(12)UK Patent Application (19)GB (11) 2 121 053 A

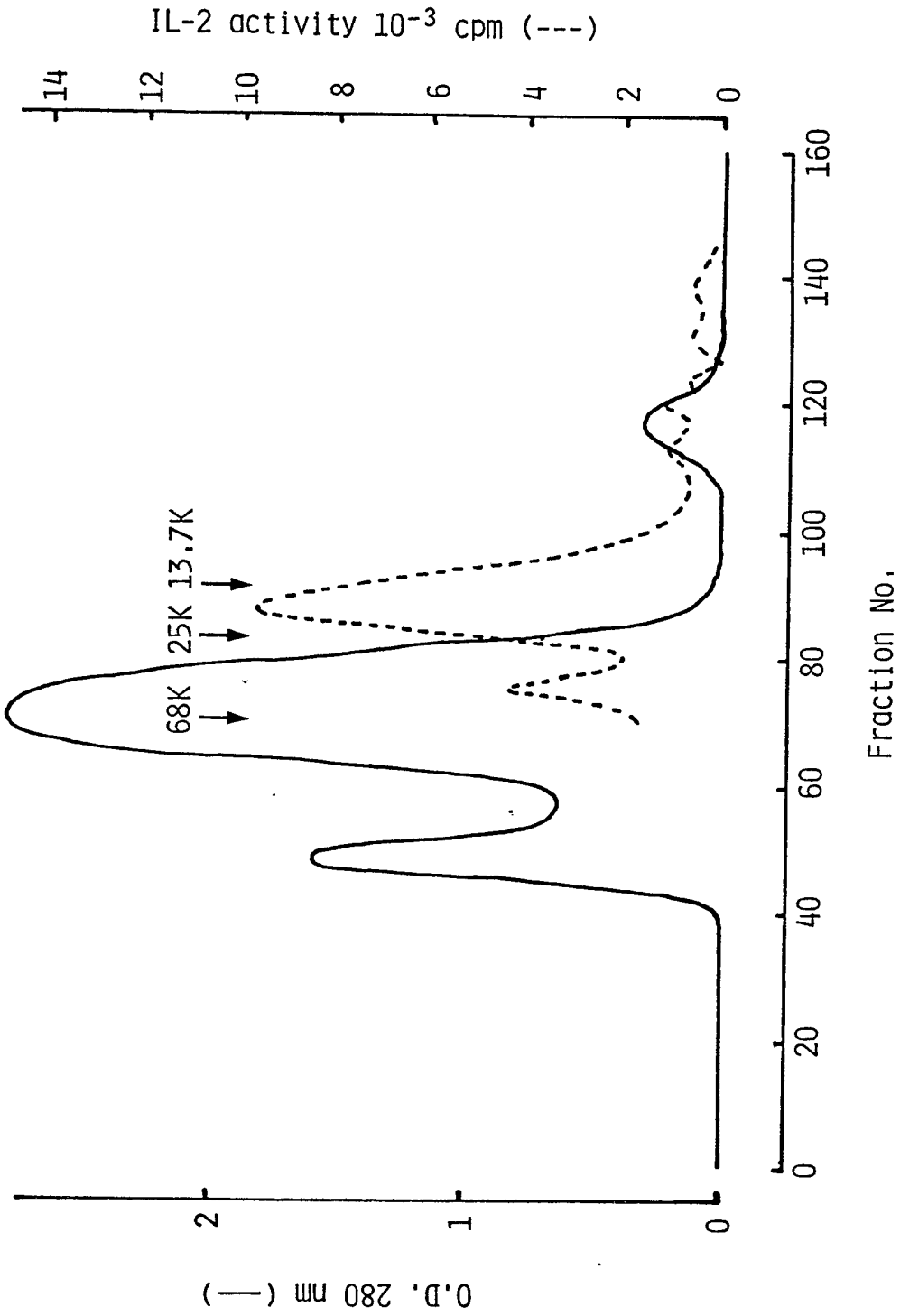
(21) Application No **8312725**
(22) Date of filing
9 May 1983
(30) Priority data
(31) **57/080697**
(32) **12 May 1982**
(33) **Japan (JP)**
(43) Application published
14 Dec 1983
(51) **INT CL³ C07G 7/00**
(52) Domestic classification
C3H FX
C6Y 404
U1S 1314 C3H
(56) Documents cited
None
(58) Field of search
C3H
(71) Applicant
Shionogi Seiyaku KK
(Japan)
12 3-chome
Dosho-machi
Higashi-ku
Osaka
Japan
(72) Inventor
Katsuo Kumagai
(74) Agent and/or Address for Service
Boult Wade and Tennant
27 Furnival Street
London EC4A 1PQ

(54) **Production of interleukin and its analogous immune regulating factors**

(57) Interleukin-2 and macrophage activating factor (MAF) are produced by incubation of human peripheral blood cells in the presence of human lymphoblast cells and an interleukin inducer. Interleukin-2 and MAF are useful in the treatment of infectious diseases caused by viruses or bacteria or in the treatment of malignant tumours.

GB 2 121 053 A

1/1


IL-2 activity 10^{-3} cpm (---)
14
12
10
8
6
4
2
0
68K
25K
13.7K
O.D. 280 nm (——)
2
1
0
0
20
40
60
80
100
120
140
160
Fraction No.

SPECIFICATION

Production of interleukin and its analogous immune regulating factors

The present invention relates to a process for producing interleukin and its analogous immune regulating factors by utilizing human peripheral blood cells.

The term "interleukin" is a generic name for a group of substances which are produced by macrophages and lymphocytes, a substance presently known as "interleukin-1" being produced by macrophages, and "interleukin-2" and "interleukin-3" being produced by lymphocytes. The macrophages and lymphocytes, in addition to the above substances, produce many liquid factors relating to the immune system; in particular, the macrophage activating factor which accelerates differentiation of macrophages and has been generally called "lymphokine", is included in the term "interleukin" in a broad sense. When the living body is infected by a virus or bacteria or is influenced by the growth of cancer, the macrophages and lymphocytes produce interleukin (lymphokine) in the body fluids upon stimulation with those antigens. They are essential components in the living body which are required for the immune response.

In order to produced interleukin from human lymphocytes or macrophages by artificial induction a method exists in which human spleen cells are incubated with irritation under appropriate conditions [Y. Tanaka et al, Microbiol. Immunol., *25*, 1077 (1981)]. According to this method, interleukin from mice or rats can be produced in a yield sufficient to conduct experiments on a test animal level. However, in the case of human beings, it is extremely difficult to obtain spleen cells. Therefore, human peripheral leukocytes have been used in place of spleen cells, but the yield of interleukin is very poor. Thus, for example, interleukin-2 is produced at a rate of from 0.01 to 0.05 unit per 1×10^6 cells of the peripheral blood. This value is extremely low as can be deduced from the possibility that interleukin-2 is produced by the spleen cells of mice or rats at a rate of from 20–30 units per 1×10^6 cells. It has not yet been elucidated why the yield of interleukin is so low.

The present invention is based upon the finding that interleukin-2 and macrophage activating factor are produced by human peripheral blood cells in a very high concentration upon incubation in the presence of human lymphoblast cells and an interleukin inducer. The resulting interleukin-2 and macrophage activating factor are very useful in the treatment of infectious diseases caused by viruses or bacteria in the treatment of various types of malignant tumours. Furthermore, they enhance the preventive or therapeutic effect of other lymphokines, including interferon, when used in combination with them.

Thus, the invention provides a process for producing interleukin and/or analogous immune regulating factors which comprises incubating human peripheral blood cells in the presence of human lymphoblast cells and an interleukin inducer and optionally recovering interleukin-2 and/or macrophage activating factor from the resulting incubation broth.

The human peripheral blood cells may consist of a monocyte component, mainly comprising lymphocytes and macrophages, which is separated from heparin-treated blood by Ficoli-Isopaque density gradient centrifugation, or alternatively, they may be provided by the total cell component (including polymorphonuclear leukocytes) separated by lymphopheresis.

As lymphoblast cells, it is appropriate to use lymphoblast cells of the B cell type, particularly BALL-1 cells, which have been isolated from human acute lymphoblastic leukemia and confirmed to be derived from B lymphocytes and to have surface immunoglobulin [I. Miyoshi et al., Nature, *267*, 843 (1977)]. These cells are adapted to a RPMI-1640 medium containing 10% bovine foetal serum on which the cells grow; for example, in order to secure live cells the cells may be subcultured 2–3 times a week on an RPMI-1640 medium containing 5–10% bovine foetal or calf sera. The cells may be preserved in an ultra-low temperature container at − 80°C, and revived in a state appropriate for incubation when required.

Other B cell types may be used as the human lymphoblast cells, particularly other subline cells derived from BALL-1 cells, or cells of the T cell type may be used. These human lymphoblast cells have been widely known to researchers in this field and are also obtainable by cell formation by incubation.

The interleukin inducers which may be employed in the present invention include plant agglutinins, bacterial toxins and enzymes, all of which are known as interleukin inducers. The plant agglutinins include, for example, concanavalin A (Con A) and phytohemagglutinin (PHA); bacterial toxins include staphylococcal enterotoxin (SEA); and preferred enzymes are neuraminidase (NA) or galactose oxidase. Additionally, substances which are generally used in the induction of γ-interferon exhibit the effect of interleukin inducers.

The process of the present invention may, for example, be carried out by mixing the human peripheral blood cells with the human lymphoblast cells in an appropriate ratio, adding an interleukin inducer thereto, and incubating the mixture under appropriate conditions.

As for the culture medium, it is appropriate to use an RPMI-1640 medium, usually containing from 2–5% calf or bovine foetal sera. It is also possible to employ Dulbecco's MEM medium

and other conventional media for tissue culture.

The incubation is preferably conducted under from 5–8% CO_2 atmosphere at from 35–37°C for from 12–48 hours. These conditions are not, however, essential to the incubation which may also be effected outside the above ranges.

In mixing the human peripheral blood cells with the human lymphoblast cells, the latter (e.g. BALL-1 cells) may used in an amount of from 1×10^5–2.5×10^5 cells/ml to 1×10^6–2×10^6 cells/ml of the former. The mixing ratio, however, is not necessarily of absolute importance. The preferred concentration of the interleukin inducers varies with each case and may be easily determined or is known in the art. Thus, for example, Con A may be used in a concentration of 2–5 — 5 mcg/ml. When the inducer acts or may possibly act as an inhibitor to the culturing of tissue or in the production of interleukin, it may be used for a predetermined relatively short period of time and then removed from the culture medium, and the culturing thereafter continued in the absence of inducer. Thus, for example, when NA and GO are employed, and they may be added to the medium at an amount of about 0.05 unit/ml and about 2 unit/ml, respectively, and the mixture is stirred well and incubated at about 37°C for about 30 minutes; the suspended cells then being collected and washed, another medium added to them and incubation continued further under the same conditions as mentioned above.

The objective interleukin-2 and macrophage activating factor are released into the culture medium. After termination of incubation, the cells may be removed by centrifugation or other standard operations, and the supernatant may then be worked up by means of conventional procedures such as precipitation, dialysis, gelfiltration, liquid chromatography, high performance liquid chromatography or affinity chromatography for purification and concentration purposes, and then the product lyophilized to yield interleukin-2 and macrophage activating factor as high potency products. In practice it is not necessary to separate these products from each other.

The advantage of the present invention is that the productivity or yield of interleukin-2 is markedly increased when the incubation of human peripheral blood cells stimulated by an interleukin inducer is carried out in the presence of human lymphoblast cells. Thus, for example, the yield of interleukin-2 from 1L of culture broth containing 1×10^6 cells/ml of human peripheral blood cells is only about 30 units upon stimulation with Con A. In contrast, the yield of interleukin-2 reaches 20,000–30,000 units or more in the presence of BALL-1 cells. Similarly, the yield of macrophage activating factor is increased by, for example, about 10 times.

The present invention will now be further explained by the following Examples, which are illustrative and not limiting. In the Examples, the identification and assay of the objective interleukin-2 was effected according to a method developed by the present inventor [Kumagi et al., Conference of the Japanese Society of Immunology, *11th*, 277 (1981)]. Other assay methods are also applicable [T. Shaw et al., J. Immunol., *120*, 1967 (1978)].

This invention includes a pharmaceutical formulation comprising interleukin and/or its analogous immune regulating factors made by the present process and formulated for pharmaceutical use. Such formulations may be in unit dosage form and/or may comprise a pharmaceutically acceptable diluent, carrier or excipient; in either case, in accordance with the ordinary knowledge of the skilled man.

Example 1

In 100 ml of an RPMI-1640 medium are suspended peripheral mononuclear cells (about 1×10^8), at a rate of 1×10^6 cells/ml, which are isolated from 100 ml of human peripheral blood containing heparin by means of Ficoll-Isopaque density gradient centrifugation. BALL-1 cells which have previously been cultured and grown are admixed therewith at a rate of 2×10^5 cells/ml. and then Con A is added thereto at a concentration of 25 mcg/ml and mixed well. Then 2% bovine fetal serum is added thereto and 20 ml portions of the resulting mixture are each placed in a 100 ml bottle and incubated at 37°C in an incubator containing 8% CO_2 under stationary conditions for 48 hours, during which time the cells are agitated occasionally in the bottle. The ratio of the cell suspension to the upper gas phase in the bottle is about 2:8 by volume.

After termination of incubation, the cells are removed by centrifugal separation at 1,500 rpm for 5 minutes at room temperature (20°C). The resulting supernatant is filtered through a Millipore filter of 0.45 mμ, and the content of interleukin in the filtrate is determined. As a reference experiment of comparison, the same operation is performed in the absence of BALL-1 cells.

The activity of interleukin-2 was determined as follows. The DNA synthesis of natural killer (NK) clone cells which are derived from NK cells of mouse spleen, the growth of which is dependent upon the presence of interleukin-2, is observed by the degree of incorporation of ^3H-labelled thymidine. The cells of an NK clone strain are suspended in an RPMI-1640 medium containing 5% bovine fetal serum, to which a test sample of each serial dilution is added at 50% concentration, and incubated at 37°C under 8% CO_2 for 24 hours. Then, 0.5 μ Ci of ^3H-

thymidine is added thereto and, after incubation for 24 hours the radio-activity due to ^3H-thymidine incorporated in the cells is measured by a scintillation counter. One unit is represented by the reciprocal of the dilution concentration of the test sample by which 50% of the maximum incorporation is achieved.

The activity of macrophage activating factor (MAF) was determined as follows. THP-1 cells (5×10^5) derived from human monocytes (macrophages) [S. Tsuchiya et al., Int. J. Cancer, *26*, 171 (1980)] are incubated on an RPMI-1640 medium containing 10% calf serum, to which after the lapse of 1 day a test sample of each serial dilution is added at 50% concentration, and further incubated at 37°C for 5 days.

MAF activates the THP-1 cells and gives them a phagocytotic activity, which may be determined as follows. To cells which have been cultured for 5 days is added 1% heat-treated yeast, and the phagocytic rate is determined. One unit is represented by the reciprocal of the dilution concentration of the test sample which exhibits 50% of the maximum phagocytic rate.

The results were as follows.

Control:	
Interleukin-2	3 units/100ml
Macrophage activating factor	400 units/100ml
The present invention:	
Interleukin-2	2560 units/100ml
Macrophage activating factor	3200 units/100ml

The supernatant of the culture broth showing interleukin-2 activity may be purified by Sephacryl S-200 chromatography by which the active fraction is separated from other proteins.

The supernatant of the culture broth is condensed wih Amicon YM-10 to 3 ml, which is then poured into Sephacryl S-200 (20 × 2.4 cm) preliminarily equilibrated with 0.15M-phosphate buffer and developed with the same buffer (20–30 ml/hour) at 4°C. As shown in the accompanying drawing (each fraction, 4 ml), interleukin-2 is separated from two kinds of main accompanying proteins and eluted in predetermined fractions of approximate molecular weight 15,000. The content of interluekin-2 per unit volume is concentrated by up to about 100 times by this purification procedure.

Example 2

In 40 ml of an RPMI-1640 medium are suspended peripheral mononuclear cells (about 1×10^8) at a rate of 1×10^6 cells/ml which are isolated from 100 ml of human peripheral blood by means of Ficoll-Isopaque density gradient centrifugation. BALL-1 cells which have previously been cultured and grown are admixed therewith at a rate of 2×10^5 cells/ml. Then, NA and GO are added at a rate of 0.05 unit/ml and 2 units/ml, respectively, and the mixture is warmed at 37°C for 30 minutes, and centrifuged at 1,500 rpm at room temperature (20°C) for 5 minutes. The separated cells are washed and suspended in an RPMI medium containing 5% calf serum at a rate of 1×10^6 cells/ml. The suspension is divided into 10 ml portions each incubated in an 8% CO_2-incubator at 37°C for 48 hours. After termination of incubation, the cells are removed by centrifugation at 1,500 rpm for 5 minutes at room temperature. The supernatant collected is filtered through a Milipore filter of 0.45 mμ, and the interleukin-2 content is determined.

As a reference experiment for comparison, the same operation is performed in the absence of BALL-1 cells. The results are as follows.

Control:	
Interleukin-2	30 units/100ml
The present invention:	
Interleukin-2	10,640 units/100ml

In the same way as above, human peripheral blood cells (1×10^6 cells/ml) were incubated with Con A (5 μg/ml) and BALL-1 (1×10^6 cells/ml) or with PHA (1%) and BALL-1 (1×10^6 cells/ml) at 37°C under a CO_2 atmosphere for 48 hours. The results were as follows.

	Interleukin-2 (units/100ml)
Con A + BALL 1	3,300
PHA + BALL 1	2,600
NAGO–BALL 1*	10,200

*NAGO treatment

Brief Explanation of the drawing

The accompanying drawing shows a chromatogram of the purification of Example 1. The horizontal axis gives the fraction number, the left perpendicular gives the optical density (OD) measured at 280 nm, and the right perpendicular gives the counts (cpm) of radioactivity per minute incorporated in the NK cells.

CLAIMS

1. A process for producing interleukin and/or its analogous immune regulating factors which comprises incubating human peripheral blood cells in the presence of human lymphoblast cells and an interleukin inducer and optionally recovering interleukin-2 and/or macrophage activating factor from the resulting incubation broth.

2. A process as claimed in claim 1, wherein the human peripheral blood cells are a mononuclear cell component containing human lymphocytes and macrophages.

3. A process as claimed in claim 1 or claim 2, wherein the human lymphoblast cells are B lymphoblast cells.

4. A process as claimed in any one of claims 1 to 3, wherein the interleukin inducer is a plant agglutinin, bacterial toxin or enzyme.

5. A process as claimed in claim 1 or claim 2, wherein the human lymphoblast cells are cells referred to herein in exemplification thereof.

6. A process as claimed in any one of claims 1 to 3 or claim 5, wherein the interleukin inducer is one referred to herein in exemplification of interleukin inducers.

7. A process as claimed in claim 1 and substantially as hereinbefore described.

8. A process as claimed in claim 1 and substantially as hereinbefore described in Example 1 or Example 2.

9. A pharmaceutical formulation comprising interleukin and/or its analogous immune regulating factors produced by a process as claimed in any one of claims 1 to 8 and formulated for pharmaceutical use.

10. A formulation as claimed in claim 9 and also comprising a pharmaceutically acceptable diluent, carrier or excipient.

11. A formulation as claimed in claim 9 or claim 10 and in unit dosage form.

12. A formulation as claimed in any one of claims 9 to 11 and also comprising a different lymphokine and/or interferon.

Printed for Her Majesty's Stationery Office by Burgess & Son (Abingdon) Ltd.—1983.
Published at The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.