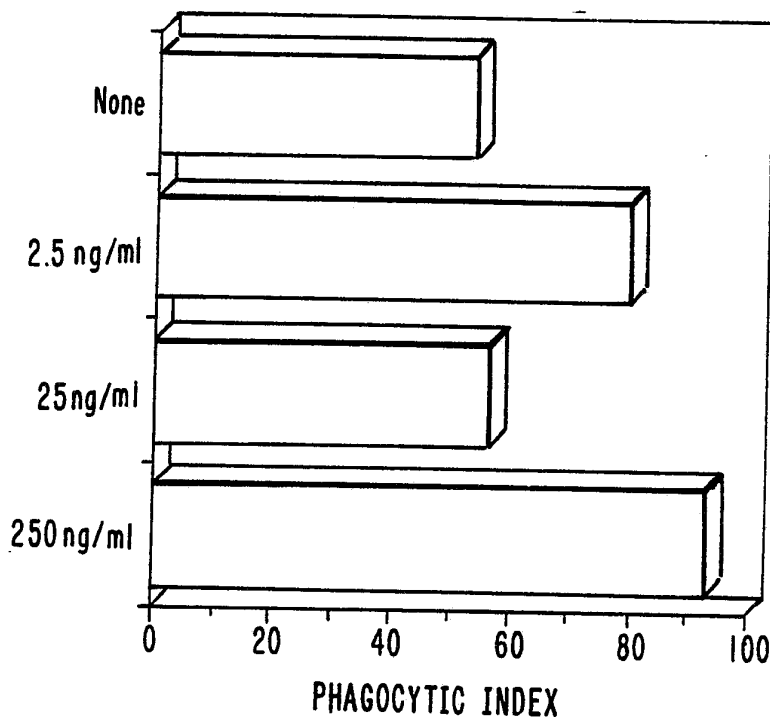




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(54) Title: METHOD FOR INCREASING AND ACTIVATING MONOCYTES AND NEUTROPHILS AND FOR INDUCING MATURATION OF MYELOID CELLS WITH INTERLEUKIN-5



(57) Abstract

Methods for increasing and activating monocytes and neutrophils and for inducing the maturation of myeloid cells in mammals by administering IL-5 are disclosed. A method for making IL-5 is also disclosed.

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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5 METHOD FOR INCREASING AND ACTIVATING MONOCYTES AND
NEUTROPHILS AND FOR INDUCING MATURATION OF MYELOID CELLS
WITH INTERLEUKIN-5

BACKGROUND OF THE INVENTION

10 This invention relates to a method for increasing and activating monocytes and neutrophils and for inducing maturation of myeloid cells in mammals by administering an effective amount of interleukin-5 (IL-5) for this purpose to a mammal in need of such treatment.

Interleukin 5 (IL-5) is a lymphokine (stimulator of the immune system) that has a broad range of immune cell stimulation in the mouse as described by Arai et al., *Annu. Rev. Biochem.*, 59: 783-836 (1990); Sanderson et al., *Immunological Rev.*, 102: 29-50 (1988); Coffman et al., *J. Immunol.*, 139: 3685-3690 (1987); Bond et al., *J. Immunol.*, 139: 3691-3696 (1987); Takatsu et al., *J. Immunol.*, 134: 382-389 (1985); McKenzie et al., *J. Immunol.*, 139: 2661-2668 (1987); Takatsu et al., *Proc. Natl. Acad. Sci. USA*, 84: 4234-4238 (1987); Lopez et al., *J. Exp. Med.*, 167: 219-224 (1988) and Dvorak et al., *Lab. Invest.*, 61(1) 116-132 (1989). For example, in mice IL-5 is disclosed as showing activity as a B-cell growth factor (II), as an enhancer of cytotoxic T-cell activity, as an eosinophil differentiation factor, and as an IgA stimulating factor. During its early development IL-5 has also been referred to as mouse B-cell Growth Factor II (BCGF II) (Swain et al., *J. Exp. Med.*, 158: 822-840 (1983)); mouse T-cell replacing factor (TRF) (Talautsu et al., *J. Immunol.*, 134: 382-389 (1985)); mouse Eosinophil Differentiation Factor (EDF) (Yamaguchi et al., *J. Exp. Med.*, 167: 1737-1793 (1988)); and mouse IgA-Stimulating Factor (Coffman et al., *supra*). The clarification and designation of the name interleukin-5 was finally proposed and adopted in 1987 [McKenzie et al., *supra*].

The human molecule of interleukin 5 has been cloned and found to retain 77% genetic homology with murine IL-5 (Azuma et al., *Nucleic Acid Res.*, 14: 9149-9158 (1986) and Lee et al., *Behring. Inst. Mitt.*,

35

83, 8-14 (1988)). Human IL-5 has been referred to as an EDF (Lopez et al., *J.Exp.Med.*, 167:219-224 (1988)). Human IL-5 has not been shown to have BCGF II activity (Clutterbuck et al., *Eur. J. Immunol.*, 17: 1743-1750 (1987); Sanderson, C., *International Journal of Cell Cloning*, 8: 147-154 (1990);
5 and Sanderson, C., *Develop. biol. Standard.*, 69: 23-29 (1988)). To our knowledge, nothing has been reported to indicate that human IL-5 has IgA stimulating activity or cytotoxic T-cell enhancing activity or that human or mouse IL-5 have any effect on neutrophils or monocytes.

IL-5 was originally thought to be important only for the co-
10 stimulation of activated B-cells, Swain et al., *supra*. It has also been shown to modulate the activity of T cells and eosinophils in mice, Takatsu et al., *supra*, and Sanderson et al., *supra*.

T cells and B cells act in later stages of an immune response. Eosinophils, while acting at earlier stages in an immune response, provide
15 protection mainly against parasitic infections. It would be highly advantageous to have an agent which would increase and activate neutrophils and monocytes which act in the early stages of infection against bacterial and fungal cells, constituting the body's first line of defense against common infection.

20 In the normal process of white blood cell hematopoiesis, cells originate in the bone marrow from a primitive immature cell known as a stem cell and differentiate through progressively more mature stages along different lineage pathways to arrive at a terminal state of differentiation as a mature monocyte, granulocyte, or lymphocyte.

25 A property of immature, undifferentiated cells is the ability to multiply rapidly. It is only when a precursor cell matures and differentiates through these multiple stages that they generally lose their capacity to proliferate and assume the role of a specialized, functional mature cell. In the normal state, cells that reach their final mature form do not proliferate to
30 any great extent, if at all.

In general, cancer is a disorder of cell differentiation. In particular, myeloid leukemias are disorders in which cells of monocytic and granulocytic lineages are blocked at an early stage of maturation and thus have not lost their proliferative capacity. Because these maturation arrested

cells continue to proliferate, they give rise to a population of immature cancer cells resulting in a diagnosis of leukemia.

There is evidence that various myeloid leukemia cells can be induced to differentiate into normal macrophages and granulocytes and that upon differentiation these cells lose their capacity to proliferate. This suggests that the induction of terminal differentiation by an agent would have usefulness as a therapy for myeloid leukemia.

SUMMARY OF THE INVENTION

10

We have now surprisingly found that administration of IL-5 increases the neutrophil and monocyte count in mammals and stimulates differentiation and activation of neutrophils and monocytes. This is very surprising since activity on monocytes and neutrophils has neither been reported previously in human nor in mouse systems. In fact, no effect of IL-5 in any system has been reported on monocytoid or on non-eosinophilic cells of the granulocytic lineage. We have thus discovered that IL-5 may be administered to a mammal to increase and activate neutrophils and monocytes and provide increased host resistance to infection or to treat infection at a very early stage. The mammal may be an immunocompromised host, e.g., any host susceptible to unwanted bacterial infection such as a patient having severe burns or ulcers, a host whose immune defenses are lowered because of radiation or chemotherapy in the treatment of cancer, and a host with a genetic or acquired immunodeficiency. These properties of IL-5 also indicate that it would be useful in topical administration to heal wounds such as open cuts or burns by stimulating neutrophil and monocyte activation and fibroblast proliferation at the wound site.

We have also discovered that IL-5 induces the maturation of myeloid and monocytoid cells. Thus, since myeloid leukemia is associated with the proliferation of immature myeloid cells, IL-5 by progressing myeloid cells to their mature state should reduce their proliferation and provide a method for treating myeloid leukemia.

Preferably, the mammals will be treated with IL-5 derived from a human source, i.e., human IL-5 such as human IL-5 produced

recombinantly from mammalian cells, such as NS-1 or CHO cells. Most preferably, the dosage for the mammals will be administered by subcutaneous intravenous injection or intravenous infusion and will be in an amount of about 0.1 microgram to about 1 milligram of IL-5 per kilogram of body weight per day. Preferably, the IL-5 is administered in an amount of about 2 to about 25 micrograms of IL-5 per kilogram of body weight per day.

The invention also involves a method for preparing recombinant human IL-5 comprising culturing a CHO cell line transfected with a vector comprising an SRalpha driven DNA sequence coding for IL-5, a human beta globin polyA signal sequence downstream of the IL-5 coding sequence, and an SV40 splice junction immediately upstream or downstream of the IL-5 coding sequence, under conditions in which the DNA sequence is expressed; and isolating the IL-5 protein from the culture. Preferably, the expression level of IL-5 is increased by dihydrofolate reductase mediated gene amplification. The IL-5 expressed by the CHO cells is preferably purified using an immunoaffinity column, e.g., an affinity chromatography on a sepharose column coupled with the TRFK 5 monoclonal antibody to murine IL-5. The vector preferably further comprises a consensus vertebrate translation initiator upstream of the 5' end of the IL-5 DNA sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration showing the construction of pSRShIL-5 as described further below.

Figure 2 is a schematic illustration showing the construction of pDSRGhIL-5 as described further below.

Figure 3 is a graphical representation showing the effect on the differentiation of HL-60 cells by human IL-5.

Figure 4 is a graphical representation showing the effect on the activation (phagocytic index) of HL-60 cells by human IL-5.

Figure 5 is a graphical representation showing the effect on the activation (phagocytic index) of U-937 cells by human IL-5.

Figure 6 is a graphical representation showing the stimulation of the hexose monophosphate shunt on U-937 cells by human IL-5.

Figure 7 is a graphical representation showing the activation (phagocytic index) of human peripheral blood neutrophils by human IL-5.

Figure 8 is a graphical representation showing the activation (phagocytic index) of human peripheral blood monocytes by human IL-5.

5

DETAILED DESCRIPTION OF THE INVENTION

Any suitable IL-5 may be employed in the present invention. Complementary DNAs (cDNAs) for IL-5 have recently been cloned and
10 sequenced by a number of laboratories, e.g., Azuma et al., *supra*; Campbell et al., *Proc. Natl. Acad. Sci. USA*, 84: 6629-6633 (1987); Tsujimoto et al., *J. Biochem.*, 106: 23-28 (1989); Lee et al., *supra*, Chou et al., *FASEB Journal*, 4(7): A2068 (1990); Tavernier et al., *DNA*, 8: 491-501 (1989); European Patent Application No. 0 267 779, WO 87/04466, and European Patent
15 Application No. 0 261 625. Moreover, non-recombinant IL-5 purified from various culture supernatants, such as those disclosed in the Takatsu et al., McKenzie et al., Coffman et al., and Yamaguchi et al. articles cited above and in Grace et al., *FASEB Journal*, 4(7): A2169 (1990), may also be employed. The disclosures of all the above articles and patent publications
20 are incorporated herein by reference for their teachings of DNA and amino acid sequences and of methods of obtaining suitable IL-5 materials for use in the present invention.

Preferably, the IL-5 used in the present invention will be a human IL-5, and most preferably it will be the human version with the
25 sequence described in SEQ. NO. 1 below.

According to this invention, mammals, e.g., humans, are administered an effective amount of an IL-5 to increase monocytes and/or granulocytes (which might be any of all of the following: polymorphonuclear cells, eosinophils and/or basophils). Such an effective
30 amount is defined as any amount of IL-5 that will enhance the phagocytic capacity of neutrophils or monocytes by at least 25 percent, preferably by at least 50%. From about 0.1 micrograms to about 1 milligrams of IL-5, preferably human IL-5 (hIL-5), per kilogram of body weight per day is preferably administered. More preferably, mammals are administered

about 2 to about 25 micrograms of hIL-5 per kilogram of body weight per day.

The amount, frequency and period of administration will vary depending upon factors such as the level of the neutrophil and monocyte count (e.g., the severity of the monocytopenia or granulocytopenia) and activity, age of the patient, nutrition, etc. Usually, the administration will be daily initially and it may continue periodically during the patient's lifetime. Dosage amount and frequency may be determined during initial screenings of neutrophil count and the magnitude of the effect of IL-5 upon the increase in neutrophil count. Dosage will be preferably aimed to increase the neutrophil and monocyte counts to an acceptable level, e.g., at least a 25% enhancement, to generate the desired biological effect, which will need to be determined individually for each patient depending on clinical circumstance. Additionally, selective manipulations may be performed, such as the enhancement of one or more subpopulations of lymphocytes.

To complement the neutrophil and monocyte increasing effect of the IL-5, it may be useful to administer it in conjunction with other biologically and/or pharmaceutically active compounds. For example, it can be combined with other white cell increasing agents [especially human colony stimulating factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and/or granulocyte-colony stimulating factor (G-CSF)]. It also might be useful to combine IL-5 with other interleukins, e.g. IL-1 and/or IL-3 and/or IL-7, for the purposes of increasing the total white blood cell and neutrophil count; IL-2 in combination with IL-5 may produce selective enhancement of specific and useful T-cell functions, and IL-5 in combination with IL-4 and/or IL-6 may be useful in specifically enhancing the function and/or numbers of normal and/or neoplastic B-cells. IL-5 may also be useful in increasing the utility of chemotherapeutic agents, including, but not necessarily limited to, alkylating agents, mitotic spindle poisons or antitumor antibiotics. By enhancing the number of white cells or the functional or differentiatonal status of specific subpopulations of cells, the efficacy of the aforementioned chemotherapeutic agents may be enhanced. The combination of an interferon, e.g. interferon-gamma or alpha (especially human interferons such as interferon alpha2B), with IL-5 may also be useful in increasing numbers and functions of certain white

cell, particularly T-cell subsets. In certain situations, to achieve the necessary biological effect, antibodies to any of the aforementioned interleukins or interferons may need to be administered instead of the native molecules.

5 Administration of the dose can be intravenous, nasal, parenteral, oral, subcutaneous, intramuscular, topical, transdermal or any other acceptable method. The IL-5 could be administered in any number of conventional dosage forms. Parenteral preparations include sterile solutions or suspensions. Inhalation administration can be in the form of a
10 nasal or oral spray, or by insufflation. Topical dosage forms can be creams, ointments, lotions, transdermal devices (e.g., of the conventional reservoir or matrix patch type) and the like.

The formulations and pharmaceutical compositions contemplated by the above dosage forms can be prepared with
15 conventional pharmaceutically acceptable excipients and additives, using conventional techniques.

At present, the IL-5 is preferably administered via the intravenous route. The solutions to be administered may be reconstituted lyophilized powders and they may additionally contain preservatives,
20 buffers, dispersants, etc.

Preferably, IL-5 is reconstituted with 10 millimolar citrate buffer, e.g., with pH > 7.2, and preservative-free sterile water with the maximum concentration not to exceed 100 micrograms per milliliter and administered by continuous intravenous infusion or by intravenous
25 injection. For continuous infusion, the daily dose can be added to 5 ml of normal saline and the solution infused by mechanical pump or by gravity.

The activation of neutrophils and monocytes by IL-5 can be determined by the following test protocols.

METHODS

A. Isolation of cells: Whole blood is drawn from a human donor. The blood is defibrinated using glass beads. Red blood cells are removed by two sequential gravity sedimentation steps over 4% dextran-phosphate buffered saline. White blood cells are washed once and layered over a 61% -76% discontinuous Percoll-phosphate buffered saline gradient. Cells are centrifuged at 1250 x g. The top layer banding cells are mononuclear cells; the bottom layer banding cells are neutrophilic cells. The top layer banding cells are used for the monocyte assays and the bottom layer banding cells for the neutrophil assays.

B. Established Cell Lines:

U-937 is a cell line (ATCC CRL 1593) established from malignant cells from a patient with diffuse histiocytic leukemia (Sunstrom, C. and Nilsson K. *Int. J. Cancer*, 17:565-577 (1976)). This cell line exhibits characteristics that indicate it is an immature monocyte.

HL-60 is a cell line (ATCC CCL 240) established from a patient with acute pro myelocytic leukemia. These cells exhibit characteristics that indicate it is an immature neutrophil.

C. Yeast Phagocytosis Assay:

HL-60 cells were routinely cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum prior to assay. For assay, cells were suspended in culture medium alone or containing between 0.1 ng/ml and 250 ng/ml IL-5 at 1×10^5 cells/ml and incubated at 37°C for 3 days (Figure 4).

U-937 cells were routinely cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum prior to assay. For assay, cells were suspended in culture medium alone or containing between 2.5 ng/ml and 500 ng/ml IL-5 at 1×10^5 cells/ml and incubated at 37°C for 3 days (Figure 5).

Isolated monocytes from one donor (Figure 8) and neutrophils from three donors (indicated as PP, LD, and DP in Figure 7) were incubated in Iscove's media containing 10% heat-inactivated fetal bovine serum alone or containing between 2.5 ng/ml and 250 ng/ml IL-5 at 1×10^6 cells/ml and incubated for 4 hours at 37°C

Following treatment, HL-60, U-937, monocyte, and neutrophil cells were used for performing phagocytic assay as follows. The phagocytic assay was performed by adding 300 ml of human serum, 50 ml of heat-killed yeast particles (10^8 organisms/ml) to 12 x 75 mm polypropylene tubes containing approximately 5×10^5 of the isolated, treated cells to be assayed (human peripheral blood, monocytes, neutrophils, HL-60 cell line or U-937 cell line). Incubation was carried out for 90 minutes in a gyratory water bath at 37°C . Following centrifugation, the cell suspension was stained with 0.4% trypan blue and 0.2% eosin Y in saline. Ingested yeast remains colorless and uningested yeast stains purple allowing for accurate determination of avidity (number of yeast ingested) as well as percent phagocytic cells. Mean values obtained for these parameters were multiplied to calculate a phagocytic index. Data were analyzed by the Student's T test comparing values from IL-5 treated cells to that of control cells receiving vehicle alone. The results are shown in Figure 4 for HL-60 cells, Figure 5 for U-937 cells, Figure 7 for human neutrophils, and Figure 8 for human monocytes.

D. NBT Assay: A brief description of the procedure is as follows:

U-937 cells were cultured and pretreated with media containing no IL-5 or IL-5 in concentrations as described above in the Yeast Phagocytosis Assay. Three-day treated cells were then submitted for NBT analysis described next.

The U-937 cells were resuspended in 0.2 ml RPMI-1640 containing 2% heat-inactivated fetal bovine serum and distributed into 12 x 75 mm polypropylene test tubes. To each tube, 0.1 ml of a 2 mg/ml solution of nitroblue tetrazolium (NBT) in 2% RPMI and 0.1 ml of a 0.25 mM stock of freshly prepared phorbol 12-myristate 13-acetate (PMA) in 2% RPMI were added to the cells. The cell suspension was incubated in a 37°C water bath for 30 minutes. After incubation, cells were centrifuged onto glass slides, e.g., using a Cytospin® slide centrifuge. Slides were air dried and fixed with 10% methanol. Cells on the slides were counter-stained using standard staining methods, e.g., using methylene blue and eosin Y by the Dif-quick® method which is a staining kit marketed by Fisher Scientific. Stained slides are counted by open field oil-emersion microscopy. Cells

stained positive for NBT reduction are identified by the presence of black granules of formazan in the cytoplasm of the cells. Data are expressed as the percent positive staining cells out of the total field. The results are shown in Figure 6.

5 The detailed protocol is as follows:

NBT (NITROBLUE TETRAZOLIUM) REDUCTION TO FORMAZAN

10 Measurement of Hexose Monophosphate Shunt Activation
Associated with Respiratory Burst.

Muller, et al., *Agents & Actions*, 11:384 (1981).

Baehner, et al., *NEJM*, 278:971 (1968).

Salin and McCord, *J. Clin. Invest.*, 54:1005 (1974).

15

Standardized for use with U-937 and HL-60 cell lines

1. Harvest target cells sufficient for 1×10^6 cells/well assay. Spin down on a centrifuge at 1200 rpm 5 min. Wash with phosphate buffered saline (PBS) and respin.
2. Bring up cells in sufficient RPMI 1640 + 2% fetal bovine serum (FBS) (2%) to aliquot 0.2 ml/well assay.
- 25 3. Aliquot cells at 0.2 ml/well in 24-well flat-bottom plate.
4. Add 0.1 ml of 20 μ M PMA in 2% (to each well).
5. Add 0.1 ml of 2 mg/ml NBT in 2% (to each well).
- 30 6. Incubate plate at 37°C for 30 min.
7. Immediately remove 0.1 ml of cells and spin down at 200 rpm (low acceleration) for 5 min. using Shandon Cytospin.

35

8. Counterstain with Dif-Quick system and mount coverslip for counting.

5 The following is a description of the methods for specific staining for differentiated neutrophils, monocytes and eosinophils. For all stains the HL-60 cell line was employed.

HL-60 cells were routinely cultured as described above in the Yeast Phagocytosis Assay. HL-60 cells were incubated for 3 days in culture media containing either no IL-5 or 250 ng/ml IL-5. After three days, the cells
10 were spun down on microscope slides to be stained for eosinophil granules (Luxol Blue), monocyte esterase (alpha-naphthyl acetate esterase) or PMN esterase (AS-D chloroacetate esterase) as described below. Figure 3 shows the results of the differential stains for media (-) or for 250 ng/ml IL-5 (+) containing media and subsequent staining for monocyte esterase
15 (mono), PMN esterase (PMN) or eosinophil granules (EOS).

NAPHTHOL AS-D CHLOROACETATE ESTERASE STAIN FOR
POLYMORPHONUCLEAR CELLS (PMNS)

- 20 Yam, et al., *Am. J. Clin. Path.*, 55:283 (1971).
Li, et al., *J. Histochem. Cytochem.*, 21:1 (1973).

Standardized for staining HL-60 cell line:

- 25 1. Spin down $1-5 \times 10^5$ cells onto microscope slide at 200 rpm (low acceleration) 5 min. using Shandon Cytospin.
2. Fix slide for 2 min. in Citrate-Acetone-CH₃OH fixative at room temperature.
- 30 3. Wash in deionized water and air-dry 20 min.
4. Stain slides in AS-D stain for 30 min. at 37°C in dark.
- 35 5. Wash three times in deionized water.

6. Counterstain in acid-hematoxylin stain for 5 min.

7. Wash three times in deionized water, air-dry, mount with coverslip.

5

Citrate-Acetone-CH₃OH Fixative:

Dilute Citrate concentrate 1:9 with deionized water.

Add 18 ml Citrate solution, 27 ml Acetone, and 5 ml absolute CH₃OH.

Store at room temperature.

10 Prepare daily.

AS-D Stain:

Dilute Trizmal 6.3 1:9 with deionized water.

Warm 50 ml dilute Trizmal 6.3 to 37°C and add with constant

15 stirring contents of 1 capsule of the dye Fast Corinth V salt. When salt is completely dissolved add 2 ml of Naphthol AS-D Chloroacetate solution. The solution will appear quite turbid. Continue stirring 15-30 min. and add to coplin jar. Do not filter.

20 AS-D Solution:

Dissolve 1 capsule of Naphthol AS-D Chloroacetate (20 mg) in 2 ml of dimethyl formamide. Prepare immediately before use.

25 All of the above reagents are provided in the Sigma kit 90 - Naphthol AS-D Chloroacetate.

ALPHA NAPHTHYL ACETATE ESTERASE STAIN FOR MACROPHAGES

Yam, et al., *Am. J. Clin. Path.*, 55:283 (1971).

30

Li, et al., *J. Histochem. Cytochem.*, 21:1 (1973).

Standardized for staining HL-60 cell line.

35 1. Spin down $1-5 \times 10^5$ cells onto microscope slide at 200 rpm (low acceleration) 5 min. using Shandon Cytospin.

2. Fix slide in Citrate-Acetone-CH₃OH Fixative 30 min. at room temperature.
- 5 3. Wash in distilled/deionized water and air-dry 20 min.
4. Stain slides in alpha naphthyl acetate esterase (NE) stain at 37°C for 30 min. in dark.
- 10 5. Wash three times in distilled/deionized water.
6. Counterstain in Mayer's Hematoxylin 5 min. at room temperature.
7. Wash in distilled/deionized water, air-dry, mount with coverslip.

15

Citrate-Acetone-CH₃OH Fixative:

Dilute Citrate concentrate 1:9 with distilled/deionized water.

Add 18 ml Citrate solution, 27 ml Acetone, and 5 ml absolute CH₃OH.

Store at room temperature.

20 Prepare daily.

NE Stain:

Dilute Trizmal 7.6 1:9 in distilled/deionized water.

- 25 Warm dilute Trizmal 7.6 to 37°C and add with constant stirring contents of 1 capsule the dye Fast Blue RR salt. When salt is completely dissolved add 2 ml of NE solution. The solution will be yellow and slightly turbid. Continue stirring for 15-20 min. and add to coplin jar. Do not filter.

30 **NE Solution:**

Dissolve 1 capsule (20 mg) of alpha naphthyl acetate to 2 ml of ethylene glycol monomethyl ether. Prepare immediately before use.

All reagents described above are provided by the Sigma kit 90

35 - alpha naphthyl acetate.

LUXOL BLUE STAIN FOR EOSINOPHILS

Johnson & Metcalf., *Exp. Haemat.*, 8(5): 549-561 (1980)

5

1. Make dry smears of HL-60 cells

2. Fix cells in B-5 Formalin & Lugol's and allow to dry for 20 minutes.

10

3. Stain slides in Luxol Blue Solution at room temperature for 2 hours.

15

4. Wash slides in deionized water and air-dry for 20 minutes.

5. Counter-stain with Harris Acid Hematoxylin for 30 seconds.

6. Wash slides in deionized water and air-dry for 20 minutes.

Count positive stained cells using open field oil -emersion

20 microscopy.

Luxol blue solution:

0.1g Luxol Blue (Kodak Solvent Blue 38)

1 L of 70% Ethanol with 500 g Urea

25

B-5 Formaline (make fresh before use):

1 ml 37% Formalin

9 ml B-5 solution

30

B-5 Solution (may be stored):

15 g Sodium Acetate

60g Mercuric Chloride

900 ml deionized water

Lugol's Solution:

1 g Iodine
2 g Potassium Iodide
100 ml deionized water

5

Procedure for B-5 Formalin & Lugol's fixation:

Incubate slide for 5 minutes in B-5 formalin.

Rinse slide in deionized water.

10 Incubate slide for 3 minutes in Lugol's solution and transfer directly into freshly made 5% sodium thiosulfate for 3 minutes.

Rinse in deionized water and air dry.

The results from the above procedures using HL-60 cells are set forth in Figure 3.

15 Figure 3 summarizes the effect of human IL-5 on the maturation of HL-60 cells. The addition of as little as 2.5 ng/ml will induce premyelocytic HL-60 cells to differentiate to mature neutrophils and monocytes as well as eosinophils. Mature monocytes are measured by specific staining for alpha-naphthyl esterase; mature neutrophils are specifically measured by AS-D chloroacetate esterase; mature eosinophils
20 are specifically measured by luxol fast blue.

Figure 4 demonstrates that human IL-5 activates premyelocytic and premonocytic cell lines as mature phagocytes. HL-60 cells can be induced to undergo Fc-mediated phagocytosis of heat-killed yeast cells by pre-incubation of the cells with human IL-5 between 1 and
25 100 ng/ml. That such phagocytosis is not only due to eosinophils is demonstrated in Figure 5 by the fact that premonocytic, not differentiable to eosinophils, U-937 cells can also be induced to undergo Fc-mediated phagocytosis with at least 25 ng/ml human IL-5. Figure 6 demonstrates that IL-5 also induces U-937 cells to become activated as measured by NBT
30 reduction. NBT reduction to NB Foramazon is indicative of upregulated hexose monophosphate shunt activity to provide energy and reducing equivalents for catabolism.

Figures 7 and 8 demonstrate that human purified neutrophils and monocytes are also activated by in vitro dosing with human IL-5. Such
35 activation is donor dependent, with a broad range of activation.

Construction of the Human IL-5 Expression plasmid pDSRGhIL-5

The construction of plasmids pCDSRalphahIL-5, pDSRG, and
 5 pSRS have been described (Yokota et al., *Proc. Natl. Acad. Sci. USA*, 84,
 7388-7392, 1987, and ATCC designation numbers 68233 and 68234). A
 human IL-5 cDNA variant was constructed by the polymerase chain
 reaction (PCR) using pCDSRalphahIL-5 as the template. Oligonucleotides
 AB790 and AB791 as shown below and in SEQ. NOS. 2 and 3,
 10 respectively, were used as the 5'-end and the 3'-end primers, respectively:

AB790:

Sal I

5'ACGCAGAAC**GTTCGACGCCGCCACCATG**AGGATGCTTCTGCATTGAGT 3'

15

Translational initiator

AB791:

EcoR I

20 3'TGGCTCACCTATTATCTTTCA**ACTTA**AGATTGACCA 5'

Stop

codon

This resultant cDNA from the PCR contains essentially the identical coding
 25 region as the parental cDNA and modifications in the non-coding regions
 as follows to facilitate expression plasmid construction and high level gene
 expression in eucaryotic cells. At the 5'-end of the cDNA, a restriction site
 Sal I (shown in bold letters) was added in AB790 and the translational
 initiator (underlined) was changed (as shown above) to conform to the
 30 consensus context of vertebrate mRNAs (Kozak, *Nucleic Acids Res.*, 15,
 8125-8148, 1987) except that in position +4 (SEQ. NO. 2, one nucleotide
 downstream from the ATG initiation codon (shown in bold letters in AB790
 above)) the nucleotide A was retained to preserve the authentic coding
 sequence for the leader peptide. Also, at the 3'-end of the cDNA, the
 35 untranslated region (UTR) of the parental cDNA was eliminated and a EcoR

I restriction site (as shown in bold letters in AB791 above) was added at the stop codon during the PCR (see SEQ. NO. 3). The PCR generated hIL-5 cDNA was purified by agarose gel electrophoresis and then restricted with Sal I and EcoR I to produce overhang ends. This Sal I/EcoR I fragment, 5 421 bp in length, was ligated to Sal I/EcoR I restricted pSRS (ATCC 68234) to produce pSRShIL-5 as shown in Figure 1.

The expression vector pDSRG (ATCC 68233) was used to construct plasmid pDSRGhIL-5 as shown in Figure 2 by replacing the Hind III/EcoR I fragment of pDSRG (containing the SRalpha promoter) with the 10 small Hind III/EcoR I fragment of the pSRShIL-5 (containing the SRalpha promoter and the hIL-5 cDNA) as shown in Figure 2. The final human IL-5 expression plasmid, pDSRGhIL-5, contains the following elements, counterclockwise from the Xba I site:

1. Ampicillin resistance gene and origin of replication from pBR322.
- 15 2. MMTV LTR driven dhfr expression unit from pMTVdhfr (Lee et al. *Nature*, 294, 228-232, 1981).
3. SRalpha promoter.
4. SV40 derived splice junction.
5. Human IL-5 cDNA.
- 20 6. Human beta-globin derived polyadenylation signal.

The coding sequence of hIL-5 present in the plasmid is the same as in pCDSRalphIL-5 given in Yokota et al., *Proc. Natl. Acad. Sci. USA*, 84, 7388-7392, 1987.

25

DHFR Gene Amplification and Selection of CHO-1 line

Chinese hamster ovary cell mutants deficient in dihydrofolate reductase activity (CHO-dhfr) are widely used for overproduction of various 30 recombinant proteins. (Kaufman et al., *Molecular and Cellular Biology*, 5, 1750-1759, 1985). CHO-dhfr mutants have an auxotrophic requirement for hypoxanthine, thymidine and proline. Expression vectors incorporating a dhfr marker may be used to complement this mutation: selection is achieved by growing cells in the absence of the required media cofactors 35 described above. Gene amplification (increase in copy number up to 1,000

X) may be accomplished by growing cells in increasing concentration of the folate analog methotrexate (MTX). The amplification of the integrated recombinant dhfr locus in the genome results in a concomitant increase in copy number of the expression unit for the gene of interest (Ringhold et al.,
5 *J. Mol. Applied Genetics*, 1, 165-175, 1981; and Kaufman et al., *EMBO J.*, 6, 187-193, 1987).

The plasmid (pDSRGhIL-5) having the coding sequence for dhfr and human IL-5 was constructed as described as above. Transfection of pDSRGhIL-5 into the CHO-dhfr-cell line was carried out by the calcium
10 phosphate precipitation method described by Chen and Okayama, *BioTechniques*, 6, 632-637, 1988. Transformants were selected in a selection medium (DMEM, Dulbecco's Modified Eagle's Medium) that lacks hypoxanthine and thymidine. The survival clones were pooled and treated with stepwisely increased concentration of methotrexate in the following
15 orders: 60 nM, 300 nM, and finally 1 μ M. A clone designated CHO-1 was chosen for the large scale propagation.

Culture Propagation

20 In order to prepare a Master Cell Bank (MCB), three original culture flasks containing the CHO-1 cells were used. The cells were propagated in the complete growth medium (DMEM high glucose, supplemented with non-essential amino acids, glutamine, and 5% fetal bovine serum). To prepare the frozen stocks of CHO-1 cells, cells were
25 collected by trypsinization, washed, resuspended in freezing medium, and aseptically dispensed in 1 ml aliquots into 1.5 ml sterile cell storage vials (freezing medium is basal medium plus 20% fetal bovine serum and 8% dimethylsulfoxide). The vials were slowly frozen at -70°C and stored in liquid nitrogen. The cells were thawed and propagated in T flasks for 5-10
30 generations in the growth medium and the frozen procedures were repeated to constitute the Master Cell Bank (MCB).

hIL-5 production was carried out in roller bottles with 850 cm² in surface area. To start production, one frozen vial from the MCB was thawed and inoculated into a T-75 flask. The culture was expanded in T-
35 flasks and finally inoculated into roller bottles. After the cell density reaches

approximately 90% confluency, the cells are washed with the serum free medium (complete medium without the addition of serum) and subjected to two batch media changes for production of hIL-5. No methotrexate was used throughout the production process. The conditioned media was
5 collected and filtered to remove any cells that may be present. The filtrate, which contained crude hIL-5, was forwarded to the purification.

Purification of hIL-5 from the crude CHO hIL-5 media was carried out by performing an affinity chromatography on a sepharose column (Affi-gel 10, BioRad) coupled with the TRFK 5 monoclonal antibody
10 (ATCC No. HB9897) to murine IL-5 (Coffman et al., *Science*, 245, 308-310 (1989) and Schumacher et al., *J. Immunology*, 141: 1576-1581 (1988)). After the crude material was loaded onto the column, the column was washed sequentially once with phosphate buffered saline (ph 7.4) (PBS), PBS plus 0.5 M NaCl, and once with PBS, to remove non-specifically
15 bound proteins. hIL-5 was then eluted with 0.2 M glycine, pH 2.95 and immediately neutralized with 1M Tris-HCl, pH 8. The purity of the hIL-5 protein was examined first by SDS-polyacrylamide gel electrophoresis and then NH₂-terminal sequencing. A uniform polypeptide sequence was consistently observed, indicating greater than 99% purity of the hIL-5
20 protein.

While the present invention has been described in connection with certain specific embodiments, it will be evident to one of ordinary skill in the art that many alternatives, modifications and variations may be made. All such alternatives, modifications and variations are intended to be
25 included within the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Bober, Loretta A.
Chou, Chuan-Chu
Grace, Michael J.

(ii) TITLE OF INVENTION: Method For Increasing And
Activating Monocytes And Neutrophils And For Inducing
Maturation Of Myeloid Cells With Interleukin-5

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Macintosh 6.0.5

(D) SOFTWARE: Microsoft Word 4.00B

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

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(viii) ATTORNEY/AGENT INFORMATION:

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(C) TELEX: 219165

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 115 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Ile	Pro	Thr	Glu	Ile	Pro	Thr	Ser	Ala	Leu	Val	Lys	Glu	Thr	Leu	5	10	15
Ala	Leu	Leu	Ser	Thr	His	Arg	Thr	Leu	Leu	Ile	Ala	Asn	Glu	Thr	20	25	30
Leu	Arg	Ile	Pro	Val	Pro	Val	His	Lys	Asn	His	Gln	Leu	Cys	Thr	35	40	45
Glu	Glu	Ile	Phe	Gln	Gly	Ile	Gly	Thr	Leu	Glu	Ser	Gln	Thr	Val	50	55	60
Gln	Gly	Gly	Thr	Val	Glu	Arg	Leu	Phe	Lys	Asn	Leu	Ser	Leu	Ile	65	70	75
Lys	Lys	Tyr	Ile	Asp	Gly	Gln	Lys	Lys	Lys	Cys	Gly	Glu	Glu	Arg	80	85	90
Arg	Arg	Val	Asn	Gln	Phe	Leu	Asp	Tyr	Leu	Gln	Glu	Phe	Leu	Gly	95	100	105
Val	Met	Asn	Thr	Glu	Trp	Ile	Ile	Glu	Ser						110	115	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACGCAGAACG TCGACGCCGC CACCATGAGG ATGCTTCTGC ATTTGAGT 48

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACCAGTTTAG AATTCAACTT TCTATTATCC ACTCGGT 37

CLAIMS

WHAT IS CLAIMED IS:

- 5 1. The use of IL-5 for increasing and activating neutrophils and monocytes in a mammal.
2. The use of IL-5 for treating neutrophilic and/or monocytic leukemia in a mammal.
3. The use of IL-5 for inducing maturation of immature
10 neutrophil and/or monocyte cells in a mammal.
4. The use of IL-5 for the manufacture of a medicament for increasing and activating neutrophils and monocytes in a mammal.
5. The use of IL-5 for the manufacture of a medicament for treating neutrophilic and/or monocytic leukemia in a mammal.
- 15 6. The use of IL-5 for the manufacture of a medicament for inducing maturation of immature neutrophil and/or monocyte cells in a mammal.
7. A method of treatment for increasing and activating neutrophils and monocytes in a mammal comprising administering to said
20 mammal an effective amount of IL-5 for such purpose.
8. The method of treatment of claim 7 in which the IL-5 is administered in combination with at least one other white blood cell increasing agent.
9. The method of treatment of claim 7 in which the IL-5 is
25 administered in combination with at least one chemotherapeutic agent.
10. A method of treating neutrophilic and/or monocytic leukemia in a mammal comprising administering to said mammal an effective amount of IL-5 for such purpose.
11. A method of inducing maturation of immature neutrophil
30 and/or monocyte cells in a mammal comprising administering to said mammal an effective amount of IL-5 for such purpose.