



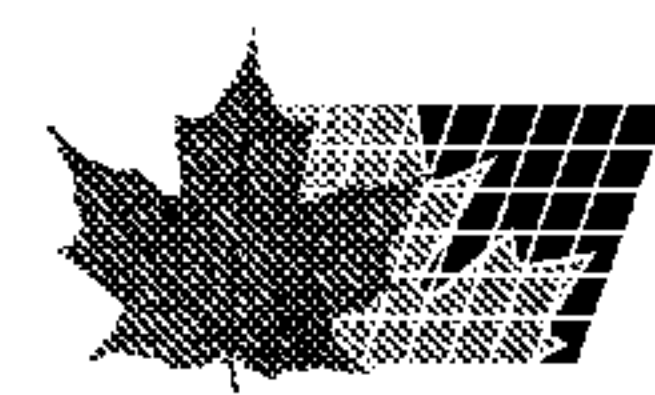
(86) Date de dépôt PCT/PCT Filing Date: 1999/11/26
 (87) Date publication PCT/PCT Publication Date: 2000/06/08
 (45) Date de délivrance/Issue Date: 2009/03/10
 (85) Entrée phase nationale/National Entry: 2001/05/25
 (86) N° demande PCT/PCT Application No.: JP 1999/006615
 (87) N° publication PCT/PCT Publication No.: 2000/033069
 (30) Priorité/Priority: 1998/11/27 (JP10/353926)

(51) Cl.Int./Int.Cl. *G01N 33/15* (2006.01),
G01N 33/50 (2006.01)
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(54) Titre : SUBSTANCE CONTENANT UN EXTRAIT D'HYPES DU CHAMPIGNON SHIITAKE DESTINEE A CRIBLER L'ACTIVITE LAK, ET PROCEDE METTANT EN OEUVRE LADITE SUBSTANCE POUR CRIBLER L'ACTIVITE LAK
 (54) Title: LAK ACTIVITY-SCREENING MATERIALS CONTAINING LENTINUS EXTRACT OF EDODES MYCELIUM AND LAK ACTIVITY-SCREENING METHODS USING THE EXTRACT

(57) **Abrégé/Abstract:**

It is an object of the present invention to provide inexpensive materials and methods for screening immunotherapeutic agents exhibiting antitumor and/or anticancer activity. The present invention provides a method for determining in vitro a material having an LAK activity-enhancing effect suitable for a subject, comprising the steps of: (a) collecting peripheral blood from the subject to prepare lymphocyte fractions, (b) preparing an LAK-induced sample and a control sample by adding or not a screening material of the present invention to said lymphocyte fractions, and (c) measuring the LAK activities of said induced sample and said control sample and comparing the results to determine the in vitro LAK activity-enhancing effect of the screening material for said subject.



ABSTRACT

It is an object of the present invention to provide inexpensive materials and methods for screening immunotherapeutic agents exhibiting antitumor and/or anticancer activity.

The present invention provides a method for determining in vitro a material having an LAK activity-enhancing effect suitable for a subject, comprising the steps of:

(a) collecting peripheral blood from the subject to prepare lymphocyte fractions,

(b) preparing an LAK-induced sample and a control sample by adding or not a screening material of the present invention to said lymphocyte fractions, and

(c) measuring the LAK activities of said induced sample and said control sample and comparing the results to determine the in vitro LAK activity-enhancing effect of the screening material for said subject.

SPECIFICATION

LAK ACTIVITY-SCREENING MATERIALS
CONTAINING LENTINUS EXTRACT OF EDODES MYCELIUM AND
LAK ACTIVITY-SCREENING METHODS USING THE EXTRACT5 FIELD OF THE INVENTION

The present invention relates to the field of tumor immunology. Specifically, the present invention relates to materials and methods for screening immunotherapeutic agents having an antitumor and/or anticancer activity.

10 More specifically, the present invention relates to screening materials and methods for determining in vitro whether or not an LAK (Lymphokine Activated Killer) cell activity-enhancing effect can be obtained in vivo.

15 PRIOR ART

It is well known in the field of tumor immunology, that tumor cells contain tumor antigens. Tumor antigens expressed by tumor cells include tumor-specific antigens (TSA) which are expressed in tumor cells but not in normal

20 cells, and tumor-associated antigens (TAA) which are expressed in normal cells as well as tumor cells, but at very low levels unless they are upregulated by malignant transformation. These tumor antigens are newly expressed when genetic alteration takes place as a result of

25 malignant transformation of normal cells or as a result of variation in expression regulation resulting from such genetic alteration. The most commonly employed therapy for treating cancer in which tumor cells exist which have an

altered antigenic expression is immunotherapy. This kind of therapy may involve either the immunization of a patient with a tumor antigen or the use of a drug which enhances the patient's immune function. It is now accepted that among the various cells functional in the immune system, Nk (natural killer) cells exhibit a particularly potent anti tumor-cell effect. It is also recognized that NK cell activity can be enhanced by employing immunotherapy. NK cells are non-T/non-B cytotoxic lymphocytes present in normal individuals, and they are known to have a MHC antigen-nonrestricted cytotoxicity effect against not only tumor cells, but also virus-infected cells and other cells which do not express or decrease expressing MHC class I molecules. However, there have now been identified tumor cells resistant to even NK cells.

Dr. S. Rosenberg of the National Cancer Institute (NCI) in the US found that incubation of peripheral lymphocytes with interleukin 2 (IL-2) can induce the production of killer cells showing cytotoxicity against a wide range of target cancer cells including autologous cancer cells and that these killer cells can kill even NK cell-resistant cancer cells, (see Japanese Patent Public Disclosure No. 116518/87). These killer cells were named lymphokine activated killer (LAK) cells. LAK cells do not consist of a cytologically homogeneous population and are known to include NK cells and killer T-cells. Recently, adoptive immunotherapy has been attempted wherein peripheral lymphocytes from a subject are activated with

IL-2 in a cell culture system and then LAK cells showing antitumor activity are reinfused into the subject (LAK therapy). It has been reported that remission from terminal cancer has been achieved or tumor growth-inhibited by the use of adoptive immunotherapy involving repeated administration of such LAK cells. However, LAK therapy exerts different effects in different individuals and sometimes has almost no effect. It also involves a number of problems such as the physical stress imposed on the subject associated with the isolation of a great number of leukocytes from the patient, the high cost of performing mass culture of isolated leukocytes, etc, and moreover, LAK therapy involving direct administration of IL-2 causes serious side effects due to the administration of IL-2 at a high concentration.

Specifically, it is known that LAK adoptive immunotherapy using IL-2 causes side effects such as general prostration, chills, fever, hypoalbuminemia, anemia, eosinophilia and that these side effects are more serious than those caused by administration of IL-2 alone. More notably, some important side effects are associated with the cytotoxicity of LAK cells against normal cells. It is also reported that such cytotoxicity of LAK cells against hematopoietic stem cells induces anemia and thrombocytopenia, as well as causing in vitro damage to lymphocytes, macrophages and vascular endothelial cells. Moreover, IL-2 administered via the oral route is poorly absorbed and must therefore mainly be administered via

injection for direct administration at the present time.

Thus, it would be desirable to determine in vitro whether or not LAK activity can be enhanced by direct administration and to avoid the application of LAK therapy which is liable to cause side effects because of the uncertainty of the effects. However, screening in vitro using IL-2 has the disadvantage of being too expensive.

Some bacteria, foods and other naturally occurring substances are known to have anticancer properties. Bacteria and food-type substance are preferential for use as anti-cancer agents due to their generally benign nature and low sideeffect profile. Many attempts have been made to cure cancer by using bacteria, as shown in reports relating to Coley's toxin consisting of a culture filtrate of *Serratia marcescens* and *Streptococcus pyogenes* (1964); treatment of leukemia with BCG (Mathe, G., *Adv. Cancer Res.*, 14, 1, 1971); tumor regression in guinea pigs (Zbar, B., et al., *J. Natl. Cancer Inst.*, 48, 831, 1971); and effectiveness of administration of yeast cell wall polysaccharide against transplanted tumor cells such as sarcoma 180, for example.

Especially, a great amount of research has been conducted into the anticancer effect of polysaccharides derived from yeast such as yeast glucan and yeast mannan, from other bacteria, from lichens and from basidiomycetes. Among them, commercial products currently available on the market as anticancer immunopotentiators include Krestin derived from the cultured mycelia of kawaratake (*Coriolus*

versicolor, Basidiomycetes: Polyporaceae) (booster of immune function of hosts, Kureha Chemical Industry and Sankyo Co.Ltd.), a polysaccharide derived from shiitake (Lentinus edodes) called lentinan and a polysaccharide
5 derived from suehirotake (Schizophyllum Commune).

Lentinus edodes (Shiitake) is a common edible mushroom found in Japan and China, and has been cultivated in Japan for about 300 years. It has been recently elucidated for its pharmacological effects and effective
10 ingredients and reported to have various effects, such as the growth inhibition effects on transplanted tumor cells in the large bowel and liver in rats and mice (Sugano N. et al., Cancer Letter, 27:1, 1985; Suzuki Y. et al., Journal of the Japan Society of Coloproctology, 43:178, 1990);
15 mitogenic effect (Tabata T. et al., Immunopharmacology, 24:57, 1992; Hibino et al., Immunopharmacology, 28:77, 1994), etc.

The present inventors researched the LAK activity-enhancing effect (antitumor and/or anticancer activity) of
20 Lentinus edodes with a view to providing materials and methods for screening the LAK activity-enhancing effect in vivo demonstrated by direct administration of an extract of Lentinus edodes mycelium.

In a conventional method, the LAK activity-enhancing
25 effect was tested by actually administering an LAK activity enhancer to the host or reinfusing into the host the activated lymphocytes which are prepared by isolating a large amount of autologous lymphocytes followed by

activating them in vitro with an LAK activity enhancer. This method involves a number of problems such as the physical stress imposed on the subject, the high cost of the therapy. Therefore, the establishment of an in vitro
5 screening method to confirm whether or not an LAK activity enhancer actually has an effect in vivo may make it possible to reduce physical stress and the high cost.

DISCLOSURE OF THE INVENTION

10 The inventors of the present invention found that an extract of the mycelium, which is a precursor to the edible fruiting body of *Lentinus edodes*, has a far higher immunopotentiating activity, antitumor activity and/or anticancer activity than the fruiting body. We also found
15 that said extract can be used as an alternative to IL-2 to induce a LAK activity in vitro. We accomplished the present invention on the basis of the finding that the antitumor effect in vivo and/or anticancer effect, especially LAK activity-enhancing effect shown by direct in
20 vivo administration of said extract can be screened in vitro.

More specifically, the present inventors found that the in vivo cytotoxicity, which is exerted by the direct administration of an antitumor or anticancer agent,
25 especially an LAK activity enhancer containing *Lentinus edodes* mycelium extract, has a positive correlation with the cytotoxicity which is exerted when lymphocytes prepared from a subject are activated with said LAK activity

enhancer. The present invention provides a method for determining in vitro a material having an LAK activity-enhancing effect suitable for a subject, comprising the steps of:

5 (a) isolating peripheral blood from the subject to prepare lymphocyte fractions,

(b) preparing an LAK-induced sample, which is produced by treating said lymphocyte fractions with a screening material of the present invention, and a control
10 sample, which is produced in the absence of the screening material, and

(c) measuring and comparing the LAK activities of said induced sample and said control sample to determine the in vitro LAK activity-enhancing effect of the screening
15 material for said subject. The present invention also provides a screening material containing the extract of *Lentinus edodes* mycelium which can be used in said in vitro screening method to screen whether or not the in vivo LAK activity can be enhanced. Therefore, the present invention
20 relates to screening materials containing the extract of *Lentinus edodes* mycelium and screening methods using said materials capable of determining in vitro before administration of the LAK activity enhancer whether or not the in vivo LAK activity-enhancing effect can be expected
25 from an LAK activity enhancer. Screening materials and screening methods of the present invention can be applied to humans as well as to domestic animals.

As used herein, "LAK activity" means the anti-tumor

cytotoxic activity of cytotoxic T-lymphocytes, which attack tumors unrecognizable by lymphocytes having NK activity, but which have little influence on autologous normal cells. "LAK activity-enhancing" refers to the effect of enhancing this LAK activity, that is inducing the production of LAK cells from lymphocytes or further enhancing the antitumor activity of existing LAK cells.

Enhancement of LAK activity increases antitumor activity of LAK cells, which leads to an improvement in the function of the cell-mediated immune system. Thus, the present invention can be applied not only to treatments for improving antitumor activity but also to treatments for improving the immune function.

One aspect of the invention relates to a screening material containing an extract of *Lentinus edodes* mycelium for use in the *in vitro* screening method of the invention to determine the *in vitro* LAK activity-enhancing effect of the extract of *Lentinus edodes* mycelium in a subject.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a bar graph corresponding to the data of Table 1 showing the results of screening for LAK activity enhancement with the extract of *Lentinus edodes* mycelium of the present invention.

THE MOST PREFERRED EMBODIMENTS OF THE INVENTION

In one embodiment, there is provided a method for

determining whether an extract of *Lentinus edodes* mycelium has an *in vitro* Lymphokine Activated Killer (LAK) activity-enhancing effect suitable for a subject, comprising the steps of:

- 5 (a) isolating peripheral blood from the subject to prepare lymphocyte fractions,
- (b) preparing an LAK-induced sample, which is produced by treating said lymphocyte fractions with the extract of *Lentinus edodes* mycelium, and a control sample,
- 10 which is produced in the absence of the extract of *Lentinus edodes* mycelium, and
- (c) measuring and comparing the LAK activity of said LAK induced sample and said control sample to determine the *in vitro* LAK activity-enhancing effect of the extract of
- 15 *Lentinus edodes* mycelium for said subject.

The present invention provides screening materials containing an extract of *Lentinus edodes* mycelium and screening methods using said materials capable of *in vitro* determining before administration of said extract whether

20 or not an LAK activity-enhancing effect can be expected by direct *in vivo* administration of an antitumor agent or an anticancer agent, especially an LAK activity-enhancing

formulation containing the extract of *Lentinus edodes* mycelium. As used herein, "screening materials" refers to materials used for testing in vitro the LAC activity-enhancing effect obtained by in vivo administration. "An
5 extract of *Lentinus edodes* mycelium" used as a screening material in the present invention refers to an extract prepared by crushing and decomposing mycelia grown from *Lentinus edodes* cultured on a solid medium or a solid medium itself containing said mycelia in the presence of
10 water and an enzyme.

The extract of *Lentinus edodes* mycelium as used herein is preferably obtained by, but not limited to, the following process. *Lentinus edodes* spawn is inoculated on a solid medium based on bagasse (sugar cane residue) and
15 defatted rice bran to grow mycelia, and then the solid medium containing the grown mycelia is delignified to enable about 30% by weight or less to pass through a 12-mesh sieve. To this delignified solid medium are added water and one or more enzymes consisting of a carbohydrase,
20 protease or combination thereof, and said solid medium is maintained at a temperature of around 30-55°C to crush and grind. The enzymes used in this step include, but not limited to, cellulase, protease or glucosidase. The solid medium crushed and ground in said step is adapted so that
25 at least 70% by weight of bagasse fiber is able to pass through a 12-mesh sieve, and then the solid medium is heated to a temperature of 95°C to ensure inactivation of the enzyme and sterilization. Finally, the resulting

suspension is filtered to give an extract of *Lentinus edodes* mycelium.

The extract of *Lentinus edodes* mycelium may be directly used in screening materials or immunotherapeutic agents of the present invention, but conveniently can be concentrated and freeze-dried into a powder to be stored and used in various forms. The freeze-dried product is a brown powder with hygroscopic characteristics and has a peculiar taste and odor.

The extract of *Lentinus edodes* mycelium of the present invention can be directly added to lymphocyte fractions prepared from peripheral blood. When the extract of *Lentinus edodes* mycelium is directly added to lymphocyte fractions, they are contained in screening materials of the present invention preferably at a concentration of 1 ng/ml - 100 mg/ml, more preferably 1 µg/ml - 100 µg/ml, most preferably 10 µg/ml - 50 µg/ml. The extract of *Lentinus edodes* mycelium of the present invention is preferably sterilized with acetone before it is added to cultured cells or directly added to peripheral blood.

Screening methods using screening materials of the present invention can be performed according to the method of Takagi et al. (Clinical Immunology, 19:245-249, 1987) with exception that a screening material such as the extract of *Lentinus edodes* mycelium of the present invention is used in place of IL-2.

Accordingly, LAK activity enhancement-screening methods of the present invention are methods for

determining in vitro a material having an LAK activity-enhancing effect suitable for a subject, comprising the steps of:

(a) isolating peripheral blood from the subject to
5 prepare lymphocyte fractions,

(b) preparing an LAK-induced sample, which is produced by treating said lymphocyte fractions with a screening material of the present invention, and a control sample, which is produced in the absence of the screening
10 material, and

(c) measuring and comparing the LAK activities of said induced sample and said control sample to determine the in vitro LAK activity-enhancing effect of the screening material for said subject.

15 To induce LAK cells, lymphocytes are isolated from peripheral blood of the subject. Heparin is added to peripheral blood from the subject, and monocytes at the interface are separated by density gradient centrifugation on Ficoll-Conray solution (s.g. = 1.077). The separated
20 monocytes are washed with PBS (pH 7.4, without Ca and Mg) 2-3 times and then suspended in a culture medium (preferably, RPMI 1640 medium (Gibco) containing FBS (inactivated fetal bovine serum) and/or antibiotics, if desired) at a density of 1×10^6 cells /ml. This
25 suspension is transferred to a Petri dish which has been precoated with autoserum (plasma) at 37°C for 15 minutes, and incubated at 37°C for 1 hour. Unattached cells are recovered as lymphocyte fractions.

LAK-induced samples are, for example, prepared by the following procedure. Lymphocyte fractions prepared by the procedure above are suspended in a culture medium at a final concentration of 1×10^5 - 1×10^6 cells/ml and 100
5 μ l of the medium containing suspended cells is added to each well at a density of 1×10^4 to 1×10^5 cells/well. The number of cells per well can be appropriately determined by those skilled in the art on the basis of the activity of effector cells used, and the sensitivity of
10 target cells to effector cells, etc. Said suspending solution contains the extract of *Lentinus edodes* mycelium as a screening material at a final concentration in a range of 1 ng/ml - 100 mg/ml in accordance with the experimental design.

15 Thus, lymphocytes of the subject are cultured in the presence of the extract of *Lentinus edodes* mycelium of the present invention at various concentrations (including zero) to prepare effector cells. As used herein, the term effector cells refer to cells treated under the culture
20 condition for 3 days and include both lymphocytes cocultured with an LAK activity enhancer (lymphocytes treated with an LAK activity enhancer) and lymphocytes cultured in the medium alone without LAK activity enhancer (lymphocytes treated under the extract-free condition).

25 Control samples are prepared by the same procedure as for LAK-induced samples with exception that sterilized recombinant IL-2 (rIL-2; 2000 U/ml) is added in place of the screening material.

LAK activity can be determined by ^{51}Cr release assay, [^3H] uridine assay or the like. In terms of convenience and objectivity, the ^{51}Cr release assay is preferably used in the present invention. The ^{51}Cr release assay is one of
5 methods for determining in vitro the cytotoxicity against target cells of LAK cells induced from lymphocytes treated with an LAK activity enhancer. The ^{51}Cr release assay is a method for determining the cytotoxicity of effector cells against target cells, which comprises the steps of:

10 (i) adding ^{51}Cr -labeled sodium chromate to the target cells to label the target cells,

(ii) reacting the target cells with effector cells (such as killer T cells or LAK cells) stimulated with a screening material or rIL-2 as a control, and

15 (iii) measuring the amount of ^{51}Cr released into the cell culture supernatant from the target cells bursted by the effector cells.

Subcultured cells used as target cells in the ^{51}Cr release assay are preferably Daudi cells or Raji cells.
20 Target cells cultured in a culture flask are recovered, labeled with ^{51}Cr and then divided into each well of a microtiter plate after labeling. It is preferable to use a culture media suitable for the growth of the cells as a culture media for the target cells. The liquid media
25 include, for example, RPMI 1640 appropriately supplemented with serum, antibiotics, etc.

Target cells are labeled by adding 100-150 μCi ^{51}Cr -sodium chromate per 10^6 cultured target cells followed by

stirring thoroughly and incubating at 37°C for 1-2 hours. Cultured cells are washed with PBS three times, and then suspended in RPMI 1640 medium containing 10% FBS at 1 x 10⁶ cells/ml. The labeled cells are washed with the medium
 5 which is used for culture or phosphate buffer (PBS), and adjusted to a final concentration of 1 x 10⁶ cells/ml in the medium containing 10% fetal bovine serum (FBS) or fetal calf serum (FCS) for assay. Target cells at a density of 5 x 10⁴ cells/well are added to each well of a microtiter
 10 plate in an amount of 50 µl.

In the assay for determining cytotoxicity, each well containing said target cells is further supplied with 100 µl of 1N HCl for maximum dissociation, 100 µl of the medium alone for natural dissociation, or effector cells in 100 µl
 15 of the medium at a density of 1 x 10⁵ - 1 x 10⁶ cells/ml stimulated with the extract of *Lentinus edodes* mycelium of the present invention at various concentrations or 2000 U/ml rIL-2 as a control for experimental dissociation. Then, the microtiter plate is centrifuged at 800 rpm for 5
 20 minutes on a plate centrifuge to collect cells at the bottom of the well, and then incubated in a 5% CO₂ incubator at 37°C for 3.5 hours.

Cytotoxicity to target cells in the ⁵¹Cr release assay is calculated by the equation below.

25

$$\text{LAK activity\%} = \frac{\text{Experimental dissociation(cpm)} - \text{Natural dissociation(cpm)}}{\text{Maximum dissociation(cpm)} - \text{Natural dissociation(cpm)}} \times 100$$

In vitro LAK activity-inducing ability of the

screening material can be determined by comparing the LAK activities of the induced sample and control sample calculated by the equation above.

At the step of determining maximum dissociation,
5 natural dissociation and experimental dissociation, target cells are incubated under 5% CO₂ at 37°C. Those skilled in the art may appropriately determine a culture period in accordance with the purpose of the experiment, the number of cells used or other conditions; for example, 3.5 hours
10 in the present invention.

The radioactivity of ⁵¹Cr released in the culture supernatant can be measured using a scintillation counter or the like.

In a preferred embodiment of the present invention,
15 various steps are performed as follows, though it will be appreciated by those skilled in the art that suitable changes and modifications can be made.

The culture supernatant in each well is collected from the incubated plate to measure radioactivity in a
20 scintillation counter.

The extract of *Lentinus edodes* mycelium which exhibited an in vitro LAK-inducing activity in the above screening method was administered at 3600 mg/day for 7 days to induce LAK activity in vivo. Lymphocyte fractions
25 collected by the above lymphocyte recovery method were used to determine LAK activity % under the same conditions as for said LAK-induced samples, showing that the in vivo LAK activity-enhancing effect has a positive correlation with

the in vitro result.

The following examples further illustrate the present invention but should not be taken as limiting the scope of the invention thereto. It will be appreciated by those skilled in the art that various changes and modifications can be made without departing from the spirit of the present invention.

EXAMPLES

10 Example 1: Preparation of an extract of *Lentinus edodes* mycelium

A solid medium consisting of 90 parts by weight of bagasse and 10 parts by weight of rice bran was soaked with an appropriate amount of pure water, and then inoculated with *Lentinus edodes* spawn and allowed to stand in an incubator at controlled temperature and humidity to grow mycelia. After mycelia spread over the solid medium, the bagasse base was delignified to enable 24% by weight or less to pass through a 12-mesh sieve. To 1.0 kg of this delignified medium were added 3.5 L of pure water and 2.0 g of purified cellulase while maintaining the solid medium at 40°C to prepare a medium-containing mixture.

Then, the medium-containing mixture was circulated by a variable speed gear pump, during which the solid medium was crushed and ground at the gears for about 200 minutes so that about 80% by weight of bagasse fiber is able to pass through a 12-mesh sieve. The medium-containing mixture was crushed and ground while the temperature of

said mixture was gradually increased. Then, the medium-
containing mixture was further heated to 90°C to ensure
deactivation of the enzyme and sterilization and allowed to
stand at 90°C for 30 minutes. The resulting medium-
5 containing mixture was filtered through a 60-mesh filter
cloth to give an extract solution of Lentinus edodes
mycelium, which was concentrated and then converted into a
freeze-dried powder.

The extract of Lentinus edodes mycelium as prepared
10 above contained 25.3% (w/w) carbohydrates determined by the
phenol-sulfuric acid method, 19.7% (w/w) proteins
determined by the Lowry method and 2.6% (w/w) polyphenols
determined by the Folin-Denis method using gallic acid as
standard. The extract of Lentinus edodes mycelium further
15 contains 8% crude fat, 22% crude ash and about 20% soluble
nitrogen-free materials other than carbohydrates.

The extract of Lentinus edodes mycelium had a sugar
composition (%) as follows: Xyl 15.2, Ara 8.2, Man 8.4, Gul
39.4, Gal 5.4, GlcN 12.0, GluUA 11.3.

20

Example 2: Determination of LAK activity

Initially, peripheral blood was collected from
subjects A, B and C before administration of the extract of
Lentinus edodes mycelium and after oral administration of
25 1200 mg the extract of Lentinus edodes mycelium three times
daily for one week to each subject. Lymphocyte fractions
isolated from these peripheral bloods by the method below
can be screened for the correlation between the in vivo

lymphocyte-activating ability of the extract of the present invention and the in vitro lymphocyte-activating ability of the extract.

Heparin was first added to the peripheral bloods, and
5 monocytes at the interface were separated by density
gradient centrifugation on Ficoll-Conray solution (s.g. =
1.077), then the separated monocytes were washed with PBS
(pH 7.4, without Ca and Mg) twice and then suspended in
RPMI 1640 medium (Gibco) containing 10% FBS (inactivated
10 fetal bovine serum) at a density of 1×10^6 cells /ml. The
cells isolated by the method above were transferred to a
culture dish which had been precoated with autoserum
(plasma) at 37°C for 15 minutes followed by incubation at
37°C for 1 hour, and then unattached cells were recovered
15 as lymphocyte fractions.

Target cells (Daudi cells) subcultured in RPMI 1640
medium containing 10% FBS were recovered by centrifugation,
and incubated with 100-150 $\mu\text{Ci}/10^6$ cells of ^{51}Cr -sodium
chromate (New England Nuclear) in a 5% CO_2 incubator at
20 37°C for 1 hour. Cultured cells labeled with ^{51}Cr were
washed with PBS three times, and then suspended in RPMI
1640 medium containing 10% FBS at 1×10^6 cells/ml.

A 50 μl aliquot (5×10^4 cells/well) of target cells
labeled as above was added to each well of a microtiter
25 plate, and 100 μl of 1N HCl was further added to each well
of the maximum dissociation group (positive control), 100
 μl of RPMI 1640 medium containing 10% FBS was further added
to each well of the natural dissociation group (negative

control), and effector cells stimulated with 10 µg/ml of the extract of *Lentinus edodes* mycelium of the present invention or 2000 U/ml of rIL-2 as a control were further added to each well of the experimental dissociation group (each 100 µl (1 x 10⁴ cells/well)). The plate was centrifuged at 800 rpm for 5 minutes on a plate centrifuge to collect cells at the bottom of the well, and then incubated in a 5% CO₂ incubator at 37°C for 3.5 hours.

The culture supernatant in each well was collected by SOKEN-PET Σ-96 from the incubated plate, and the radioactivity was measured in a γ-scintillation counter.

LAK activity was calculated by the equation below.

$$\text{LAK activity\%} = \frac{\text{Experimental dissociation(cpm)} - \text{Natural dissociation(cpm)}}{\text{Maximum dissociation(cpm)} - \text{Natural dissociation(cpm)}} \times 100$$

The results are shown in Table 1 and Fig. 1.

Table 1:

Test No.		LAK activity		
		A	B	C
1	Before administration	13%	27%	14%
2	Screening using extract (final concentration: 10 µg/ml)	21%	34%	15%
3	After administration of extract	40%	43%	15%

INDUSTRIAL APPLICABILITY

The extract of *Lentinus edodes* mycelium was orally administered to subjects A and B to exhibit an enhancement

of LAK activity in vivo(see Table 1, Test No. 3). The in vitro screening with the extract of the present invention exhibited an LAK activity-enhancing effect when lymphocytes isolated from peripheral blood of subjects A and B were
5 stimulated with the extract of the present invention and said effect had a positive correlation with the LAK activity-enhancing effect obtained by directly orally administering the extract of the present invention to subjects A and B (see Table 1, Test No. 2). Thus, it was
10 found that the LAK activity-enhancing effect of the extract of Lentinus edodes mycelium of the present invention obtained by oral administration can be predicted by in vitro screening.

The extract of Lentinus edodes mycelium was actually
15 orally administered to subject C to exhibit unenhancement of LAK activity even in vivo (see Table 1, Test No. 3). The in vitro screening with the extract of the present invention showed no LAK activity-enhancing effect even when lymphocytes collected from peripheral blood of subject C
20 were stimulated with the extract of the present invention (see Table 1, Test No. 2). This example also demonstrated that the LAK activity-enhancing effect of the extract of Lentinus edodes mycelium of the present invention obtained by oral administration can be predicted by in vitro
25 screening.

Therefore, it was found that the LAK activity-enhancing effect of LAK activity enhancers of the present invention obtained by oral administration can be exactly

predicted from in vitro screening results. Thus, the in vivo LAK activity-enhancing effect of the direct administration of LAK activity enhancers containing the extract of *Lentinus edodes* mycelium of the present invention can be conveniently determined in vitro before said LAK activity enhancers are actually directly administered. As a result, LAK activity enhancers can be effectively and rapidly administered to subjects promising for LAK activity-enhancing effect and, moreover, useless administration of said LAK activity enhancers to subjects unpromising for LAK activity-enhancing effect can be prevented.

Moreover, physical stress on the subject is greatly lessened in methods of the present invention because the in vivo therapeutic effect of LAK activity enhancers can be screened in vitro without the need to collecting a large number of lymphocytes from the blood of the subject.

CLAIMS

1. A method for determining whether an extract of *Lentinus edodes* mycelium has an *in vitro* Lymphokine Activated Killer (LAK) activity-enhancing effect suitable for a subject, comprising the steps of:

(a) isolating peripheral blood from the subject to prepare lymphocyte fractions,

(b) preparing an LAK-induced sample, which is produced by treating said lymphocyte fractions with the extract of *Lentinus edodes* mycelium, and a control sample, which is produced in the absence of the extract of *Lentinus edodes* mycelium, and

(c) measuring and comparing the LAK activity of said LAK induced sample and said control sample to determine the *in vitro* LAK activity-enhancing effect of the extract of *Lentinus edodes* mycelium for said subject.

2. The method of Claim 1, wherein the extract of *Lentinus edodes* mycelium is prepared by the method comprising the steps of: crushing and delignifying a solid medium containing *Lentinus edodes* mycelia in the presence of water and one or more of additive enzyme(s) selected from cellulase, protease and glucosidase to prepare a suspension, wherein said solid medium is based on bagasse and defatted rice bran; and

raising the temperature of said suspension to ensure inactivation of the enzyme(s).

3. A screening material containing an extract of *Lentinus edodes* mycelium for use in the *in vitro* screening method of claim 1 to determine the *in vitro* LAK activity-enhancing effect of the extract of *Lentinus edodes* mycelium in a subject.

4. The screening material of Claim 3, wherein, the extract of *Lentinus edodes* mycelium is prepared by the method comprising the steps of:

crushing and delignifying a solid medium containing *Lentinus edodes* mycelia in the presence of water and one or more of additive enzyme(s) selected from the group consisting of cellulase, protease and glucosidase to prepare a suspension, wherein, said solid medium is based on bagasse or defatted rice bran; and

raising the temperature of said suspension to ensure inactivation of the enzyme(s).

Fig. 1

