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IMPROVED PROCESS FOR PREPARING ACTIVATED KILLER CELLS
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- (57) This invention relates to a process for preparing activated killer cells, particularly lymphokine activated killer (LAK) cells.

CLAIM

1. In a process wherein peripheral blood mononuclear cells or lymphocytes from tumor-tissue are cultured to produce a population of cells which are cytotoxic for natural killer cell resistant tumor cells, the improvement comprising culturing at a cell concentration of at least about 1.0×10^6 cells/mL a population of cells selected from the group consisting of peripheral blood mononuclear cells, peripheral blood lymphocytes resulting therefrom, and lymphocytes obtained from tumor tissue in a closed container made from a copolymeric film material having an oxygen permeability of at least about $1.8 \times 10^5 \mu\text{m}^3 (\text{STP})/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$; and a thickness of from about 0.04 mm to about 0.23 mm; said material being selected from the group consisting of

(a) copolymers of ethylene and an α -olefin of 4-10 carbon atoms having a density of from about 0.915-0.925 g/cm³;

(b) copolymers of ethylene and methacrylic

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acid;

(c) ionomers; and

(d) laminates or coextrudates of an
ionomer/polyester elastomer and a linear low density
polyethylene elastomer.



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Form 10

PATENTS ACT 1952-1973

COMPLETE SPECIFICATION

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Complete Specification for the invention entitled: IMPROVED PROCESS FOR PREPARING ACTIVATED
KILLER CELLS

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

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width,
on tough white paper of good quality and it is to be inserted inside this form.

TITLE**IMPROVED PROCESS FOR PREPARING
ACTIVATED KILLER CELLS**5 BACKGROUND OF THE INVENTIONField of the Invention

This invention relates to a process for preparing activated killer cells, particularly lymphokine activated killer (LAK) cells.

10 Description of Related Art

Rayner, et al., Cancer 55, 1327-1333 (1985), disclose that lymphokine-activated killer (LAK) cells can be generated by incubating fresh peripheral blood lymphocytes in interleukin-2. The authors disclose that LAK cells kill fresh autologous and allogeneic human tumor cells in vitro. The LAK cells can be generated from peripheral blood lymphocytes of normal individuals and tumor-bearing patients. They report that pure, recombinant interleukin-2 (rIL-2) generates LAK cells capable of killing a wide variety of tumors including sarcomas and cancers of the colon, pancreas, adrenal gland, and esophagus. They report that the use of LAK cells, possibly with the systemic administration of rIL-2, represents a promising future approach to the immunotherapy of human cancer. The reference discloses activation of the peripheral blood lymphocytes in 25-cm² flasks at a cell concentration of 10⁶ cells/mL for 5 days in 5% CO₂ at 37° C.

30 Rosenberg, et al., The New England Journal of Medicine 313, 1485-1492 (1985), disclose preliminary results of the systemic administration of autologous lymphokine-activated killer cells and rIL-2 to patients with advanced cancer. They treated 25 patients with metastatic cancer in whom standard therapy had failed. Objective regression of cancer

(more than 50 percent of volume) was observed in 11 of the 25 patients. Complete tumor regression occurred in one patient with metastatic melanoma and had been sustained for up to 10 months after therapy. The reference discloses activation or culturing of the cells in 2.5-liter roller bottles at a cell concentration of 1.5×10^6 cells/mL for 3-4 days at 37° C. Patients were infused with up to about 2×10^{11} LAK cells.

Rosenberg, et al., Science 233, 1318-1321 (1986), disclose that adoptive transfer of tumor-infiltrating lymphocytes (TIL) expanded in interleukin-2 to mice bearing micrometastases from various types of tumors showed that TIL are 50 to 100 times more effective in their therapeutic potency than LAK cells. The TIL were prepared by harvesting tumors aseptically and mincing them into 1-2 mm pieces which were then stirred in 40 mL of a specified saline solution to obtain a cell suspension which was filtered, washed and suspended in complete medium containing rIL-2 until about a 100-fold expansion of the original cells was obtained.

U.S. Patent 3,928,294, issued on December 23, 1975 to Crawford, et al., discloses a permselective, biocompatible membrane article having high gaseous permeability, said membrane consisting of a poly(alpha-olefin-sulfone) derived from the series C_8-C_{18} alpha-olefins and sulfur dioxide, the membrane having an oxygen transmission rate of about 1 to 4×10^4 cc mil/in² yr atm and a CO₂ transmission rate of about 4 to 17×10^4 cc mil/in² yr atm. Articles which can be fabricated from the membrane material include blood storage bags.

Belgian Patent 862,772 discloses a medical device wherein at least the part which comes into contact with a bodily fluid and/or a liquid

medication is made from a copolymer which is crosslinked 20 to 75% by irradiation and has a specified formula. The copolymer is prepared by polymerization of an ester having an acrylic radical and an ethylene compound, both of a specified formula. Included among the devices disclosed is a blood bottle. The patent is directed to materials having low permeability to gases.

U.S. Patents 4,496,361 and 4,588,401, issued to Kilksen on January 29, 1985 and May 13, 1986, respectively, disclose a platelet storage container made of a copolymeric film material capable of being heat sealed at a low temperature and having

an oxygen permeability of from about $1.8 \times 10^5 \mu\text{m}^3 (\text{STP})/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$ [100 cc (STP)/(24 hr-atm-100 in²)],

a carbon dioxide permeability of from about 4.4×10^5 to about $8.0 \times 10^5 \mu\text{m}^3 (\text{STP})/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$ [250 to about 450 cc (STP)/(24 hr-atm-100 in²)],

a tensile strength of at least about 8 mPa, a seal strength of at least about 1000 g/cm,

a stiffness low enough so that the container can be extended by liquid to a capacity of about 275 mL,

a durability sufficient to provide a dart drop value of at least about 100, and

a thickness of from about 0.08 mm to about 0.23 mm.

Blood is stored at 4° C and platelets are stored at about 22° C.

U.K. Patent Application Publication 2,065,067 discloses a bag for use in freezing physiological solutions, food or chemicals comprising a laminated sheet heat sealed to form a bag, the sheet comprising an inner layer of unstretched film

of a random copolymer of an α -olefin with ethylene containing from 1 to 20 mol % of α -olefin of over 1×10^4 average molecular weight, having a density less than 0.936 and having 4 to 18 carbon atoms, and an outer layer of a heat-resistant polymerized film whose glass transition temperature is above room temperature. The α -olefin can be, amongst others, 1-butene, 4-methyl-1-pentene, or 1-octene, and the outer layer can be materials such as ethylene-tetrafluoroethylene copolymer.

In the effort to further investigate and refine LAK cells, TIL and rIL-2 as an adoptive immunotherapies for cancer, improvements which allow activation at a higher concentration of cells or otherwise increased attractiveness of the therapies are most desirable. For example, culturing at a concentration of about 1.5×10^6 cells/mL when about 1.8×10^{10} to 1.8×10^{11} cells are needed would require total culture volumes of from about 12 to 120 L. The benefits of a technique which would permit the use of significantly lower volumes are apparent.

SUMMARY OF THE INVENTION

The present invention provides an improved process for preparing lymphokine activated killer (LAK) cells or TIL. In a process wherein peripheral blood mononuclear cells or lymphocytes from tumor-tissue are cultured to produce a population of cells which are cytotoxic for natural killer cell resistant tumor cells, the improvement comprises culturing a population of cells selected from the group consisting of peripheral blood mononuclear cells, peripheral blood lymphocytes resulting therefrom, and lymphocytes obtained from tumor tissue in a closed container made from a copolymeric film material having an oxygen permeability of at least about $1.8 \times 10^5 \mu\text{m}^3 (\text{STP})/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$; and a thickness

of from about 0.04 mm to about 0.23 mm; said material being selected from the group consisting of

(a) copolymers of ethylene and an α -olefin of 4-10 carbon atoms having a density of from about 0.915-0.925 g/cm³;

(b) copolymers of ethylene and methacrylic acid;

(c) ionomers; and

(d) laminates or coextrudates of an ionomer/polyester elastomer and a linear low density polyethylene elastomer.

DETAILED DESCRIPTION OF THE INVENTION

As used herein:

"Interleukin-2" (IL-2) means human interleukin-2 which is a glycoprotein with a molecular weight of approximately 15,000 daltons and consists of a 133 amino acid polypeptide containing a disulfide bridge. The term, as used herein, includes natural and recombinant interleukin-2 (rIL-2) and biologically functional equivalents thereof.

"Biologically functional equivalents" mean polypeptides having the same or very similar biological activity, such as rIL-2 muteins described in U.S. Patent 4,518,584 and rIL-2 proteins having a methionine replacing the NH₂-terminal alanine found in native IL-2. The disclosure of U.S. Patent 4,518,584 in regard to rIL-2 muteins is incorporated herein by reference. These muteins are rIL-2 molecules wherein the cysteine at position 125, numbered in accordance with native human IL-2, is deleted or replaced by a neutral amino acid and said mutein exhibits the biological activity of native human IL-2.

"Lymphokine activated killer (LAK) cells" mean a cytotoxic population of cells which are capable of lysing autologous tumor cells and natural

killer (NK) cell resistant tumor cell lines and are generally prepared by culturing peripheral blood mononuclear cells with interleukin-2.

5 "Peripheral blood lymphocytes" means peripheral blood mononuclear cells from which monocytes have been depleted.

"Lower alkyl" means alkyl of 1-4 carbon atoms.

10 "Copolymeric film material" means a film material in which each layer is made from a single copolymer or homopolymer and at least one layer is made from a single copolymer.

"STP" means standard temperature and pressure.

15 "RPMI 1640" refers to a culture medium which is well known in the art and whose composition is given in "Culture of Animal Cells", Freshney, 72-73, Alan R. Liss, Inc., N.Y.

20 The process of the invention enables LAK-cell activation at concentrations of up to about 5×10^7 cells/mL without altering the extent or range of LAK-cell activity. At these cell concentrations the requisite number of LAK cells for adoptive immunotherapy can be obtained with culture volumes of from about 0.2 to about 2 L. Although the invention will be described in specific with reference to human peripheral blood mononuclear cells or human peripheral blood lymphocytes, it is to be understood that the invention applies to the corresponding cells of other mammals. Preferably, the process of the invention is used with human cells. The process of the invention is also useful for activation of TIL.

30 Peripheral blood mononuclear cells (PBMC) required for the process of the invention are obtained by repeated leukapheresis using procedures well known in the art to collect up to about 5×10^{10}

mononuclear cells. In general, blood is removed intravenously from a human donor and passed to an instrument which separates mononuclear cells from red blood cells, platelets and plasma, these latter components being returned to the donor. The mononuclear cells are collected in a plastic bag. The PBMC so obtained are fractionated by Ficoll-Hypaque density gradient separation. The cells are then washed with a suitable salt solution, such as Hanks' balanced salt solution (HBSS), which is obtainable from Gibco, Grand Island, NY, and then resuspended in a suitable medium, such as RPMI 1640 medium, which is also obtainable from Gibco, supplemented with 1 mM L-glutamine solution, 1% penicillin-streptomycin solution, 1% gentamycin solution, and 2-10% heat inactivated pooled human serum (HIS) (percentages are v/v unless otherwise specified).

The resuspended PBMC are preferably, prior to density gradient separation, treated with an L-amino acid lower alkyl ester or hydrogen chloride salt thereof. This optional treatment can also be effected after density gradient separation. Suitable L-amino acid lower alkyl esters are those wherein the amino acid is selected from phenylalanine, glutamic acid, glutamine and tyrosine. Preferably, the L-amino acid is phenylalanine or tyrosine and most preferably is phenylalanine. The lower alkyl group can be methyl, ethyl, propyl, isopropyl, butyl, isobutyl or t-butyl but is preferably methyl or ethyl and is most preferably methyl. The PBMC can be resuspended in any suitable medium such as serumless RPMI 1640 or HBSS without Ca and Mg, but the latter is preferred. Preferably, the hydrogen chloride salt of the L-amino acid lower alkyl ester is dissolved in RPMI and the pH of the resulting solution is adjusted

to about 7.4 prior to adding the resulting solution to the suspension of PBMC. The amino acid lower alkyl ester is present at a concentration of from about 1 mM to about 5 mM (based on the total volume of the combined PBMC suspension and the amino acid lower alkyl ester solution). Contacting of the PBMC and ester is preferably carried out at a temperature of from 20° C to about 25° C. Treatment of the PBMC with L-amino acid lower alkyl ester causes depletion of monocytes and also confers on the ultimate LAK cell product enhanced activity after culturing with IL-2 at higher cell concentration. This L-amino acid lower alkyl ester treatment is further described in assignee's copending U.S. patent application Serial No. 868,697, filed on May 30, 1986, which is incorporated herein by reference. Monocytes can also be depleted from PBMC by using conventional techniques such as the passage of the PMBC over glass beads. The resulting peripheral blood lymphocytes obtained in this manner are preferably then treated with the amino acid lower alkyl ester as set forth above.

In the process of the invention the peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom are cultured with IL-2 for an incubation period of from about 2 to 7 days, preferably about 3 to 5 days, preferably at a temperature of from about 35° C to about 39° C, most preferably 37° C. Culturing is conducted in a closed container made from a copolymeric film material having an oxygen permeability of at least about $1.8 \times 10^5 \mu\text{m}^3 (\text{STP})/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$ [$100 \text{ cc (STP)}/(24 \text{ hr-atm-100 in}^2)$]; and a thickness of from about 0.04 mm to about 0.23 mm. The copolymeric material is selected from the group consisting of

(a) copolymers of ethylene and an α -olefin

of 4-10 carbon atoms having a density of from about 0.915-0.925 g/cm³ (a so-called linear low density polyethylene); typical α -olefins include 1-butene, hexene, 1-octene and 1-decene;

5 (b) copolymers of ethylene and methacrylic acid;

(c) ionomers, such as sodium or zinc neutralized copolymers of ethylene and acrylic or methacrylic acid or derivatives thereof; and

10 (d) laminates or coextrudates of an ionomer/polyester elastomer and a linear low density polyethylene elastomer. Preferably, the copolymeric film material is a copolymer of ethylene and an α -olefin of 4-10 carbon atoms having a density of
15 from about 0.915-0.925 g/cm³. These copolymers of ethylene and an α -olefin of 4-10 carbon atoms have about 3-5 mol percent of the latter component. Preferably the α -olefin is 1-butene or 1-octene, most preferably 1-octene. Suitable copolymeric materials
20 should be capable of being heat sealed at a low temperature, i.e., from about 125° C to about 140° C. Fabrication of containers is facilitated by materials which meet this requirement

The thickness of the copolymeric film
25 material selected for use in constructing the container will depend on the permeability per millimeter and the mechanical durability of the copolymeric material. For instance, if the material has relatively high oxygen, then a relatively high
30 thickness can be used. On the other hand, if the oxygen permeability of the material at a particular thickness is marginally low while the mechanical durability is rather high, then a lower thickness can be employed. For a copolymer of ethylene and
35 1-butene or 1-octene having a density of from 0.915 to 0.925 g/cm³, the film material will have a

thickness of from about 0.04 mm to about 0.14 mm. The copolymeric film material should be sufficiently mechanically durable to withstand the moderate stresses encounter during use and is preferably translucent. The container should have sufficient volume to accommodate from about 0.2 to about 5 liters of cell suspension. Preferably, the container is a flexible bag. The container is preferably equipped with suitable access ports or tubes which can be closed by methods well known in the art, such as by use of clamps, etc., to maintain a reasonable level of protection from contamination. In a preferred embodiment the tubes are closed by heat sealing.

The copolymeric film material must also be compatible with the PBMC or PBL and the culture medium. By "compatible" it is meant that the material does not interact with the cells or medium or leach into them chemicals which are deleterious to the cells or recipient.

In the process of the invention the PBMC or peripheral blood lymphocytes (PBL) in suspension are placed in the container along with suitable medium and IL-2, the container is closed to maintain sterility, and the resulting suspension is incubated for the time period prescribed earlier herein. The container holding the PBMC or PBL, culture medium and IL-2 is preferably maintained in the presence of a from about 3% to about 10% by volume CO₂ atmosphere, most preferably 5% CO₂. The PBMC or PBL are preferably suspended at a cell concentration during culturing of from about 1.0×10^6 to about 5×10^7 cells/mL, most preferably from about 5×10^6 to about 1.5×10^7 cells/mL. Suitable media include RPMI 1640, supplemented with serum or with human serum albumin (HSA). Preferably, the medium is RPMI

supplemented with HSA, most preferably supplemented with therapeutic grade HSA. Preferably, the IL-2 concentration is from about 5×10^2 to about 5×10^4 pM, most preferably, from 1000 pM to about 2000 pM.

5 Preferably, the IL-2 is rIL-2, and, most preferably, the rIL-2 is a rIL-2 composition consisting essentially of water, rIL-2, and optionally, a polyol, said composition having an IL-2 specific activity of at least about 120,000 units/mg, said
10 specific activity being at least 40% of that of Jurkat IL-2. This rIL-2 composition is described in copending U.S. patent application Serial No. 825,133, filed on January 31, 1986 and assigned to E. I. du Pont de Nemours and Company. The relevant
15 disclosure of this application is incorporated herein by reference. This rIL-2 composition is prepared by a process comprising (a) mixing with water to generate a suspension rIL-2 which has been lyophilized after purification by high performance
20 liquid chromatography, and (b) heating the resulting suspension at a temperature of from about 25° C to about 95° C for at least about two hours.

In the process of the invention, during culturing the cell suspension is maintained at a
25 depth that will allow adequate oxygen to reach all the cells. For instance, when the container is fabricated in the form of a blood bag, the bag is maintained in a horizontal position on a rack that will allow gases to enter the side contacting the
30 rack. In this situation, if the cell density is low, the suspension depth can be up to about 4 cm; whereas, if the cell density is high the depth will be about 0.5 cm or less.

Previously hereto, the PBMC or PBL were
35 cultured in conventional rigid containers, such as roller bottles, T-flasks, petri dishes, cluster

dishes and polypropylene tubes. In a preferred embodiment of the invention the container used for culturing has one or more access tubes which are heat-sealed shut prior to sterilization of the container. In this embodiment, access to the container is obtained through use of a sterile connection device such as than described in U.S. Patent 4,369,779. The PBMC or PBL, culture medium and IL-2 are placed in the container through an access tube which is then sealed shut by use of the sterile connection device. After the incubation period, the LAK cells can be removed by using the sterile connection device to connect the access tube to the desired transfer container. In this manner sterility of the system can be maintained.

The LAK cells prepared by the process of the invention can be suspended in a pharmaceutically acceptable carrier, such as saline, saline containing 5% normal human serum albumin, or Hank's balanced salt solution, to provide a composition which can be infused into a patient afflicted with a tumor. The patient is concurrently treated with rIL-2 as further described by Rosenberg, et al., The New England Journal of Medicine 313, 1485-1492 (1985). In that modality, the patient's blood is withdrawn, subjected to leukapheresis and harvested cells are immediately cultured for 3 days to generate LAK cells. The LAK cells are then infused into the patient. Typically, about 3×10^{10} to 14×10^{10} LAK cells are infused in 4-9 doses. Interleukin-2 is administered every eight hours at doses such as 10,000, 30,000 or 100,000 units per kilogram of weight. The treatment consists of a two-week regime of leukapheresis and reinfusion and generally repetition starting the third week. Recombinant IL-2 can be included in the LAK cell composition.

Although the process of the invention has been described with specific reference to LAK cells, it is also useful for preparing TIL. These cells can be obtained by the procedure described in Rosenberg, et al., Science 233, 1318-1321 (1986). In this procedure a suspension of cells is obtained from 1-to 2-mm pieces of tumor tissue. This cell suspension can be then cultured pursuant to the process of the invention but without the optional treatment with an L-amino acid lower alkyl ester.

The present invention can be carried out by the herein-described specific embodiments but is not limited thereto. The invention is further illustrated by the following examples in which all percentages are by volume and temperatures are in degrees Celsius, unless otherwise stated.

Cytotoxicity Assay

Unless otherwise stated, a 4 hour ^{51}Cr release assay was used to measure cytotoxicity of LAK cells for tumor cells. Tumor cells at a concentration of about 2×10^6 to 10×10^6 were incubated with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ in 0.4 mL of Tris-phosphate buffered saline for 1 hour at 37° . The cells were washed 3 times with RPMI 1640 containing 5% or 10% fetal calf serum (FCS) and resuspended to 10^5 cells/mL in RPMI-20% FCS or RPMI-10% FCS. The effector cells (LAK cells) were suspended to various concentrations and 0.1 mL was added to wells in round bottom microliter plates. The ^{51}Cr labelled target cells (0.1 mL) were added to all wells and the plates were centrifuged at 200 xg for 5 minutes. After 4 hours of incubation at 37° , the plates were centrifuged again and 0.1 mL of resulting supernatant was removed from each well and counted in a gamma counter. Percent cytotoxicity is calculated from the following formula:

%cytotoxicity=

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Each variable was tested in triplicate and the resulting data are expressed as % cytotoxicity. This cytotoxicity test is further described in "Selected Methods in Cellular Immunology", Mishell and Shiigi, eds., 124-137, W. H. Freeman and Co., San Francisco (1980).

In other experiments, the results of the assays are presented as "Lytic Units" (LU or LU30) which are the number of target cells per 100 effector cells when 30% of the target cells are killed when LAK cells and target cells are incubated together for 4 hours at 37° C. The calculation of LU is based upon the method of Pross, et al., Journal of Immunological Methods 68, 235-249 (1984). The greater the number of LU, the greater the potency of the LAK cell preparation.

EXAMPLES 1-9 AND COMPARISONS A-F

PBMC were obtained by Ficoll-Hypaque separation of ACD-anticoagulated venous blood drawn from two healthy consenting human donors. Cells taken from one donor were treated with L-phenylalanine methyl ester whereas cells from the other donor were not. The methyl ester treatment was effected by washing the cells three times with HBSS and resuspending the resulting washed cells in RPMI 1640 medium supplemented with 1 mL L-glutamine, 0.1% gentamycin solution, 10% FCS and 5 mM L-phenylalanine methyl ester. The resulting cell suspension was incubated at ambient temperature (about 22° C) for 40 minutes after which the cells were washed three times with HBSS.

The PBMC or PBL resulting from monocyte depletion with L-phenylalanine methyl ester were cultured in 10 mL of RPMI 1640 supplemented with 10%

FCS in T Flask (T-25) (Comparisons) or closed bags constructed of a copolymer of ethylene (about 97 mol %) and 1-octene (LLDPE) having a density of 0.918 g/cm³ and a nominal volume of 200 mL. A 0.12 mm thick LLDPE material of similar composition is known from U.S. Patent 4,496,361, Example 5, to have an oxygen permeability of $2.6 \times 10^5 \mu\text{m}^3 \text{ (STP) / m}^2 \cdot \text{sec} \cdot \text{Pa}$. Culturing was conducted in 5% CO₂ atmosphere at 37° in the presence of 10 units/mL of rIL-2. One unit of IL-2 is defined as that quantity of IL-2 needed to stimulate 50% uptake of radiolabelled (tritium) thymidine by murine tumor-specific cytotoxic T-cell lines (CTL) standardized against Jurkat IL-2 as set forth more fully in Gehmans, et al., Journal of Immunological Methods 74, 39-47 (1984), which is incorporated herein by reference.

After the culture period, the resulting LAK cells were harvested and resuspended in RPMI supplemented with 10% FCS for cytotoxicity assay using as target cells (T) Daudi cells (D) (an NK resistant cell line), melanoma cells (M), and lymphoma cells (L). The results are presented in Table 1 where the "E" refers to "effector cells", i.e., LAK cells. Experiments where the PBMC were treated with phenylalanine methyl ester (PME) are so indicated.

TABLE 1

Example	Cell			E:T Ratio	% ⁵¹ Cr Release
	No. or Comparison	PME	Conc. (no./mL) x10 ⁻⁶		
30	1	no	D	1.5	75.6
				20:1	71.7
				10:1	59.2
				5:1	44.6
35	2	no	D	4	73.8
				20:1	

16

					10:1	68.3
					5:1	48.7
					2.5:1	33.0
	3	yes	D	5	20:1	82.2
5					10:1	73.9
					5:1	57.2
					2.5:1	35.5
	Comp. A	no	D	1.5	20:1	80.5
					10:1	73.9
10					5:1	60.1
					2.5:1	44.5
	Comp. B	yes	D	1.5	20:1	72.3
					10:1	63.0
					5:1	44.1
15					2.5:1	23.3
	4	no	M	1.5	20:1	54.7
					10:1	37.2
					5:1	39.4
					2.5:1	27.1
20	5	no	M	4	20:1	49.2
					10:1	48.6
					5:1	35.5
					2.5:1	20.5
	6	yes	M	5	20:1	34.0
25					10:1	25.6
					5:1	25.5
					2.5:1	17.7
	Comp. C	no	M	1.5	20:1	44.4
					10:1	31.1
30					5:1	25.6
					2.5:1	17.7
	Comp. D	yes	M	1.5	20:1	29.8
					10:1	23.1
					5:1	10.0
35					2.5:1	9.2
	7	no	L	1.5	20:1	41.7

16

					10:1	35.1
					5:1	25.4
					2.5:1	17.9
5	8	no	L	4	20:1	49.0
					10:1	36.2
					5:1	24.7
					2.5:1	15.3
10	9	yes	L	5	20:1	33.1
					10:1	32.2
					5:1	19.2
					2.5:1	7.8
15	Comp. E	no	L	1.5	20:1	46.1
					10:1	39.4
					5:1	31.3
	Comp. F	yes	L	1.5	2.5:1	23.2
					20:1	28.4
					10:1	21.2
					5:1	14.0
					2.5:1	17.5

20 EXAMPLE 10-12 AND COMPARISON G

PBMC were collected from healthy consenting human donors by means of either leukapheresis or American Red Cross whole blood specimens. In both cases the PBMC were obtained from venous blood prevented from clotting by means of ACD anticoagulant. The PBMC were separated from other blood components by Ficoll-Hypaque density gradient centrifugation. Some of PBMC preparations were treated with L-phenylalanine methyl ester (PME) to deplete them of monocytes. The methyl ester treatment was accomplished by washing the cells three times with HBSS and resuspending the resulting washed cells in RPMI 1640 medium supplemented with 1 mM L-glutamine, 0.1% gentamycin solution, 10% FCS and 5 mM L-phenylalanine methyl ester. The cell suspension so obtained was incubated at ambient temperature for

40 minutes after which the resulting cells were washed three times with HBSS and resuspended in RPMI 1640 containing L-glutamine, gentamycin and 10% FCS.

The PBMC or PBL resulting from monocyte depletion with methyl ester were cultured in 10 mL T-flask (Comparison G) or in closed bags constructed from a film of a copolymer of ethylene (97 mol %) and 1-octene (LLDPE) having a density of 0.918 g/cm³, a nominal volume of 200 mL, and a film thickness of 0.13 mm (5 mil). Culturing was conducted in 5% CO₂ atmosphere at 37° C in the presence of 10 units/mL of rIL-2.

After the culture period the resulting LAK cells were harvested and resuspended in RPMI 1640 supplemented with 10% FCS for cytotoxicity assays using as target cells the Raji cell line. The results are presented in Table 2 where "SD" is the standard deviation. It is believed that high standard deviations reflect donor variability.

TABLE 2

Example No. or Comparison	Cell Conc. (no./mL) $\times 10^{-6}$	No. of Donor Samples	Lytic Units	
			mean	SD
25 G	no 1.5	10	20.1	11.8
10	no 1.5	10	24.4	13.8
11	yes 1.5	17	25.0	16.8
12	yes 10	19	24.4	15.1

EXAMPLES 13

These experiments were conducted using procedures similar to those set forth in Examples 10-12. PBMC from six donors were prepared for culture in T-flask (comparisons) or LLDPE bags. None of the PBMC were treated with L-phenylalanine methyl ester. The results are given in Table 3 using Lytic Units as measured by cytotoxicity assays with Raji

cells as targets. The abbreviation "nt" indicates that the cells were not tested in the particular device.

TABLE 3

5		Cell Conc. (no./mL)	Lytic Units	
	Donor No.	$\times 10^{-6}$	T-flask	Bags
10	1	1.0	6.3	nt
		2.0	20.0	nt
		5.0	25.0	nt
	2	1.0	7.7	14.3
		1.5	9.5	20.8
	3	1.0	15.2	38.5
5.0		18.5	25.0	
15		4	10	1.0
	15		0.5	<0.1
	3.0		nt	34.5
	5	5.0	nt	25.0
		10	nt	0.7
		20	nt	0.1
20	6	5.0	nt	35.7
		10	nt	1.5
		20	nt	0.3

EXAMPLE 14

25 Except as otherwise stated these experiments were conducted using procedures similar to those described in Examples 10-12. PBMC from four donors were in each case split into two equal portions. In each case one portion was treated with

30 L-phenylalanine methyl ester (PME) and the other was subjected directly to culturing. Culturing was performed in closed LLDPE bags. The results are presented in Table 4.

35

TABLE 4

	<u>Donor No.</u>	Cell Conc. (no./mL)	Lytic Units	
		$\times 10^6$	PME	Untreated
5	1	12	4.7	0.7
	2	13	15.1	<0.1
	3	14	15.2	2.9
	4	15	3.6	1.7

EXAMPLE 15

- 10 Except as otherwise indicated these experiments were conducted using procedures similar to those described in Examples 10-12. PBMC from three donors were used to compare LAK cell activation in T-flask with activation in LLDPE bags at
- 15 relatively high cell densities, e.g. 1×10^6 to 1×10^7 . The results are presented in Table 5.

TABLE 5

	<u>Donor No.</u>	Cell Conc. (no./mL)	PME	Lytic Units	
		$\times 10^6$		T-flask	Bags
20	1	1.0	no	2.3	nt
		5.0		4.0	27.0
		10		0.1	nt
25	2	1.0	yes	3.7	nt
		5.0		2.9	11.9
		10		16.1	nt
	3	5	yes	3.8	18.5
		10		6.9	nt

EXAMPLES 16-18 AND COMPARISON H

- 30 Using procedures similar to those described in Examples 1-9 LAK cells are generated by culturing PBMC for four days in bags about 10 in² in size constructed of a copolymer of (a) ethylene (about 97 mol %) and 1-octene having a density of 0.918 g/cm³,
- 35 (b) ethylene-methacrylic acid copolymer (Nucrell 0903 polymer, a product of the Du Pont Company), (c)

ionomer (Surlyn copolymer, a product of the Du Pont Company), and polyvinyl chloride (PVC) (Comparison).

The PBMC cells were present in a concentration of 5×10^6 cells/mL and 50 mL of culture fluid were

5 employed. Raji cells were used as target cells. The results are presented in Table 6 where cytotoxicity is indicated in terms of counts per minute.

TABLE 6

Example	Bag				Counts
10 No. or	Thickness,				Per
<u>Comparison</u>	<u>mm (mil)</u>	<u>Material</u>	<u>E:T Ratio</u>	<u>Minute</u>	
16	0.13 (5)	a	1.25	82	
			2.5	106	
			5	138	
15			10	206	
			20	239	
			40	251	
	H	0.36 (14)	PVC	1.25	27
				2.5	36
20			5	57	
			10	76	
			20	41	
			40	32	
	17	0.13 (5)	b	1.25	52
25				2.5	55
			5	67	
			10	109	
			20	145	
			40	185	
30	18	0.05 (2)	c	1.25	58
				2.5	60
			5	95	
			10	133	
			20	178	
35			40	200	

EXAMPLE 19

Tests in these experiments were run in a film-test chamber permitting the evaluation of films of different compositions and thicknesses. The

5 chamber was constructed on polycarbonate cylinders with an outside diameter of four inches, inside diameter of two inches, and a depth of two inches. The chamber was steam sterilized. Next a radiation-sterilized test film was mechanically
10 clamped over one end and pressure sealed to prevent liquid loss. With the chamber placed film end down, a suspension of cells and media to be cultured were added. A second sterile film of the same composition and thickness as the first was then placed over the
15 top of the chamber and clamped tight to prevent leakage. Using procedures similar to those described in Examples 1-9, PBL were cultured in such chambers.

Tests were run which showed that the chamber was equivalent to a bag run with the same
20 film, cell density and media depth. In a typical run LLDPE film used with the chamber gave an LU30 of 6.5 whereas a bag of the same composition gave 5.4. LAK activation was conducted in these chambers using Surlyn 1601 ionomer films (a product of the Du Pont
25 Company) of 0.05, 0.13, 0.18 and 25 mm (2, 5, 7.5, 10 mil) thicknesses. These films had transmission rates of about 340, 136, 88 and 68 cc/24 hr-atm-100 in², respectively. LU30 results were 15, 11, 7.4 and 3
30 respectively.

35

CLAIMS

The claims defining the invention are as follows:-

1. In a process wherein peripheral blood mononuclear cells or lymphocytes from tumor-tissue are cultured to produce a population of cells which are cytotoxic for natural killer cell resistant tumor cells, the improvement comprising culturing at a cell concentration of at least about 1.0×10^6 cells/mL a population of cells selected from the group consisting of peripheral blood mononuclear cells, peripheral blood lymphocytes resulting therefrom, and lymphocytes obtained from tumor tissue in a closed container made from a copolymeric film material having an oxygen permeability of at least about $1.8 \times 10^5 \mu\text{m}^3 \text{ (STP)}/(\text{m}^2 \cdot \text{sec} \cdot \text{Pa})$; and a thickness of from about 0.04 mm to about 0.23 mm; said material being selected from the group consisting of
 - (a) copolymers of ethylene and an α -olefin of 4-10 carbon atoms having a density of from about 0.915-0.925 g/cm³;
 - (b) copolymers of ethylene and methacrylic acid;
 - (c) ionomers; and
 - (d) laminates or coextrudates of an ionomer/polyester elastomer and a linear low density polyethylene elastomer.
2. A process according to Claim 1 wherein the population of cells which is cultured is selected from peripheral blood mononuclear cells and peripheral blood lymphocytes resulting therefrom.
3. A process according to Claim 2 wherein said peripheral blood mononuclear cells or peripheral blood lymphocytes are human cells.
4. A process according to Claim 3 wherein the peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom are cultured

for 2-7 days in the presence of recombinant interleukin-2 at a concentration of from about 500 pM to 50,000 pM.

5 5. A process according to Claim 4 wherein the the peripheral blood mononuclear cells or peripheral blood lymphocytes are cultured at a temperature of from about 35° to about 39° C.

10 6. A process according to Claim 5 wherein the peripheral blood mononuclear cells or peripheral blood lymphocytes are cultured in the presence of from about 3% to about 10% by volume CO₂ atmosphere.

7. A process according to Claim 6 wherein peripheral blood lymphocytes are used.

15 8. A process according to Claim 7 wherein the concentration of peripheral blood lymphocytes is from about 1×10^6 cells/mL to about 5×10^7 cells/mL.

9. A process according to Claim 8 wherein the peripheral blood lymphocytes are cultured for 3-5 days.

20 10. A process according to Claim 9 wherein the recombinant interleukin-2 concentration is from about 1000 pM to about 2000 pM.

25 11. A process according to Claim 10 wherein the copolymeric film material consists essentially of a copolymer of ethylene and an α -olefin of 4-10 carbon atoms, said copolymer having a density of from about 0.915-0.925 g/cm³.

30 12. A process according to Claim 11 wherein the α -olefin of 4-10 carbon atoms is 1-butene or 1-octene and said copolymeric film material has a thickness of from about 0.04 mm to about 0.14 mm.

13. A process according to Claim 12 wherein the α -olefin of 4-10 carbon atoms is 1-octene.

35 14. A process according to Claim 13 wherein the container is closed by heat sealing.

15. A process according to Claim 14 wherein

the concentration of peripheral blood lymphocytes is from about 5×10^6 to about 1.5×10^7 cells/mL.

16. A process according to Claim 15 wherein the peripheral blood lymphocytes are cultured in RPMI 1640 medium supplemented with human serum albumin.

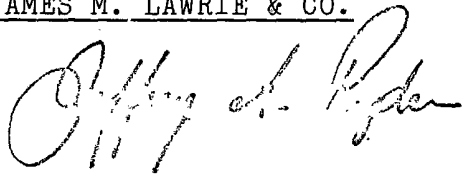
17. A process according to any one of Claims 1 to 16 wherein, prior to culturing, the peripheral blood mononuclear cells or peripheral blood lymphocytes are contacted with a L-amino acid lower alkyl ester or hydrogen chloride salt thereof, said L-amino acid being selected from the group consisting of phenylalanine, glutamic acid, glutamine and tyrosine and mixtures thereof.

18. A process according to Claim 17 wherein the L-amino acid lower alkyl ester is phenylalanine methyl ester.

19. A process according to Claim 1, substantially as hereindescribed with reference to any one of the Examples.

DATED this 20 day of January 1988

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