healthy volunteer using a heparinized syringe and diluted 2-fold with PBS. The dilution was overlaid on a Ficoll, and the resultant preparation was centrifuged to obtain a high density layer of lymphocytes. The lymphocytes were suspended in RPMI 1640 medium (pH 7.2) containing 10 μg/ml kanamycin, $5x10^{-5}$ M 2-mercaptoethanol and 10 v/v fetal bovine serum, and the suspension was distributed to a 12-well microplate in a volume of 0.5 ml/well. A purified protein obtained by the method of Experiment 1 was appropriately diluted with a fresh preparation of the same buffer, and the dilution was distributed to the microplate in a volume of 1.5 ml/well, followed by adding 0.5 ml/well of a fresh preparation of the same buffer either with or without 50 units/ml of a recombinant human interleukin 2 to the microplate, incubating the microplate at 37°C for 24 hours in a 5 v/v % CO_2 incubator, and washing the resultant cells with PBS to obtain cultured lymphocytes containing NK cells as an effector cell. $lx10^4$ cells/well aliquots of K-562 cells (ATCC CCL243), derived from human chronic myelocytic leukemia as an NK-cell susceptive target cell, which had been labelled with ^{51}Cr in a conventional manner, were distributed to a 96-well microplate, and mixed with the

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above NK cells in a ratio of 2.5:1, 5:1 or 10:1 (=(effector cells):(target cells)). The microplate was incubated at 37°C for 4 hours in a 5 v/v % CO_2 incubator, followed by counting the radio activity of each supernatant to count the dead target cells. In each system, the percentage (%) of the dead target cells with respect to the target cells used in this experiment was calculated for evaluating cytotoxicity. The results are shown in Table 2.

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Table 2

Protein concentration (pM)	Concentration of interleukin 2 (Unit/ml)	Cytotoxicity		
		Effector cells/Target cells		
		2.5/1	5/1	10/1
0	0	19	36	59
0	10	28	44	61
0.5	0	22	41	63
0.5	10	31	54	69
5	0	28	49	66
5	10	36	58	71
50	0	29	53	67
50	10	42	62	72
500	0	33	56	84
500	10	57	78	96

Note : In the table, the symbol "pM" means 10^{-12} M, and the term "protein" means the present protein.

The results in Table 2 show that the protein according to the present invention has the property of enhancing the cytotoxicity of NK cells. As is evident from the results, cytotoxicity is further enhanced by the simultaneous presence of interleukin 2.

Experiment 4-3

Induction of LAK cell formation

$1x10^4$ cells/well aliquots of Raji cell (ATCC CCL86), a human Burkitt's lymphoma as an NK-cell non-susceptive target cell labelled with ^{51}Cr in a conventional manner were distributed to a 96-well microplate, and mixed with a cell suspension of the target cells and cultured lymphocytes containing LAK cells as an effector cell, prepared in a similar manner to the method of Experiment 4-2 except for culturing 72 hours, in a ratio of 5:1, 10:1 or 20:1 (=(effector cells):(target cells)), followed by incubating the microplate at 37°C for 4 hours in a 5 v/v % CO_2 incubator and counting the radio activity of each supernatant in a conventional manner. Thereafter, the cytotoxicity (%) was calculated in a similar manner to the method used in Experiment 4-2. The results are shown in Table 3.

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AUSTRALIAN PATENT OFFICE

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Table 3

Protein concentration (pM)	Concentration of interleukin 2 (Unit/ml)	Cytotoxicity		
		Effector cells/Target cells		
		5/1	10/1	20/1
0	0	12	23	31
0	10	14	25	35
0.5	0	14	24	34
0.5	10	18	32	42
5	0	16	26	37
5	10	21	36	50
50	0	22	41	49
50	10	26	52	56
500	0	27	44	61
500	10	33	59	72

Note : In the table, the symbol "pM" means 10^{-12}M, and the term "protein" means the present protein.

The results in Table 3 show that the protein of the present invention induces LAK-cell formation. As is evident from these results, this induction is enhanced in the presence of interleukin 2.

Experiment 5

Acute toxicity test

A purified protein obtained by the method of Experiment 1 was injected percutaneously, orally or intraperitoneally into 8-week-old mice in a conventional manner. As a result, the LD_{50} of the protein was about one mg/kg mouse or higher independent of these administration routes. This shows that the present protein is safe to incorporate into medicaments which are administrable to humans.

It is well known that IFN-γ is closely related to the inhibition of bacterial infection and the propagation of malignant tumors, the regulation of human biophylaxis through its immunoregulatory function, and to the inhibition of immuno-globulin E antibody production. As described above, IFN-γ is now commercially available and used as an agent for human susceptive diseases, and the diseases to be treated, dose, administration, and safety have almost been established. *"Cytokines in Cancer Therapy"*, edited by Frances R. Balkwill, translated by Yoshihiko WATANABE, published by Tokyo-Kagaku-Dojin, Tokyo, Japan (1991) discloses that treatments using killer cells such as NK- and LAK-cells are used as an antitumor immunotherapy and applied to human diseases, and reports that most of them exert a satisfactory therapeutic effect. Recently focussed is the relationship between the therapeutic effect and the

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augmentation of killer cell cytotoxicity or the induction of killer cell formation using cytokines. For example, T. Fujioka et al. reported in *"British Journal of Urology"*, Vol.73, No.1, pp.23-31 (1994) that interleukin 2 strongly induced the formation of LAK cells in an antitumor immunotherapy using LAK cells and interleukin 2, and exerted a satisfactory effect on the metastasis of human cancer without substantially inducing serious toxicity and side effects.

Thus it is revealed that IFN-γ and killer cells are closely related to the treatment and the prevention of human diseases for complete cure and remission. Against this background, as shown in the results in Experiments 4 and 5, the fact that the present protein induces IFN-γ production by immunocompetent cells, enhances NK cell cytotoxicity, and induces LAK cell formation indicates that the present agent containing the protein can be administered to humans over a relatively long period of time and exert a satisfactory therapeutic effect on the treatment and the prevention of IFN-γ and/or killer cell related diseases without substantially inducing serious side effects.

The following Examples explain the preferred embodiments of the present invention in more detail. Examples A-1 to A-8 are the preferred embodiments for the preparation of the present protein, and Examples B-1 to B-6 are the preferred embodiments for the present agent for susceptive diseases:

Example A-1

Preparation of protein

New born hamsters were immunosuppressed

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in a conventional manner by injecting a rabbit antiserum to hamster thymus into the hamsters. About $5x10^5$ cells/hamster of THP-1 cells (ATCC TIB202), a myelomonocytic cell line of a human acute leukemia, were transplanted to their dorsal subcutaneous tissues and the hamsters were fed for 3 weeks in a conventional manner. Tumor masses, about 15 g weight each, subcutaneously formed in each hamster were extracted, suspended in physiological saline in a conventional manner, and washed with PBS.

In accordance with the method of Matthew J. Kostura et al. in *"Proceedings of the National Academy of Sciences of the United States of America"*, Vol.86, pp.5,227-5,231 (1989), the suspended cells were washed with 10-fold volumes of cold 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, 0.1 mM disodium ethylenediaminetetraacetate, allowed to stand in 3-fold volumes of a fresh preparation of the same buffer, allowed to stand for 20 min under ice-chilled conditions, lyophilized at -80°C, and thawed to disrupt the cells. The disrupted cells were centrifuged, and the supernatant was fed to a column packed with **"DEAE-SEPHAROSE"**, a gel for ion-exchange chromatography commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by washing the column with 10 mM phosphate buffer (pH 6.6), feeding it with a gradient buffer of sodium chloride increasing stepwise from 0 M to 0.5 M, and collecting fractions eluted at about 0.2 M sodium chloride.

The fractions were pooled, dialyzed against 10 mM phosphate buffer (pH 6.8), fed to a column packed with "DEAE 5PW", a gel for ion-exchange chromatography commercialized by

AUSTRALIAN

Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM phosphate buffer (pH 6.8), fed with a linear gradient buffer of sodium chloride increasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.8), and fractions eluting at about 0.2-0.3 M sodium chloride were collected.

The resulting fractions were pooled and dialyzed against PBS. The dialyzed solution was fed to a cylindrical plastic column prepared by first packing a gel for immunoaffinity chromatography with a monoclonal antibody, which had been prepared according to the method disclosed in Japanese Patent Application No.58,240/95 applied for by the present applicant (EP 0712 931 published 22 May 1996), then washing with PBS. One hundred mM glycine-HCl buffer (pH 2.5) was fed to the column to effect fractionation, followed by collecting fractions containing a protein which induces IFN-γ production by immunocompetent cells from the eluate, dialyzing the fractions against sterile, distilled water, concentrating the dialyzed inner solution with a membrane filter, and lyophilizing the concentrate to obtain a solid purified protein. The yield was about 50 ng per hamster.

Example A-2

Preparation of protein

New born nude mice were injected in their dorsal subcutaneous tissues with about $1x10^6$ cells/nude mouse of KG-1 cells (ATCC CCL246), a myelomonocytic cell line derived from human acute myelomonocytic leukemia, and fed for 4 weeks in a conventional manner. Tumor masses, about 20 g weight each, formed subcutaneously in each nude mouse were extracted and dispersed in physiological saline in a conventional manner. The

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cells were washed and disrupted in a similar fashion to that described in Example A-1, and the resulting mixture was purified to obtain a purified protein which induces IFN-γ production by immunocompetent cells in a yield of about 20 ng per nude mouse.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the methods of Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near or at its N-terminus and a similar molecular weight and biological activity to the protein described in Experiment 1.

Example A-3

Preparation of protein

Cells of HL-60 (ATCC CCL240), a myelomonocytic cell line derived from human acute promyelocytic leukemia, were suspended in RPMI 1640 (pH 7.4) placed in an about 10-ml plastic cylindrical diffusion chamber in which was installed a membrane filter with a diameter of 0.5 μm, then the chamber was intraperitoneally embedded in an aged rat. The rat was fed for 4 weeks in a conventional manner, then the chamber was removed. The propagated cells in the chamber were collected, washed with physiological saline, and disrupted in a similar fashion to that described in Example A-1, followed by purifying the resulting mixture to obtain a purified protein which induces IFN-γ production by immunocompetent cells. The yield was about 20 ng per rat.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the methods described in Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near or at its N-terminus and has a similar

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molecular weight and biological activity to the protein of Experiment 1.

Example A-4

Preparation of protein

Cells of THP-1 (ATCC TIB202), a myelomonocytic cell line derived from human acute monocytic leukemia, were suspended in RPMI 1640 medium (pH 7.2) supplemented with 10 v/v % fetal bovine serum to give a cell density of about $3x10^5$ cells/ml, and cultured at 37°C for 3 weeks in a 10 v/v % $C0_2$ incubator while replacing the medium with fresh medium. The propagated cells were separated from the resulting culture, washed with physiological saline, and disrupted in a similar fashion to that described in Example A-l, followed by purifying the resulting mixture to obtain a purified protein which induces IFN-γ production in a yield of about 10 ng per litre of culture.

A portion of the purified protein was analyzed for amino acid sequence in accordance with the methods described in Experiments 2-4, revealing that the protein has the partial amino acid sequence of SEQ ID NO:1 near or at its N-terminus and has a similar molecular weight and biological activity to the protein described in Experiment 1.

Example A-5

Preparation of protein

New born hamsters were immunosuppressed by injection, in a conventional manner, with a rabbit antithymus serum, injected with about $5x10^5$ cells/head of A-253 cells (ATCC HTB41), an epidermoid carcinoma, of the human submaxillary gland, in their dorsal subcutaneous tissues and fed for 3 weeks in the usual manner. Thereafter,

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tumor masses formed subcutaneously, about 10 g weight in each hamster, were extracted, dispersed in physiological saline, and washed with PBS.

The propagated cells thus obtained were washed with 20 mM Hepes buffer (pH 7.4) containing 10 mM potassium chloride, 1.5 mM magnesium chloride, and 0.1 mM disodium ethylenediaminetetraacetate, suspended in a fresh preparation of the same buffer to give a cell density of about $2x10^7$ cells/ml, disrupted by a homogenizer, and centrifuged to remove cell debris to obtain a supernatant, followed by concentrating the supernatant with an ultrafiltration membrane to obtain a cell extract containing a protein which induces interferon-γ production by immunocompetent cells. The extract was purified in a similar fashion to the method described in Example A-1, concentrated, and lyophilized to obtain a solid purified protein in a yield of about 3 μg per hamster.

The purified protein was sampled and analyzed in accordance with the methods described in Examples 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 near or at its N-terminus and has a similar molecular weight and biological activities to those of the protein described in Experiment 1.

Example A-6

Preparation of protein

A seed culture of A-253 cells was inoculated into RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum and cultured in a conventional manner at 37°C until a monolayer of cells was formed. Thereafter, the cells were detached from the surface of the culture vessel with "TRYPSIN-EDTA",

AUSTRALIAN PATENT OFFICE

a trypsin commercialized by Gibco BRL, NY, USA, and washed with PBS. In accordance with the method in Example A-l, the cells were disrupted, and the disrupted cells were purified and centrifuged to obtain a supernatant which was then incubated at 37° C for 6 hours, purified, concentrated, and lyophilized to obtain a solid purified protein which induces IFN-γ production by immunocompetent cells in a yield of about one μg per 10^7 cells.

The supernatant was sampled and analyzed in accordance with the methods in Experiments 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 near or at its N-terminus and has a similar molecular weight and biological activities to those of the protein described in Experiment 1.

Example A-7

Preparation of protein

A seed culture of A-253 cells was inoculated into RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum and cultured in a conventional manner at 37° C until a monolayer of cells was formed. Thereafter, the culture medium was replaced with serum-free RPMI 1640 medium (pH 7.4) supplemented with 10 IU/ml of a natural IFN-γ derived from KG-1 cells as an IFN-γ inducer, and incubated at 37°C for 48 hours. The culture was centrifuged to obtain a supernatant which was then purified by the method in Example A-l, concentrated, and lyophilized to obtain a solid purified protein which induces IFN-γ production by immunocompetent cells in a yield of about 5 ng per 10^7 cells.

The supernatant was sampled and analyzed in accordance

STRALIAN

with the methods described in Experiments 2-4 revealing that it has the amino acid sequence of SEQ ID NO:1 near or at its N-terminus and has similar molecular weight and biological activities to those of the protein described in Experiment 1.

Example A-8

Preparation of protein

A purified protein obtained by the method in Example A-1 was dissolved in an adequate amount of sterile, distilled water, and the solution was fed to a column packed with "**ASAHIPAK®** C4P-50 4E", a gel for high-performance liquid chromatography commercialized by Showa Denko K.K., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetic acid, followed by washing the column with 0.1 v/v % aqueous trifluoroacetic acid and feeding a linear gradient solution of acetonitrile increasing from 0 v/v % to 90 v/v % in a mixture of trifluoroacetic acid and acetonitrile to the column at a flow rate of 60 ml/hour. Fractions containing a protein which induces IFN-γ production by immunocompetent cells were collected from the eluted fractions, pooled, neutralized with 1 M aqueous Tris solution (pH 11.2), and concentrated in a conventional manner, followed by removing acetonitrile from the resulting concentrate to obtain a concentrated protein with a purity of at least 95% in a yield of about 10% by weight with respect to the proteinaceous material, d.s.b.

In accordance with the method in Experiment 2, the concentrated protein was sampled and analyzed for molecular weight, resulting in a single protein band, which induces

AUSTRALIAN PATENT OFFICE

IFN-γ production, at a position corresponding to a molecular weight of 18,400±1,000 daltons. Another fresh sample was analyzed for amino acid sequence in accordance with the methods in Experiments 3 and 4, revealing that it has the amino acid sequence of SEQ ID NO:3 near or at its C terminus and the sequence of SEQ ID NO:1 near or at its N-terminus. More particularly, it has the sequence of SEQ ID NO:7, and further it has the amino acid sequence of SEQ ID NOs:4 and 5 as internal fragments and exhibits a similar biological activity to the protein of Experiment 1 even when concentrated to a relatively high level.

Example B-1

Liquid

A purified protein obtained by the method in Example A-1 was dissolved in physiological saline containing one w/v % human serum albumin as a stabilizer, followed by sterile filtering the solution to obtain a liquid.

The product has a satisfactory stability and can be used as an injection, collunarium or nebula to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

Example B-2

Dried injection

A purified protein obtained by the method in Example A-2 was dissolved in physiological saline containing one w/v % of a purified gelatin as a stabilizer, and the solution was sterile filtered in a conventional manner. The sterile solution was distributed to vials in one ml aliquots and lyophilized, then the vials were cap sealed.

AUSTRALIAN PATENT OFFICE

The product has a satisfactory stability and can be used to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

Example B-3

Dry injection

A solid pharmaceutical was prepared by a similar method to that described in Example B-2 except for using a purified protein obtained by the method in Example A-5 and "TREHAOSE", a crystalline trehalose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan, as a stabilizer.

The product has a satisfactory stability and can be advantageously used as a dry injection for treating and/or preventing malignant tumors, viral diseases, bacterial infections, and immunopathies.

Example B-4

Ointment

"HI-BIS-WAKO 104" a carboxyvinylpolymer commercialized by Wako Pure Chemicals, Tokyo, Japan, and "TREHAOSE", a crystalline trehalose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan, were dissolved in sterile distilled water in respective amounts of 1.4 w/w % and 2.0 w/w %, and the solution was mixed to homogeneity with a purified protein obtained by the method in Example A-3, then adjusted to pH 7.2 to obtain a paste containing about one mg of purified protein per g of the paste.

The product has a satisfactory spreadability and can be used to

AUSTRALIAN PATENT OFFICE

treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

Example B-5

Tablet

A purified protein obtained by the method in Example A-4 and' "LUMIN (1-1'-1' '-triheptyl-11-chinolyl(4).4.4'-penthamethinchynocyanine-l-l"-dijodide)" as a cell activator were mixed to homogeneity with "FINETOSE®", an anhydrous crystalline α-maltose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan. The mixture was tabletted in a conventional manner to obtain tablets of about 200 mg weight each, containing the purified protein and LUMIN in an amount of one mg each.

The product has a satisfactory swallowability, stability and cell-activating activity and can be used to treat and/or prevent susceptive diseases such as malignant tumors, viral diseases, bacterial infections, and immunopathies.

Example B-6

Agent for adoptive immunotherapy

Human monocytes were separated from the peripheral blood of a patient with malignant lymphoma, suspended in RPMI 1640 medium (pH 7.2), which had been supplemented with 10 v/v % human AB serum and preheated at 37°C, to give a cell density of about $1x10^6$ cells/ml, mixed with about 10 ng/ml of a purified protein obtained by the method in Example A-1 and about 100 units/ml of a recombinant human interleukin 2, and incubated at 37°C for one week, followed by centrifugally collecting LAK cells.

The LAK cells exerted strong cytotoxicity on lymphoma cells when introduced into the patient, and the therapeutic effect was significantly higher than that of conventional adoptive immunotherapy using interleukin 2 alone. Cytotoxic T-cells, obtained by treating a patient's tumor tissue invasive lymphocytes instead of the patient's monocytes, showed a similar effect to the LAK cells when reintroduced into the patient. The agent for adoptive immunotherapy is suitable to apply to solid tumors such as malignant nephroma, malignant melanoma, large intestinal cancer, and lung cancer.

As described above, the present invention was made based on a novel protein which induces IFN-γ production by immunocompetent cells and the discovery of human cells which produce the protein. The protein, for which the amino acid sequence has been partly revealed stably induces IFN-γ production by immunocompetent cells. Therefore, the protein can be used widely as an IFN-γ inducer for IFN-γ production by culturing cells, and a therapeutic and/or prophylactic agent for IFN-γ susceptive diseases such as viral diseases, malignant tumors, and immunopathies which are susceptible to IFN-γ. The present agent for susceptive diseases which contains the protein as an effective ingredient exerts an outstanding effect on the treatment of inveterate diseases such as malignant tumors.

Because the protein has strong IFN-γ production inducibility and has a relatively low toxicity, it generally induces a desired level of IFN-γ production with only a small amount and does not substantially cause serious side effects even when administered to patients at a relatively high dose.

AUSTRALIAN PATENT OFFICE

Therefore, the protein is advantageous in that it quickly induces a desired level of IFN-γ production without strictly controlling the dose. Especially, the present protein of human cell origin is advantageous in that it causes fewer side effects and induces fewer antibodies when administered to humans in the form of a pharmaceutical composition as compared with polypeptides produced artificially by recombinant techniques.

The protein of the present invention having these satisfactory properties can be produced in a desired amount by the process of the present invention using human cells.

Thus the present invention with these significant functions and effects is a significant invention which greatly contributes to this field.

While what are at present considered to be the preferred embodiments of the invention have been described, it will be understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications as fall within the true spirit and scope of the invention.

Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

AUSTRALIAN PATENT OFFICE

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO:1:

(i)SEQUENCE CHARACTERISTICS:

(A) LENGTH:10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:1:

SEQ ID NO:1:

Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser
1 5 10

INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A)LENGTH:50 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:N-terminal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:2:

SEQ ID NO:2:

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Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                   10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser
    50
```

INFORMATION FOR SEQ ID NO:3:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:10 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:C-terminal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:3:

SEQ ID NO:3

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Ser Ile Met Phe Thr Val Gln Asn Glu Asp
1               5                   10
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INFORMATION FOR SEQ ID NO:4:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:14 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:4:

SEQ ID NO:4

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Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg
1               5                   10
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INFORMATION FOR SEQ ID NO:5:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:17 amino acids
(B)TYPE:amino acid

(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:5:

SEQ ID NO:5

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Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr
1               5                  10                  15
Lys
```

INFORMATION FOR SEQ ID NO:6:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:157 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:protein
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:6:

SEQ ID NO:6

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Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Cys Glu Lys Ile Ser Xaa Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
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INFORMATION FOR SEQ ID NO:7:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:13 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE: N-terminal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:7:

SEQ ID NO:7

AUSTRALIAN PATENT OFFICE

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg
1               5                   10
```

INFORMATION FOR SEQ ID NO:8:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:25 amino acids
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:8:

SEQ ID NO:8

```
Ile Ile Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile
1               5                   10                  15
Gln Ser Asp Leu Ile Phe Phe Gln Lys
            20                  25
```

INFORMATION FOR SEQ ID NO:9:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:18
(B)TYPE:amino acid
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:9:

SEQ ID NO:9

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Gln Pro Val Phe Glu Asp Met Thr Asp Ile Asp Gln Ser Ala Ser Glu
1               5                   10                  15
Pro Gln
```

INFORMATION FOR SEQ ID NO:10:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:471 base pairs
(B)TYPE:nucleic acid
(C)strandedness:double
(D)TOPOLOGY:linear
(ii)MOLECULE TYPE:cDNA to mRNA
(vi)ORIGINAL SOURCE:
(A)ORGANISM:mouse
(B)INDIVIDUAL ISOLATE:liver
(ix)FEATURE:
(A)NAME/KEY:mat peptide
(B)LOCATION:1..471
(C)IDENTIFICATION METHOD:S
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:10:

SEQ ID NO:10

```
AAC TTT GGC CGA CTT CAC TGT ACA ACC GCA GTA ATA CGG AAT ATA AAT 48
Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1               5                  10                  15
GAC CAA GTT CTC TTC GTT GAC AAA AGA CAG CCT GTG TTC GAG GAT ATG  96
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
ACT GAT ATT GAT CAA AGT GCC AGT GAA CCC CAG ACC AGA CTG ATA ATA 144
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
TAC ATG TAC AAA GAC AGT GAA GTA AGA GGA CTG GCT GTG ACC CTC TCT 192
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
    50                  55                  60
GTG AAG GAT AGT AAA AYG TCT ACC CTC TCC TGT AAG AAC AAG ATC ATT 240
Val Lys Asp Ser Lys Xaa Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
TCC TTT GAG GAA ATG GAT CCA CCT GAA AAT ATT GAT GAT ATA CAA AGT 288
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
GAT CTC ATA TTC TTT CAG AAA CGT GTT CCA GGA CAC AAC AAG ATG GAG 336
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
TTT GAA TCT TCA CTG TAT GAA GGA CAC TTT CTT GCT TGC CAA AAG GAA 384
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
        115                 120                 125
GAT GAT GCT TTC AAA CTC ATT CTG AAA AAA AAG GAT GAA AAT GGG GAT 432
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
AAA TCT GTA ATG TTC ACT CTC ACT AAC TTA CAT CAA AGT             471
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
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The claims defining the invention are as follows:

1. A purified protein of human cell origin, which has the amino acid sequence of SEQ ID NO: 1 near or at the N-terminus and induces interferon-gamma production by an immunocompetent cell, said protein excluding a protein having the amino acid sequence of SEQ ID No:6 where Xaa is isoleucine or threonine.

2. The protein of claim 1, which has the amino acid sequence of SEQ ID NO:2 at the N-terminus.

3. The protein of claim 1, which has the amino acid sequence of SEQ ID NO:3 near or at the C-terminus.

4. The protein of claim 1, which has the amino acid sequences of SEQ ID Nos:4 and 5 as internal fragments.

5. The protein of claim 1, which has a molecular weight of 14,000-24,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

6. The protein of claim 1, which enhances the cytotoxicity of and induces the formation of killer cells.

7. The protein of claim 1, which is derived from a human hematopoietic cell.

8. The protein of claim 1, wherein one or more amino acids in SEQ ID NO: 1 are replaced with other amino acid(s), or one or more amino acids are added to the amino acid sequence of SEQ ID NO: 1 without altering the function of the protein.

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9. The protein of claim 2, wherein one or more amino acids in SEQ ID NO:2 are replaced with other amino acid(s), or one or more amino acids are added to the amino acid sequence of SEQ ID NO:2 without altering the function of the protein.

10. The protein of claim 3, wherein one or more amino acids in SEQ ID NO:3 are replaced with other amino acid(s) or one or more amino acids are added to the amino acid sequence of SEQ ID NO:3 without altering the function of the protein.

11. A process for producing the protein of claim 1, which comprises propagating a human cell which produces the protein, and collecting the produced protein from the propagated cells.

12. The process of claim 11, wherein said human cell is a human hematopoietic cell.

13. The process of claim 11, which comprises transplanting the human cell to a warm-blooded animal excluding a human, and propagating the cell while allowing it to receive the body fluid of the animal.

14. The process of claim 13, wherein said animal is a rodent.

15. The process of claim 11, wherein the propagated cells are disrupted, then the protein is collected from the resulting mixture.

16. The process of claim 11, wherein the propagated cells are subjected to the action of an inducer.

17. The process of claim 16, wherein the inducer is a member selected from the group consisting of mitogens and IFN-γs.

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18. The process of claim 11, wherein the protein is collected by salting out, dialysis, filtration, concentration, separatory sedimentation, gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography, absorption chromatography, affinity chromatography, chromatofocusing, gel electrophoresis and/or isoelectrophoresis.

19. An agent which comprises the protein of claim 1 as an effective ingredient and a pharmaceutically- acceptable carrier.

20. The agent of claim 19, which additionally comprises interleukin 2.

21. The agent of claim 19, which contains serum albumin and/or gelatin as a stabilizer.

22. The agent of claim 19, when used as an antioncotic agent.

23. The agent of claim 22, when used as an agent for antitumor immunotherapy.

24. The agent of claim 19, when used as an antiviral agent.

25. The agent of claim 19, when used as an antibacterial agent.

26. The agent of claim 19, when used as an agent for immunopathy.

27. The agent of claim 26, which further contains interleukin 12.

28. The agent of claim 27, when used for treating atopic diseases.

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29. The agent of claim 19, which contains 0.000001-100 w/w % of the protein, on a dry solid basis.

30. The protein of any one of claims 1 to 10 substantially as herein described with reference to any one of the Examples.

31. The process of any one of claims 11 to 18 substantially as herein described with reference to any one of the Examples.

32. The agent of any one of claims 19 to 29 substantially as herein described with reference to any one of the Examples.

DATED this 31st day of July, 2000

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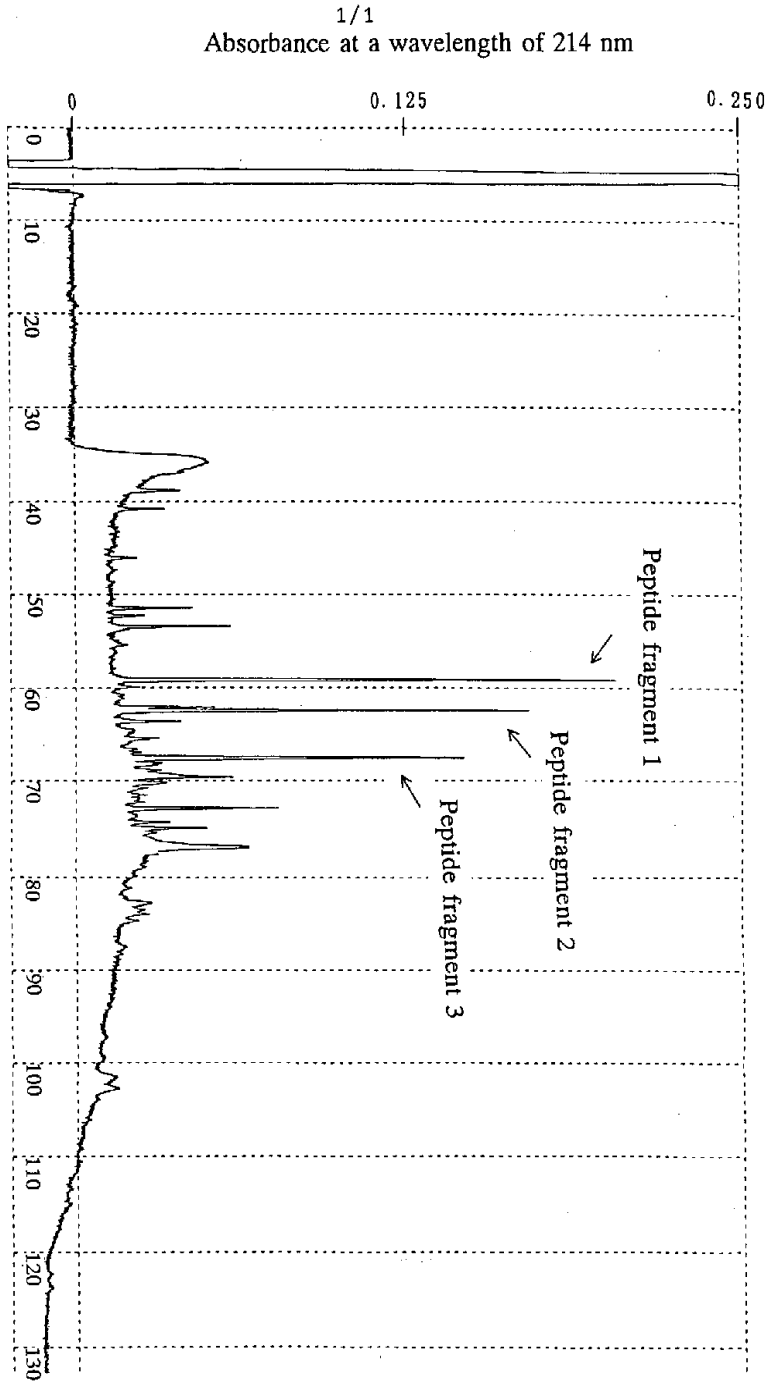
Absorbance at a wavelength of 214 nm
0
0.125
0.250
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Peptide fragment 1
Peptide fragment 2
Peptide fragment 3
Time (min)

FIG.1