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FORM 1

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA  
PATENTS ACT 1952  
APPLICATION FOR A STANDARD PATENT

Abbott Laboratories, of One Abbott Park Road, Abbott Park, Illinois, 60064-3500, UNITED STATES OF AMERICA, hereby apply for the grant of a standard patent for an invention entitled:

Rat Monoclonal Antibodies Directed Against Human Antigens and Processes for Preparation Thereof

which is described in the accompanying complete specification.

Details of basic application(s):-

<u>Basic Applic. No:</u>	<u>Country:</u>	<u>Application Date:</u>
299,694	US	23 January 1989


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DATED this TWENTY THIRD day of JANUARY 1990

Abbott Laboratories

By:



Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS  
OUR REF: 118277  
S&F CODE: 50000

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5845/2

DECLARATION IN SUPPORT OF A  
CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a  
patent for an invention entitled:  
RAT MONOCLONAL ANTIBODIES DIRECTED AGAINST HUMAN  
ANTIGENS AND PROCESSES FOR PREPARATION THEREOF

~~4651.AU.01~~

Title of Invention

Full name(s) and  
address(es) of  
Declarant(s)

I/We Charles M. Brock, Assistant Secretary  
of Abbott Laboratories  
One Abbott Park Road  
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do solemnly and sincerely declare as follows:-

Full name(s) of  
Applicant(s)

- ~~1. I am/We are the applicant(s) for the patent~~  
(or, in the case of an application by a body corporate)
- 1. I am/We are authorised by  
Abbott Laboratories  
the applicant(s) for the patent to make this declaration on  
its/their behalf.
- 2. The basic application(s) as defined by Section 141 of the  
Act was/were made

Basic Country(ies)

in the United States of America

Priority Date(s)

on January 23, 1989

Basic Applicant(s)

by HERVE BAZIN; MARC DE BRUYERE

Full name(s) and  
address(es) of  
inventor(s)

- ~~3. I am/We are the actual inventor(s) of the invention referred  
to in the basic application(s)~~  
(or where a person other than the inventor is the applicant)

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(respectively)

is/are the actual inventor(s) of the invention and the facts upon  
which the applicant(s) is/are entitled to make the application are  
as follows:

Set out how Applicant(s)  
derive title from actual  
inventor(s) e.g. The  
Applicant(s) is/are the  
assignee(s) of the  
invention from the  
inventor(s)

Applicants have title to all inventions by virtue  
of a license agreement dated January 23, 1989  
between inventors Herve Bazin, Marc DeBruyere and  
Abbott Laboratories.

- 4. The basic application(s) referred to in paragraph 2 of this  
Declaration was/were the first application(s) made in a Convention  
country in respect of the invention(s) the subject of the application.

Declared at Abbott Park, this 4 day of January 1990  
Illinois

ABBOTT LABORATORIES

*Charles M Brock*

Signature of Declarant(s)  
Charles M. Brock  
Assistant Secretary



**(12) PATENT ABRIDGMENT (11) Document No. AU-B-48727/90**  
**(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 636867**

- (54) Title  
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- (57) Claim

1. A method of producing rat monoclonal antibodies that bind to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b, comprising the steps of:

- (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogen comprising CD4 antigen;
- (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
- (3) selecting hybridoma cells that produce antibody that binds to the human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b;
- (4) culturing the selected hybridoma cells; and
- (5) recovering said antibody.

5. A hybridoma that produces a rat monoclonal antibody that binds to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

10. A rat monoclonal antibody that binds to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

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19. An in vivo therapeutic method for the treatment of immune disorders, which method comprises administering to a patient an amount effective for the treatment of immune disorders of a rat monoclonal antibody according to any one of Claims 10 to 13 or 17.

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FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

Class Int Class

Complete Specification Lodged:  
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Priority:

Related Art:

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Complete Specification for the invention entitled:

Rat Monoclonal Antibodies Directed Against Human Antigens  
and Processes for Preparation Thereof

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

ABSTRACT OF THE DISCLOSURE

RAT MONOCLONAL ANTIBODIES DIRECTED AGAINST  
HUMAN ANTIGENS AND PROCESSES FOR PREPARATION THEREOF

A method of producing rat monoclonal antibodies to human T-cell lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen comprising: (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising T-cell lymphocyte markers; (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells; (3) selecting hybridoma cells that produce antibody that binds to the human T-cell lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen; (4) culturing the selected hybridoma cells; and (5) recovering said antibody. A method of producing rat monoclonal antibodies that bind to human CD4 antigen, the method comprising: (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising CD4 molecule; (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells; (3) selecting hybridoma cells that produce antibody that binds to human CD4 antigen; (4) culturing the selected hybridoma cells; and (5) recovering said antibody. A hybridoma that produces a rat monoclonal antibody that binds to human CD4 antigen. A rat monoclonal antibody that binds to human CD4 antigen.

In a preferred embodiment the rat monoclonal antibody binds to a human CD4 antigen epitope other than those epitopes defined by murine monoclonal antibodies OKT4 and Leu3a. A rat monoclonal antibody that binds to human T-cell lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen.

**RAT MONOCLONAL ANTIBODIES DIRECTED AGAINST  
HUMAN ANTIGENS AND PROCESSES FOR PREPARATION THEREOF**

**FIELD OF THE INVENTION**

The present invention relates to the production of rat monoclonal antibodies. More particularly, the present invention relates to a new, reproducible method of producing rat monoclonal antibodies that bind to human T-lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen and rat monoclonal antibodies that bind to the human CD4 antigen. The present invention also relates to the hybridomas produced by the novel methods and to the monoclonal antibodies secreted by the hybridomas. The antibodies are useful for detecting the human antigens in vitro, and in particular CD4 which is useful for determining the prognosis of AIDS infection. The antigens are expected to be useful for the treatment of diseases, such as AIDS, wherein the antigens are involved in the disease process. The antibodies are also expected to be useful in isolating the antigens.

**BACKGROUND OF THE INVENTION**

The development of monoclonal antibodies (MAbs) has offered the opportunity of studying the distribution, structure and function of T-lymphocyte membrane antigens. Over the past years a series of molecules present on the T-lymphocyte have been defined by mouse monoclonal antibodies; some have been shown to be associated with T-lymphocyte function. For instance, T-cell antigen receptor complex (Ti-T3) is



recognized by CD3 monoclonal antibodies (e.g. OKT3) and by WT311 (Kung, P.C. et al, Science, 206, 347, 1979); the receptor for Interleukin-2 is recognized by CD25 monoclonal antibodies (e.g., TAC) (Leonard, W.J. et al. Nature, 300, 267, 1982); and the T-lymphocyte receptor for sheep erythrocytes (E-receptor), which has recently been proposed as a ligand for LFA-3, is recognized by CD2 monoclonal antibodies (e.g. OKT11) (Verbi, W. et al Eur. J. Immunol., 12, 81, 1982 and Selvaraj, T. et al, Nature, 326, 400, 1987). Some antibodies to T-lymphocytes mimic the effect of natural ligands or of antigen stimulation: OKT3 and WT31 activate T-lymphocytes through the T-cell antigen receptor complex (van Wauwe, J.P. et al, J. Immunol., 124, 2708, 1980 and Meuer, S.C. et al, J. Exp. Med., 158, 988, 1983). Certain combinations of CD2 monoclonal antibodies activate T-lymphocytes through an alternative pathway (Meuer, S. et al. Cell, 36, 897, 1984). CD4 and CD8 monoclonal antibodies (e.g., OKT4 and OKT8) (Reinherz, E.L. et al, Proc. Natl. Acad. Sci. USA, 76, 4061, 1979 and Reinherz, E.L. et al, J. Immunol., 124, 1301, 1980) define distinct subpopulations of T-lymphocytes. The antigen defined by CD4 monoclonal antibodies is mainly expressed on T-helper cells and seems to recognize MHC class II gene products, while the antigen defined by CD8 monoclonal antibodies is mainly expressed to T suppressor/cytotoxic cells and seems to recognize MHC class I antigens (Meuer, S.C. et al, Proc. Natl. Acad. Sci. USA, 79, 4395, 1982 and Biddison, W.E. et al, J. Immunol., 131, 152, 1983).

In addition to their role in regulating immune function, T-cells bearing the CD4 antigen (T-4 cells) are useful in the diagnosis of disease. In particular, the Human Immunodeficiency Virus (HIV) attacks T-4 cells resulting in decline in the number of circulating T-4 cells. A decline in the number of circulating T-4 cells is a predictor of the development of Acquired Immunodeficiency Syndrome (AIDS) (Goedert, J. et al, JAMA, 257, 331-334, 1987). Therefore, the enumeration of T-4 cells is medically useful in the management of patients infected with HIV.

Antibodies against the CD4 antigen are thought to be useful in treating disorders of the immune system. For instance, in patients having rheumatoid arthritis or psoriatic arthritis, the intravenous injection of murine anti-CD4 monoclonal antibodies gave objective relief to the patients (Herzog, C. et al, The Lancet, December 19, 1987, 1461-1462).

Despite the development of these mouse monoclonal antibodies, there has been an interest in producing anti-human T-lymphocyte and especially anti-human CD4 monoclonal antibodies in a rat model because of the difference in immune repertoire between rat and mouse. Moreover, because large quantities of rat monoclonal antibodies are easily produced and purified and because some rat immunoglobulin isotypes have been shown to fix human complement and to activate human killer cells, rat monoclonal antibodies are attractive reagents for clinical therapeutic use.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention there is provided a method of producing rat monoclonal antibodies that bind to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced  
5 by a reference hybridoma LO-CD4-a or LO-CD4-b, comprising the steps of:

- (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogen comprising CD4 antigen;
- (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
- 10 (3) selecting hybridoma cells that produce antibody that binds to the human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b;
- (4) culturing the selected hybridoma cells; and
- (5) recovering said antibody.

15 According to a second embodiment of the invention there is provided a hybridoma that produces a rat monoclonal antibody that binds to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

20 According to a third embodiment of the invention there is provided a hybridoma having the identifying characteristics of hybridoma LO-CD4-a having ECACC Deposit No. 89012101.

According to a fourth embodiment of the invention there is provided a hybridoma having the identifying characteristics of hybridoma LO-CD4-b having ECACC Deposit No. 8912102.

25 According to a fifth embodiment of the invention there is provided a a rat monoclonal antibody that binds to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

30 According to a sixth embodiment of the invention there is provided a rat monoclonal antibody having the identifying characteristics of monoclonal antibody LO-CD4-a produced by hybridoma LO-CD4-a having ECACC Deposit No. 89012101.

35 According to a seventh embodiment of the invention there is provided a rat monoclonal antibody having the identifying characteristics of monoclonal antibody LO-CD4-b produced by hybridoma LO-CD4-b having



ECACC Deposit No. 89012102.

According to an eighth embodiment of the invention there is provided a binding protein having the identifying characteristics of monoclonal antibody LO-CD4-a produced by hybridoma LO-CD4-a having ECACC  
5 Deposit No. 89012101.

According to a ninth embodiment of the invention there is provided a binding protein having the identifying characteristics of monoclonal antibody LO-CD4-b produced by hybridoma LO-CD4-b having ECACC Deposit No. 89012102.

10 According to a tenth embodiment of the invention there is provided a method of producing rat monoclonal antibodies that bind to a human T-cell lymphocyte antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b comprising the steps of:

- 15 (a) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising T-cell lymphocyte markers;
- (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
- (3) selecting hybridoma cells that produce antibody that in turn  
20 binds to the human T-cell lymphocyte antigen epitope which binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b;
- (4) culturing the selected hybridoma cells; and
- (5) recovering said antibody.

25 According to an eleventh embodiment of the invention there is provided a rat monoclonal antibody produced by the method of Claim 16, wherein said monoclonal antibody binds to a human T-cell lymphocyte antigen epitope which in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

30 The present invention additionally provides an in vivo therapeutic method comprising introducing into a patient a rat monoclonal antibody produced according to the above-described methods.

The present invention also provides an in vitro diagnostic method comprising contacting a rat monoclonal antibody produced according to the  
35 above-described methods with a suitable test sample and assaying for the

antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the reactivity pattern of monoclonal antibodies LO-CD4-a and LO-CD4-b and of reference pan-T or subset T specific  
5 monoclonal antibody OKT3, T11, T12, LO-CD5-a and OKT4 with peripheral blood lymphocytes (PBL) (left panel) and



phytohemagglutinin (PHA) activated PBL (right panel). X-axis: fluorescence intensity; Y-axis: cell number.

Figure 1B shows the reactivity pattern of monoclonal antibodies LO-CD4-a and LO-CD4-b and of reference pan-T or subset T specific monoclonal antibody OKT3, T11, T12, LO-CD5-a and OKT4 with tonsil cells (left panel) and thymocytes (right panel). X-axis: fluorescence intensity. Y-axis: cell number.

Figure 2 shows patterns from double labeling with pan-T or pan-B monoclonal antibodies and rat monoclonal antibodies. Tonsil cells are labeled with monoclonal antibodies LO-CD4-a plus fluorescein-conjugated rabbit anti-rat immunoglobulin antibody (Fab')<sub>2</sub> fragments (RARA-FITC) followed by mouse anti-CD2-phycoerythrin conjugate (T11PE) or mouse anti-CD19 phycoerythrin conjugate (B4PE).

Figure 3 is double labeling patterns that show subsets of lymphocytes recognized by LO-CD4-a and LO-CD4-b. Peripheral blood mononuclear cells (PBMN) were labeled with rat monoclonal antibodies LO-CD4-a or LO-CD4-b plus RARA-FITC followed by OKT4PE or OKT8F8.

Figure 4 shows flow cytometer scans of fluorescent labeled PBLs. The X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) intensity and the Y-axis in all panels represents counts full scale. Figs. 4(A) to 4(G) show the data for monoclonal antibody LO-CD4-a and Figs. 4(H) to 4(N) show the data for monoclonal antibody LO-CD4-b.

Figs. 4(A) and 4(B) are controls showing green and red autofluorescence, respectively, for PBLs in the

absence of added antibody conjugate. Fig. 4(C) is a control showing the binding of monoclonal antibody LO-CD4-a to PBLs detected by fluorescein conjugated mouse anti-rat conjugate (MARFITC). Fig. 4(D) is a control showing the fluorescence intensity of PBLs after incubation with OKT4FITC only. Fig. 4(E) shows the fluorescence intensity of PBLs incubated with OKT4FITC after preincubation with LO-CD4-a. Fig. 4(F) is a control showing the fluorescence intensity of PBLs after incubation with Leu3aPE only. Fig. 4(G) shows the fluorescence intensity of PBLs incubated with Leu3aPE after preincubation with LO-CD4-a.

Figs. 4(H) to 4(N) parallel Figs. 4(A) to 4(G), except that where monoclonal antibody LO-CD4-a was used in Figures 4(A) to 4(G), monoclonal antibody LO-CD4-b was used in Figures 4(H) to 4(N).

Figure 5 shows flow cytometer scans of fluorescent labeled PBLs. The X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) intensity and the Y-axis in all panels represents counts full scale. Figs. 5(A) to 5(H) show the data for undepleted PBLs and Figs. 5(I) to 5(P) show the data for CD4 depleted PBLs.

Figs. 5(A) to 5(B) are controls showing green and red autofluorescence, respectively, for PBLs in the absence of added antibody conjugate. Figs. 5(C) and 5(D) show binding of monoclonal antibodies LO-CD4-a and LO-CD4-b, respectively, to undepleted PBLs detected by phycoerythrin conjugated goat anti-rat antibody (GARPE). Fig. 5(E) is a control showing immunofluorescence of PBLs after incubation with GARPE.

Figs. 5(F), 5(G) and 5(H) show the fluorescence intensity of undepleted PBLs after incubation with Leu3aPE, OKT4FITC and OKT8FITC, respectively.

Figs. 5(I) to 5(P) parallel Figs. 5(A) to 5(H), except that depleted PBLs were used in place of undepleted PBLs.

Figure 6 shows dot plot analyses of flow cytometry data of red fluorescence vs. green fluorescence for fluorescent labeled PBLs. The X-axis of each plot represents green fluorescence (Fluorescence 1), and the Y-axis of each plot represents red fluorescence (Fluorescence 2).

Figs. 6(A) to 6(D) show the data for monoclonal antibody LO-CD4-b, and Figs. 6(E) to 6(I) show the data for monoclonal antibody LO-CD4-a.

Fig. 6(A) is a control showing the dot plot for red and green autofluorescence of PBLs in the absence of added antibody conjugate. Fig. 6(B) shows the dot plot for fluorescence of PBLs incubated with monoclonal antibody conjugate Leu3aPE. Fig. 6(C) shows the dot plot for fluorescence of PBLs incubated with MARFITC after preincubation with monoclonal antibody LO-CD4-b. Fig. 6(D) shows the dot plot for fluorescence of PBLs incubated with monoclonal antibody conjugates Leu3aPE and then MARFITC after preincubation with LO-CD4-b.

Figs. 6(E) to 6(H) parallel Figs. 6(A) to 6(D), except that monoclonal antibody LO-CD4-a was used instead of monoclonal antibody LO-CD4-b. Fig. 6(I) shows the dot plot for fluorescence of PBLs incubated with MARFITC after preincubation with Leu3a.



Figure 7 shows flow cytometer scans of fluorescent labeled PBLs. The X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) intensity and the Y-axis in all panels represents counts full scale.

Figs. 7(A) and 7(B) are controls showing green and red autofluorescence, respectively, for PBLs in the absence of added antibody conjugate. Fig. 7(C) is a control showing the fluorescence for PBLs incubated only with GARPE. Fig. 7(D) shows the fluorescence intensity of PBLs incubated with OKT8 as detected by phycoerythrin conjugated goat anti-mouse antibody (GAMPE). Figs. 7(E) and 7(F) show the fluorescence intensity of PBLs incubated with OKT4 and Leu3a, respectively, as detected by GAMPE. Figs. 7(G) and 7(H) show the fluorescence intensity of PBLs incubated with LO-CD4-a and LO-CD4-b, respectively, as detected by GARPE.

#### DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this application, the following abbreviations have the following meanings:

AET: 2-aminoethylisothiuronium; ALL: acute lymphoblastic leukemia; CALL: common ALL; CLL: chronic lymphocytic leukemia; conA: concanavalin A; Ig: immunoglobulin; DMSO: dimethylsulfoxide; E: sheep erythrocyte rosette; GAM-FITC: fluorescein-conjugated goat anti-mouse Ig antibodies; IL2: interleukin-2; PE: phycoerythrin; FITC: fluorescein isothiocyanate; kDa: kilodalton; MAb: monoclonal antibody; MLR: mixed lymphocyte reaction; NBCS: newborn calf serum; NHL: non

Hodgkin lymphoma; PBS: 0.15 NaCl buffered at pH 7.2 with 8 mM phosphate; PHA: phytohemagglutinin; PWM: pokeweed mitogen; PAGE: polyacrylamide gel electrophoresis; PBMN: peripheral blood mononuclear cells; PMSF: phenyl methane sulfonyl fluoride; RARA-FITC: fluorescein-conjugated rabbit anti-rat IG antibody F(ab')<sub>2</sub> fragments; SAC: staphylococcus aureus Cowan I; SDS: sodium dodecyl sulfate; SRBC: sheep red blood cells; TT: tetanus toxoid; ECACC: European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research at Porton Down, Salisbury, Wilts. SP 4-OJG, United Kingdom.

As described above, in one embodiment the present invention provides a method of producing rat monoclonal antibodies to human T-cell lymphocyte antigen epitopes other than those defined by murine monoclonal antibodies directed to the same antigen comprising:

- (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising T-cell lymphocyte markers;
- (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
- (3) selecting hybridoma cells that produce antibody that binds to the human T-cell lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen;
- (4) culturing the selected hybridoma cells; and
- (5) recovering said antibody.

The present invention also provides a method of producing rat monoclonal antibodies that bind to human CD4 antigen, the method comprising:

- (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising CD4 molecule;
- (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
- (3) selecting hybridoma cells that produce antibody that binds to the human CD4 antigen;
- (4) culturing the selected hybridoma cells; and
- (5) recovering said antibody.

A variety of rats that are easily available to researchers in the art, such as Wistar, Sprague-Dawley, Lewis and Louvain rats, are suitable for use as the source of immunized cells for fusion.

LOU/C or LOU/M rats are a preferred source of immunized cells for fusion. These rats are syngeneic rats. These two strains of rats were originally developed by Bazin et al (J. Natl. Cancer Inst. (U.S.), 51, 1359, 1973). The strains are readily available to those working in the art.

Methods for immunizing the rats are described below in detail.

Alternatively, stimulation of antigen-specific B-cells can be accomplished in vitro. Rat immunocompetent cells are harvested from lymphatic organs removed from the rat. Procedures for harvesting and in vitro immunization are well known to artisans in the field. (C.L. Reading, Methods in Enzymology,

121:18-27, 1986; D. Gratecos et al., J. Immunological Methods 103, 169-178, 1985; B.B. Mishell, S.M. Shiigi, Selected Methods in Cellular Immunology, 1980).

The immunogen can be derived from any T-lymphocyte or any T-cell line. Human origin of the cells is preferred. Most preferably, CD4 would be obtained from circulating human T lymphocytes. Purified antigen can also be used.

In a preferred embodiment, the method is directed to producing rat monoclonal antibodies to human CD4 antigen.

CD4 was originally defined by the OKT-4 antibody (Kung, P., et al, Science, 206, 347, 1979) as an antigen on the surface of a subpopulation of T cells present in circulating peripheral blood lymphocytes. The CD4 antigen has been further characterized as a glycoprotein of 55kD with structural relationships to other members of the immunoglobulin family (Thompson, J., and Zimmermann, W., Tumor Biol., 9, 63-83, 1988). The gene for CD4 has been cloned and the antigen has been expressed on cells other than human T-cells (Maddon, P. et al, Cell, 47, 333-348, 1986). The CD4 antigen has also been expressed in soluble form, making it routine to isolate the glycoprotein in pure form (Deen, K. et al, Nature, 331, 82-84, 1988.) The CD4 antigen is also expressed on T-cell lines such as CEM and HPB-ALL as well as other non-T-cell lines such as U937 of myeloid origin.

Reference to CD4 antigen refers to the 55kD glycoprotein, or fragments thereof regardless of its form or mode of expression. This includes CD4 on

circulating T-cells as well as on circulating non-T-cells such as myeloid cells. Also included are CD4 from other species which would result in rat antibodies which cross-react with human CD4. Further included is recombinant CD4 whether expressed in a human or non-human cell. Additionally, included is purified CD4, whether from natural or recombinant sources.

One preferred example of a suitable T-cell line useful as an immunogen is the T-ALL line and derivatives thereof which are publicly available. Especially preferred is HPB-ALL. (Morikawa, S. et al, Int. J. Cancer, 21, 166, 1978).

The T-cell lines are cultured by known methods appropriate to the particular T-cell line being used.

HPB-ALL can be cultured at 37°C in the presence of 5% CO<sub>2</sub> in RPMI-1640 medium containing 10% fetal calf serum, 2 mM glutamine and streptomycin (PS).

Purified T lymphocytes can be obtained as follows.

Peripheral blood mononuclear cells (PBMC) from normal donors are isolated by centrifugation on Ficoll-Hypaque (density: 1.077). T lymphocytes are then purified by rosetting according to known methods with 2-aminoethylisothiuronium-treated sheep red blood cells (SRBC-AET). PBMC at a concentration of  $5 \times 10^6$ /ml are then mixed with 1/10 volume SRBC-AET at a concentration of about  $1.0 \times 10^9$ /ml and incubated at room temperature for 15 minutes. The incubation mixture is centrifuged at 100xg for 5 minutes and after resuspending the pellet in a physiological buffer the suspension is incubated at 4°C for 1 to 2 hours.

Rosetted cells (greater than 95% of T lymphocytes) are then separated from non-rosetted cells (less than 5% T lymphocytes) by centrifugation on Ficoll-Hypaque. T-cells are recovered from the rosette by lysing the SRBC with 0.83%  $\text{NH}_4\text{Cl}$ , and then washing with physiological buffer.

Immunization protocols for rats are antigen dependent and can be readily determined by the skilled artisan. In general, a suitable immunization procedure consists of two intraperitoneal injections, the second after a three to four week interval, and, optionally an intraperitoneal booster injection six to eight weeks later.

Concentrations of antigens suitable for immunizing the rats are well known to artisans in the field. (J.J. Langone and H.V. Vunakis, Methods in Enzymology, 1986; Frontiers in Biology, 25, F. Borex, 1981).

The immunogen may be administered along with a pharmaceutically acceptable carrier such as Freund's adjuvant or saline.

As used herein "immunized cells" refers to the sensitized spleen cells of the immunized rat. The sensitized spleen cells are isolated from the rat by conventional methods. In addition "immunized cells" refers to in vitro immunized immunocompetent cells. The immunized immunocompetent cells are harvested from immune organs by conventional methods.

To produce the hybridoma, the immunized cells or immunized rat immunocompetent cells are fused with immunocytoma cells.

For fusion with immunized cells from LOU rats, IR983F non-secreting rat immunocytoma cells are preferably used. The IR983F fusion cell line (sometimes referred to as the "983 cell line") is a permanent azaguanine-resistant in vitro cell line (Bazin, H., in *Protides of the Biological Fluids*, H. Pecters, ed., 615, Pergamon, Oxford, 1982) and is publicly available. IR983F cells can also be used as a fusion partner for immunized rat immunocompetent cells.

Other fusion partners for immunized rat cells or immunized rat immunocompetent cells are Y3-AG 1.2.3, YB2/3HL.B2.G11.16AG.20, and YB2/3.O.AG.20 cell lines, all of which are also publicly available. Fusion partners for rat immunocompetent cells may be from another species, such as mouse.

The cells are grown at 37°C in the presence of 5% CO<sub>2</sub>. The skilled artisan can readily determine suitable culture conditions for other cell lines (J.J. Langone and H.V. Vanakis, Methods In Enzymology, supra).

Fusion is performed about four days after the last injection of immunogen. Fusion is performed according to many known methods, such as PEG, electrofusion and immunochemical and biochemical methods (J. Immunological Methods, 101, 153-170, 1987, S.R. Samoilovich et al.).

When hybrid clones are observed generally about the seventh day after fusion, they are transferred to 24-well plates. When supernatants in the wells become yellow, screening for specific antibody-secreting hybrid clones can be performed by any suitable method

such as indirect immunofluorescence using the immunizing cells as target cells. After a second screening of the whole blood, those hybrid clones which secrete antibodies reacting with lymphocytes, and not with granulocytes and/or monocytes, are selected. The hybrid lines are then cloned twice by limiting dilution. The hybridoma cells are either cultured in culture medium, i.e., complete DMEM, frozen in complete DMEM-7.5% DMSO and stored in liquid nitrogen by known methods (Soding, J.W., Monoclonal Antibodies: Principles and Practice, 86, Academic Press, London, 1984), or injected subcutaneously into LOU rats or into LOU/C·IgK-1b(OKA) rats to induce solid tumors.

LOU/C·IGK-1b(OKA) rats are a congenic line to the LOU/C strain, having the kappa light chain locus from the OKA strain in LOU/C background (Bazin, H. et al, J. Immunol. Methods, 71, 9, 1984). The tumor cells can be frozen in PBS-10% FCS-7.5% DMSO and stored in liquid nitrogen by known methods (Bazin, H. et al, Int. J. Cancer, 10, 568, 1972). The tumor cells can also be injected intraperitoneally to induce ascitic tumor. The cells can be collected from the ascitic fluid and stored at -80°C.

The rat monoclonal antibodies can be produced in quantity by known methods, either in vitro (See, for example, Bodeus, M. et al, Immunol. Meth., 79, 1, 1985) or in vivo (See, for example, Bazin, H. et al, Int. J. Cancer, 10, 568, 1972 and Bazin, H., Adv. Cancer Res., 50, 279, 1987).



Purification of the rat monoclonal antibodies can also be achieved by known methods (See, for example, Bazin, H., J. Immunol. Meth., 71, 9, 1984).

According to the above described methods, hybridomas that produce rat monoclonal antibodies that bind to human CD4 antigen can be produced. Further it is possible to produce hybridomas that produce monoclonal antibodies that bind to T-cell lymphocyte antigen epitopes other than those epitopes defined by murine monoclonal antibodies directed to the same antigen or antibodies that bind to a human CD4 antigen epitope other than those epitopes defined by the well known murine monoclonal antibodies OKT4 and Leu3a.

These latter monoclonal antibodies can be identified by performing conventional inhibition assays. A description of one suitable assay is described in detail in Example 5 below.

Two preferred rat monoclonal antibodies having the identifying characteristics that: (1) they bind to human CD4 antigen, and (2) they do not bind to the same CD4 antigen epitopes defined by murine monoclonal antibodies OKT4 and Leu3a, have been isolated according to the above method.

One of these preferred monoclonal antibodies, designated LO-CD4-a, was obtained after immunization with the T-ALL line HPB-ALL. This monoclonal antibody is produced by hybridoma LO-CD4-a which has been deposited at the EPACC and has Deposit No. 89012101.

The other of the preferred monoclonal antibodies has been designated LO-CD4-b and was obtained after immunization with purified T lymphocytes. This

monoclonal antibody is produced by hybridoma LO-CD4-b which has been deposited at the EPACC and has Deposit No. 89012102.

A detailed description of the production of the hybridomas producing these antibodies and of further identifying characteristics of these antibodies is set forth in the working Examples below.

A brief summary of the further identifying characteristics of the monoclonal antibodies LO-CD4-a and LO-CD4-b follows.

Reactivity Pattern of Monoclonal Antibodies LO-CD4-a and LO-CD4-b with Normal Cells

- (1) React with:
  - (a) about 50% or less of peripheral blood lymphocytes
  - (b) about 70% of PHA-activated lymphocytes
  - (c) about 60% of tonsil cells
  - (d) about 60% of thymocytes
- (2) Essentially do not react with:
  - (a) monocytes
  - (b) granulocytes
  - (c) red blood cells
  - (d) platelets
  - (e) B-lymphocyte-enriched suspension of PBL depleted of T lymphocytes
  - (f) B-lymphocyte-enriched suspension of tonsil cells' depleted of T lymphocytes.

Specificity of Monoclonal Antibodies LO-CD4-a and LO-CD4-b as Determined by Double Labeling Experiments

- (1) T-lymphocyte specific
- (2) Recognize same subpopulation of T lymphocytes recognized by murine monoclonal antibody OKT4 and Leu3a
- (3) Recognize different subpopulation of T lymphocytes than is recognized by murine monoclonal antibody OKT8

Reactivity Patterns of Monoclonal Antibodies LO-CD4-a and LO-CD4-b with Leukemia Cells

- (1) Cross react with non-T cell line U937 (expresses the T4 antigen) (Table 3)
- (2) Essentially do not react with non-T and non-B lymphoblasts (Table 2)
- (3) React with T-ALL cell lines (Table 2)
- (4) React with CEM and HPB-ALL T-cell lines (Table 4)
- (5) React weakly with MOLT-4 T-cell line (Table 4)
- (6) Do not react with JURKAT T-cell line (Table 4)

Antigenic Specificity of Monoclonal Antibodies LO-CD4-a and LO-CD4-b

Specific only for CD4 positive peripheral blood lymphocytes

Effects of Monoclonal Antibodies LO-CD4-a and LO-CD4-b  
on Lymphocyte Proliferation

(1) LO-CD4-a

- (a) Proliferation of lymphocytes induced by tetanus toxoid or allogenic-antigen induced: Inhibited about 40-60%
- (b) Proliferation of lymphocytes induced by concanavalin A, pokeweed mitogen or T3-induced proliferation: Inhibition not significant
- (c) Proliferation of lymphocytes induced by phytohemagglutinin: No inhibition

(2) LO-CD4-b

- (a) Proliferation of lymphocytes induced by tetanus toxoid or allogenic-antigen induced: Inhibited about 70%
- (b) Proliferation of lymphocytes induced by concanavalin A, pokeweed mitogen or T3-induced proliferation: Inhibition weak, i.e., about 30 to 40%
- (c) Proliferation of lymphocytes induced by phytohemagglutinin: No inhibition

Using the first method of the present invention, one may anticipate that other rat monoclonal antibodies can be developed that recognize epitopes on human T-cell lymphocyte antigens distinct from those epitopes recognized by murine monoclonal antibodies directed to the same antigens. For example, thus far investors have only been able to identify rat monoclonal

antibodies to human CD8 marker that recognize the same epitope as that recognized by murine monoclonal antibodies such as OKT8. The present method should lead to development of monoclonal antibodies that recognize a broader range of epitopes than currently used methods, when practiced by one skilled in the art.

The present invention also includes a binding protein having the identifying characteristics of monoclonal antibodies LO-CD4-a produced by hybridoma LO-CD4-a having ECACC Deposit No. 89012101, and a binding protein having the identifying characteristics of monoclonal antibodies LO-CD4-b produced by hybridoma LO-CD4-b having ECACC Deposit No. 89012102.

Recombinant DNA techniques make it possible to construct antibodies having the binding properties of one antibody in the framework of an antibody of a different class or even from a different species. Such techniques are well known in the field (Morrison, S., et al, Proc. Natl. Acad. Sci. USA, 81, 6851-6855, 1984 and Jones, P. et. al. Nature, 321, 522-525, 1986.) Also included are the so-called "single chain" antibodies in which the binding specificity of a parent antibody is reproduced in a binding protein of about 25 kDa (Bird, R. et al, Science, 242, 423-426, 1988.) All binding proteins having the binding characteristics of LO-CD4-a and LO-CD4-b, whether of natural or recombinant sources, are included in the present invention.

The present invention also provides an in vivo therapeutic method comprising introducing into a patient a rat monoclonal antibody according to the present invention.

The rat monoclonal antibody is generally administered along with a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier that is used is not critical to the method and the skilled artisan can readily determine suitable carriers.

A pharmaceutically acceptable diluent can also be employed in the therapeutic method of the present invention. The particular pharmaceutically acceptable diluent employed is not critical thereto. Examples of such diluents include physiological saline, Ringer's solution, vitamin cocktail and amino acid vitamin cocktail.

The antibodies may be administered intravenously or by any method determined by the skilled artisan to be suitable for the particular therapy.

The pharmaceutically effective amount of the antibodies of the present invention to be administered will vary depending upon the age, weight and sex of the patient and can readily be determined by the skilled artisan.

The present invention further provides an in vitro diagnostic method comprising contacting a rat monoclonal antibody according to the present invention with a suitable test sample and assaying for the antigen.

The term "test sample" means, for example, tissue biopsies, serum, acetic fluid and spinal fluid.

In vitro detection can be carried out using any of the well known in vitro immunological assays, such as those described by Young, W.W. et al, J. Exp. Med.

150, 1008-1019, 1979 and Kannagi, R. et al, Cancer Res., 43, 4997-5005, 1983).

#### EXAMPLES

The invention will now be described by reference to the following specific examples which are for illustration only and are not intended to limit the present invention.

#### EXAMPLE 1

#### PRODUCTION OF HYBRIDOMAS LO-CD4-a AND LO-CD4-b SECRETING ANTI-CD4 MONOCLONAL ANTIBODIES

##### Isolation of lymphocytes

Peripheral blood mononuclear cells (PBMN) from normal donors were isolated by centrifugation on Ficoll-Hypaque (d: 1.077). T lymphocytes were purified by resetting with 2-aminoethylisothiuronium-treated sheep red blood cells (SRBC-AET) as follows: PBMN at  $5 \times 10^6$ /ml were mixed with 1/10 volume AET-SRBC at about  $1.0 \times 10^9$ /ml and incubated at room temperature for 15 minutes. After centrifugation at 100 g for 5 minutes and new incubation at 4°C for 1 or 2 hr, rosetted cells (>95% of T lymphocytes) were separated from non-rosetted cells (<5% T lymphocytes) by centrifugation on Ficoll-Hypaque. SRBC were lysed with 0.83%  $\text{NH}_4\text{Cl}$ .

##### Immunization

Both T cell line HPB-ALL (Morikawa, S. et al. Int. J. Cancer, 21, 166, 1978) and purified T lymphocytes

were used for immunization. For the first experiment,  $2 \times 10^7$  HPB-ALL cells suspended in Freund's Complete adjuvant were injected intraperitoneally (i.p.) in a LOU/C rat. One month later,  $2 \times 10^7$  cells and seven weeks later,  $5 \times 10^7$  cells were injected i.p.. An adjuvant was not used for the second two immunogen injections. For the second experiment,  $5 \times 10^7$  purified T lymphocytes were injected twice i.p. into a LOU/C rat at a three week-interval. For the first injection, the T-lymphocytes were suspended in Freund's Complete adjuvant. No adjuvant was used for the second injection.

HPB-ALL were cultured at  $37^\circ\text{C}$  in the presence of 5%  $\text{CO}_2$  in RPMI-1640 medium containing 10% fetal calf serum, 2 mM glutamine and PS.

#### Production of rat-rat hybridomas

For fusion, IR983F (983) non-secreting rat immunocytoma cells (Bazin, H., "Production of rat monoclonal antibodies with LOU rat non-secreting IR983F myeloma cell line", in Prot. Biol. Fluids, Peeters, H., ed., Pergamon Press, Oxford and N.Y., 1982, 615) were used. The cells were cultured in DMEM medium (Gibco) supplemented with 15% horse serum (HS), 2 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin (PS) and non-essential amino acids (complete DMEM medium). 983 cells were grown at  $37^\circ\text{C}$  in the presence of 5%  $\text{CO}_2$ .

Fusion was performed four days after the last injection.

For the fusion itself, Eagle's minimum essential medium or MEM (REGA 3) without L-glutamine (GIBCO),



buffered with 10 mM HEPES (GIBCO), and supplemented with 50 mg gentamicin/liter (GIBCO) was used.

The 983 cells grow well in DMEM without HEPES (Dulbecco's modified Eagle's medium; GIBCO) supplemented with 5% heat-inactivated (30 min at 56°C) fetal calf serum (GIBCO), 5% heat-inactivated horse serum (Flow), 1% nonessential amino acids (GIBCO), 1% sodium pyruvate 100 mM: (GIBCO), and 0.1% gentamicin sulfate 50 mg/ml (GIBCO).

All serum batches used for culture purposes were tested for 3 weeks on the 983 cell line for growth efficiency. An optimal doubling time of about 17 hr or less is required.

For the hybridoma selection media, hypoxanthine-aminopterin-thymidine (HAT) (50X) and hypoxanthine-thymidine (HT) (50X) were used. The concentrations of aminopterin (Sigma), hypoxanthine (Sigma), and thymidine (Sigma) are 20, 5, and 0.8 mM, respectively.

Fusion was carried out using PEG (polyethylene glycol).

The concentration of the PEG solution used for fusion was 41.6% in fusion medium containing 8.75 ml dimethyl sulfoxide (Merck) added to 100 g of initial PEG solution to improve the fusion efficiency. Two-milliliter final PEG solution aliquots were sterilized by autoclaving or filtration and stored in an incubator at 37° for a maximum of 3 months.

Feeder layers of rat peritoneal cells were prepared the day before fusion in HAT medium, or 1-4 days before development or cloning in HT medium. The

cells were seeded at a concentration of  $2 \times 10^5$  cells/ml on the basis of 0.1 ml/microwell (96-well plates) or 1 ml/macrowell (24-well plates). At no later stage, e.g., during mass culture or additional cloning, are peritoneal cells added. The origin of the rats used as the source of the feeder layers is not important and outbred Wistar rats were therefore used.

The fusion capacity of the 983 cells depends on the growth conditions. To ensure a high yield of hybrids, the IR983F cells used for the fusion must be in a strictly exponential growth phase for at least a week. The different ways to obtain this are either to add medium every day to the culture vessel (spinner or plastic flasks), adjust the cell concentration to  $5 \times 10^5$  cells/ml, (A. M. Lebacqz-Verheyden et al, Hybridoma, 2, 355, 1983) or adjust the cell concentration back to  $10^5$  cells/ml on days 0, 3, and 5.

After anesthesia, the immunized LOU rats were bled via the carotid artery. Their spleens were removed and immersed in EMEM without serum. The organ was teased under sterile conditions on a metal grid support by a beaker. The cell suspension was allowed to stand for 10 min and the large fragments that settled were discarded.

The 983 cells and the spleen cells were centrifuged for 5 min at 250xg. The pellet was washed twice with fusion medium EMEM without serum. Cells were mixed in a ratio of one 983 cell to every five spleen cells. After centrifugation (5 min at 250xg), the supernatant was discarded and the cell pellet was detached by gentle shaking. One milliliter of warmed

PEG solution (37°) was added dropwise to  $2 \times 10^7$  cells during 90 sec of gentle shaking. After another 30 sec of shaking, 2 ml of EMEM was added during the following 90 sec to the cell mixture. Finally, 20 ml of EMEM was progressively added to dilute the PEG solution. Centrifugation for 5 min at 180 g preceded the resuspension of all cells in the selection serum-rich HAT culture medium. The cell density was adjusted to  $10^6$  cells/ml, and the cell suspension was distributed over 24 (1 ml)- and 96 (0.1 ml)-well plates containing peritoneal cells in the selection HAT medium. The medium was renewed four times before starting the screening procedure.

From the seventh day after fusion, hybrid clones were observed and they were transferred to 24-well plates. When supernatants in the wells became yellow, screening for specific antibody-secreting hybrid clones was performed by indirect immunofluorescence using immunizing cells as target cells. After a second screening of the whole blood, those hybrid clones which secreted antibodies reacting with lymphocytes, and not with granulocytes and/or monocytes, were selected. The hybrid lines were cloned twice by limiting dilution. The hybridoma cells were either cultured, frozen in complete DMEM-7.5% DMSO and stored in liquid nitrogen, or injected subcutaneously into LOU rats to induce solid tumors. The tumor cells were frozen in PBS-10% FCS-7.5% DMSO and stored in liquid nitrogen, or if necessary, tumor cells were injected i.p. to induce ascitic tumor by known methods (Bazin, H. et al, J. Immunol. Methods, 71, 9, 1984). The sera or ascitic

fluid of tumor-bearing rats were collected and stored at -80°C.

Among the hybrid clones secreting antibodies against the immunizing cells, most cross-reacted with granulocytes and/or monocytes and were not analyzed further. Five secreted a monoclonal antibody reacting selectively with lymphocytes, and two, designated LO-CD4-a and LO-CD4-b, secreted a monoclonal antibody that reacted with CD4. Monoclonal antibody LO-CD4-a was obtained after immunization with the T-ALL line (HPB-ALL14) and monoclonal antibody LO-CD4-b was obtained after immunization with purified T lymphocytes.

#### **Production and Purification of Monoclonal Antibodies LO-CD4-a and LO-CD4-b**

Monoclonal antibodies LO-CD4-a and LO-CD4-b were produced in quantity either in vitro or in vivo.

For in vitro production of the monoclonal antibodies, hybridomas LO-CD4-a or LO-CD4-b were cultured under standard conditions described for fusion of the IR983F cell line essentially according to the method described by Bazin, H. ("Production of rat monoclonal antibodies with the LOU rat non-secreting 983F myeloma cell line" in Prot. Biol. Fluids, 1982, Peeters, E. ed., 9th colloquium, 1981, Pergamon Press Oxford and N.Y., pp. 615-618). The in vitro production of the monoclonal antibodies LO-CD4-a and LO-CD4-b in plates, flasks or roller bottles was conducted essentially according to the method described by Bodeus, M. et al, Immunol. Meth., 79, 1, 1985).

For in vivo production of the monoclonal antibodies, rats of the LOU/C or the LOU/C·IgK-lb(OKA) strain (Bekers, A. et al, Immunochemistry, 11, 605, 1974) were used. The rats are perfectly histocompatible with the hybridomas, LO-CD4-a and LO-CD4-b which are LOU/C hybridomas. Rats of the LOU/C·IgK-lb strain were preferred for facilitation of purification of the monoclonal antibodies. This is because LOU/C·IgK-lb rats produce Kappa light chains of the Kappa-lb allotype and monoclonal antibodies LO-CD4-a and LO-CD4-b are immunoglobulins with the IgK-la allotype light chains. Thus, contaminating IgK-lb immunoglobulins from the host can be easily eliminated by immunoadsorption on anti-IgK-lb antibodies by standard methods.

In order to adapt the hybridoma cell to in vivo culture, the first passage of in vitro-cultured hybridoma cells was subcutaneous. This route is more successful than other routes and uses a lower number of cells than is required for the intraperitoneal route.

The rat was anesthetized and  $5 \times 10^6$  hybridomas cells were injected subcutaneously in the right flank (for convenience). The rat was observed three times a week for tumor growth. The tumor generally appears after 2 or 3 weeks, but up to 3 months can elapse before the hybridoma cells begin to proliferate.

After adequate tumor growth, the rat was anesthetized and bled to check monoclonal antibody secretion. Thereafter the rat was killed, the fur swabbed with 70% ethanol, and a piece of tumor excised

and disrupted PBS (0.15M phosphate buffered saline; pH 7.2) with a Potter homogenizer.

These cells were then used for ascites production.

The LOU/C rat easily develops ascites after intraperitoneal injection of syngeneic immunocytoma or hybridoma cells without any priming (Bazin, H. et al, Int. J. Cancer, 10, 568, 1972 and Bazin, H., Adv. Cancer Res., 50, 279, 1987). However, the yield of ascitic fluid can be greatly enhanced by pretreatment with a 1:1 (v:v) mixture of 2,6,10,14-tetramethylpentadecane (also called pristane) and incomplete Freund's adjuvant (IFA). Thus ascites production can also be achieved by intraperitoneal injection of 2 ml of the mixture, called PIFA, or of IFA at the moment of tumor passage (Kints, J.-P. et al, Enhancement of ascites production by intraperitoneal injection of pristane and incomplete Freund adjuvant. Joint meeting of Contact Group Monoclonal Antibodies and Belgian Immunological Society, URB-VUB, Brussels, Feb. 19, 1988).

The method of producing ascites was as follows.

Oil (PIFA or IFA) and cells were prepared in separate syringes in order to avoid killing the cells in the emulsion. After being anesthetized, 2 ml adjuvant was injected intraperitoneally through the right flank and cells were also injected intraperitoneally, but through the left flank to avoid accidental intrasplenic inoculation. The abdomen was gently massaged to spread the cells and oil through the whole peritoneal cavity. The rats were kept in standard conditions with food and water ad libitum. They were checked three times a week for ascitic tumor growth.

The ascites was taken when the tumor reached its maximum development. The rat was killed in an ether bell-jar and punctured with a 18G-needle. Ascites were collected in a clean flask. After swabbing with alcohol, the peritoneal cavity was opened on the left side of the abdomen. When no more liquid passed through the needle, the remaining ascitic fluid was aspirated on the side of the liver with a 20 ml syringe without a needle.

The monoclonal antibodies were purified from in vitro culture or ascites by standard ammonium sulfate procedures. Alternatively, the monoclonal antibodies were purified to homogeneity by immunoadsorption on anti-IgK-lb antibodies according to standard procedures. (See, for example, Bazin, H. et al, J. Immunol. Meth., 71, 9, 1984, for purification from ascitic fluid.)

The hybridomas that produce monoclonal antibodies LO-CD4-a and LO-CD4-b, designated LO-CD4-a and LO-CD4-b, respectively, have been deposited with the European Collection of Animal Cell Cultures and have ECACC Deposit Nos. 89012101 and 89012102, respectively.

In all Examples below, monoclonal antibody LO-CD4-a was used from a 50% saturated ammonium sulfate precipitate from ascites. Monoclonal antibody LO-CD4-b was purified to homogeneity.

## EXAMPLE 2

### REACTIVITY PATTERN OF MONOCLONAL ANTIBODIES LO-CD4-a AND LO-CD4-b WITH NORMAL CELLS

$5 \times 10^5$  cells in 100  $\mu$ l PBS-2% new born calf serum (NBCS)-0.2%  $\text{NaN}_3$  or 100  $\mu$ l whole blood were incubated for 45 min. at 4°C with 100  $\mu$ l antibody-containing supernatant or serum (1/200 dilution). Normal rat serum was used as control. Red blood cells were lysed when whole blood was used. Cells were washed once and incubated for 45 minutes at 4°C with 100  $\mu$ l of rabbit anti-rat immunoglobulin (Ig) antibody F(ab')<sub>2</sub> fragments labeled with fluorescein isothiocyanate (RARA-FITC), or goat anti-mouse Ig conjugated with FITC (GAM-FITC). Cells were washed once. Immunofluorescence intensity was examined either on a microscope or an EPICS cytofluorograph (Coulter).

Peripheral blood lymphocytes were isolated from blood of normal donors by a single step 1.077 density Ficoll-Hypaque separation. Lymphocytes were activated with PHA according to known methods. Tonsil cells and thymocytes were obtained by known methods. B lymphocyte-enriched suspensions of PBL and tonsil cells were prepared by depleting the suspensions of T-lymphocytes by CRBC rosetting by known methods.

Reference monoclonal antibodies OKT3 (CD3) and OKT4 (CD4) were obtained from Ortho Diagnostics. Reference monoclonal antibodies T11 (CD2) and T12 (CD6) were purchased from Coulter. Reference monoclonal



antibody LO-CD5-a (CD5) was produced by the present inventors.

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

TABLE 1  
REACTIVITY WITH NORMAL HEMATOPOIETIC CELLS

Cells	Mabs	
	LO-CD4-a	LO-CD4-b
Blood		
Lymphocytes		
Total	54% +++	53% +++
E+	68% +++	67% +++
E-	-	-
PHA-PBL	69% +++	74% +++
Monocytes	-	-
Granulocytes	-	-
RBC	-	-
Platelets	-	-
Tonsil cells		
Total	59% +++	60% +++
E-	-	-
Thymocytes	64% ++	66% ++

The monoclonal antibodies LO-CD4-a and LO-CD4-b reacted only with T lymphocytes (Table 1). No reaction was found with monocytes, granulocytes, red blood cells, platelets or with a B lymphocyte-enriched suspension prepared by depleting T lymphocytes from PBL and tonsil by SRBC rosetting. Monoclonal antibodies LO-CD4-a and LO-CD4-b reacted strongly with around 55% of PBL and about 70% of T lymphocyte-enriched PBL. Thus, these two monoclonal antibodies are specific for a subset of T lymphocytes.

Typical reactivity patterns of monoclonal antibodies LO-CD4-a and LO-CD4-b and of reference pan-T or subset-T specific monoclonal antibody OKT3, T11, T12, LO-CD5-a and OKT4 are shown in Figure 1.

In Fig. 1A, the left panel shows the reactivity patterns with PBLs and the right panel shows the reactivity patterns with P. activated PBLs. In Fig. 1B, the left panel shows reactivity patterns with tonsil cells and the right panel shows reactivity with thymocytes. The X-axis represents fluorescence intensity and the Y-axis represents cell number.

The profiles of monoclonal antibodies LO-CD4-a and LO-CD4-b were identical to that of CD4 monoclonal antibody OKT4, whatever kind of cell was concerned. Monoclonal antibodies LO-CD4-a and LO-CD4-b reacted strongly with around 65% of thymocytes.

### EXAMPLE 3

#### DOUBLE LABELING

For double immunofluorescence, tonsil cells or PBMNs were labeled with rat monoclonal antibodies and RARA-FITC as described in Example 2 above. Then the cells were incubated with phycoerythrin-conjugated monoclonal antibodies for 45 minutes at 4°C.

Tonsil cells were obtained by known methods. PBMNs from normal donors were isolated as described in Example 1. centrifugation on Ficoll-Hypaque (d:1.077).

Reference monoclonal antibodies T11 (CD4) and B4 (CD19) were purchased from Coulter and reference monoclonal antibodies OKT4 (CD4) and OKT8 (CD8) were purchased from Ortho Diagnostics.

In order to confirm that LO-CD4-a and LO-CD4-b are T lymphocyte specific and to find out if the subpopulation of T lymphocytes recognized by monoclonal antibodies LO-CD4-a and LO-CD4-b is indeed the one recognized by OKT4, tonsil cells (Figure 2) or PBMN (Figure 3) were first labeled with rat monoclonal antibodies LO-CD4-a and LO-CD4-b and RARA-FITC, followed by phycoerythrin-conjugated mouse reference monoclonal antibodies T11 (CD4), B4 (CD19) (Figure 2), OKT4 (CD4) or OKT8 (CD8) (Figure 3). Cells that labeled with monoclonal antibody LO-CD4-a made up about 78% of T11+ lymphocytes (Figure 2). In contrast, the percentage of lymphocytes double-labeled with B4 and rat monoclonal antibody LO-CD4-a remained low (4-6%) (Figure 2). This weak positivity could be due to a specific binding to Fc receptors on B cells. Regarding

subsets of peripheral T lymphocytes, more than 99% of T4+ lymphocytes were also labeled with LO-CD4-a or LO-CD4-b and less than 2% of PBL was labeled with OKT4 alone or with the rab monoclonal antibody alone (Figure 3). Less than 6% of PBL cells was double-labeled with OKT8 and LO-CD4-a or LO-CD4-b (Figure 3). Hence, rat monoclonal antibodies LO-CD4-a and LO-CD4-b are T lymphocyte specific. Further, the subpopulation of T lymphocytes recognized by LO-CD4-a and LO-CD4-b is exactly that recognized by OKT4, not by OKT8 (T4+, T8-).

#### EXAMPLE 4

#### REACTIVITY PATTERNS OF LO-CD4-a AND LO-CD4-b WITH LEUKEMIC CELLS

In order to detect cross-reactions with immature B or myeloid cells and to delimit at which stage of differentiation the different T antigens appear or disappear, leukemias, lymphomas (Table 2) and the leukemia-derived cell lines (Tables 3 and 4) were studied.

Indirect immunofluorescence was performed as described in Example 2.

Leukemia and lymphoma cells T-ALL, T-CLL, B-CLL, B-NHL and CALL were obtained from leukemic patients by known methods. Leukemia-derived cell lines of B lineage (Nalm-1, KM3, Nalm-6, Raji and Daudi), Myeloid line (U937, HL-60 and K562) and CCRF-CEM, HPB-ALL, MOLT-4 and JURKAT are all publicly available.

HPB-ALL, CCRF-CEM, MOLT-4, JURKAT, Raji, Daudi, Nalm-1, Nalm-6, KM3, U937, K562 and HL-60 were cultured at 37°C in the presence of 5% CO<sub>2</sub> in RPMI-1640 medium containing 10% fetal calf serum, 2 mM glutamine and PS.

Monoclonal antibodies OKT3 (CD3) and OKT4 (CD4) were purchased from Ortho Diagnostics. Monoclonal antibodies T11 (CD2) and T12 (CD6) were purchased from Coulter. Monoclonal antibody Leu3a was purchased from Becton-Dickenson. Monoclonal antibody LO-CD5-a (CD5) was produced by the present inventors and used as an example of an anti-CD5 monoclonal antibody.

TABLE 2

REACTIVITY WITH LEUKEMIA CELLS

Cells	Mabs	
	LO-CD4-a	LO-CD4-b
T-ALL		
1	94% +++	97% +++
2	56% +++	64% +
3	24% ±	24% ±
4	20% ±	19% ±
5	89% ++	89% ++
6	54% ++	61% ++
T-CLL	94% ++	93% ++
B-CLL		
1	3% ++	5% ++
2	4% ++	4% ++
3	2% ++	3% ++
4	-	-
B-NHL	4% +	5% +
cALL		
1	8% ++	7% ++
2	2% +	2% +
3	-	-

TABLE 3

REACTIVITY WITH NON-T CELL LINES

Cells	Mabs	
	LO-CD4-a	LO-CD4-b
B lineage		
Nalm-1	-	-
KM3	-	-
Nalm-6	-	-
Raji	-	-
Daudi	-	-
Myeloid lines		
U937	++	++
HL-60	±	±
K562	-	-

TABLE 4

REACTIVITY WITH T CELL LINES

Monoclonal Antibodies	CEM	HPB-ALL	MOLT-4	JURKAT
OKT3	-	++	-	±
T11	+++	+++	+++	++
T12	+	+	+	-
LO-CD5-a	++	++	++	±
Leu3a	+++	+++	±	-
OKT4	+++	+++	±	-
LO-CD4-a	+++	+++	±	-
LO-CD4-b	+++	+++	±	-



No cross-reaction of monoclonal antibodies LO-CD4a and LD-CD4-b with non-T cell lines, listed in Table 3, was found, except that with U937 just as OKT4 and Leu3a did (data not shown). This cell line is known to express the T4 antigen.

Non-T non-B lymphoblasts were not labeled by monoclonal antibody LO-CD4-a or LO-CD4-b. (Table 2). The detection of 2-12% positive cells can be assigned to the presence of residual normal lymphocytes. The 2-10% cells brightly labeled with LO-CD4-a and LO-CD4-b are probably due to residual normal cells. LO-CD-4-a and LO-CD4-b stained all T-ALL cell lines tested (Table 2) with variable intensity as did OKT4 (data not shown).

Table 4 shows that the LO-CD4-a and LO-CD4-b antigen is present on CEM and HPB-ALL T-cell lines, weakly expressed on the MOLT-4 T-cell line and not expressed on the JURKAT T-cell line. This is the same pattern exhibited by monoclonal antibodies Leu3a and OKT4.

#### EXAMPLE 5

#### INHIBITION OF OKT4 AND Leu3a BINDING

Monoclonal antibodies LO-CD4-a and LO-CD4-b recognized exactly the same subset of T lymphocytes as OKT4 (Figure 3). Therefore blocking of binding of mouse CD4 monoclonal antibodies by LO-CD4-a and LO-CD4-b was investigated to determine if LO-CD4-a and

LO-CD4-b complete for the same epitopes as either OKT4 or Leu3a.

Lymphocytes were isolated from peripheral blood by a single step 1.077 density Ficoll-Hypaque separation (PBLs). Mouse monoclonal antibody OKT4 (CD4) was purchased from Ortho Diagnostics and monoclonal antibody Leu3a (CD4) was purchased from Becton-Dickinson. Mouse-anti rat monoclonal antibody was obtained from Jackson Immunoresearch Laboratories. OKT4 mouse anti-CD4 FITC conjugate (OKT4FITC) was purchased from Ortho Diagnostics, Leu3a mouse anti-CD4 phycoerythrin conjugate (Leu3aPE) was purchased from Bectin-Dickenson, and mouse anti-rat FITC conjugate (MARFITC) was purchased from Jackson, Immunoresearchb Laboratories.

The PBL concentration was adjusted to  $1 \times 10^7$  cells/ml in Assay Buffer (AB) (1% BSA, 0.1%  $\text{NaN}_3$ , 6 $\mu\text{M}$  colchicine in PBS, pH 7.4). Monoclonal antibodies LO-CD4-a and LO-CD4-b were diluted to 100  $\mu\text{g}/\text{ml}$  protein in AB. Then 100  $\mu\text{l}$  of cell suspension was incubated with 10  $\mu\text{l}$  of either diluted rat monoclonal antibody LO-CD4-a or LO-CD4-b for 30 min. at 4°C with occasional agitation. The cells were washed by resuspending in 2.0 ml of AB and then centrifuged at 400xg for 5 min. Washing and centrifugation was repeated two more times. Next the washed cells in 100  $\mu\text{l}$  AB were incubated with 10  $\mu\text{l}$  (100  $\mu\text{g}/\text{ml}$  protein) of MARFITC, OKT4FITC, or Leu3aPE for 30 min. at 4°C with occasional agitation. Alternatively, the cells were incubated with 10  $\mu\text{l}$  of OKT4FITC or Leu3aPE without preincubation with monoclonal antibodies LO-CD4-a or LO-CD4-b. The cells

were washed twice as described above and fixed in 0.8 ml of formalin in PBS. The cells were analyzed on a Becton Dickinson FACScan flow cytometer.

The data are shown in Figure 4.

In Figure 4, the X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) and the Y-axis in all panels represents counts full scale. Figs. 4(A) to 4(G) show the data for monoclonal antibody LO-CD4-a and Figs. 4(A) to 4(N) show the data for monoclonal antibodies LO-CD4-b.

Figs. 4(A) and 4(B) are controls showing green and red autofluorescence for PBLs in the absence of added antibody conjugate, respectively. Fig. 4(C) is a control showing the binding of monoclonal antibody LO-CD4-a to PBLs detected by MARFITC. Fig. 4(D) is a control showing the fluorescence intensity of PBLs after incubation with OKT4FITC only. Fig. 4(E) shows the fluorescence intensity of PBLs incubated with OKT4FITC after preincubation with LO-CD4-a. Fig. 4(F) is a control showing the fluorescence intensity of PBLs after incubation with Leu3aPE only. Fig. 4(G) shows the fluorescence intensity of PBLs incubated with Leu3aPE after preincubation with LO-CD4-a.

Figs. 4(H) to 4(N) parallel Figs. 4(A) to 4(G), except that where monoclonal antibody LO-CD4-a was used in Figs. 4(A) to 4(G), monoclonal antibody LO-CD4-b was used in Figs. 4(H) to 4(N).

The data in Figure 4 is summarized in Table 5 below.

TABLE 5

EFFECT OF MONOCLONAL ANTIBODIES LO-CD4-a and LO-CD4-b  
ON BINDING OF OKT4 AND Leu3a TO PBLs

---

	Relative Fluorescence Intensity
OKT4 FITC only	100
OKT4FITC after preinc. with LO-CD4-a	97
Leu3aPE only	100
Leu3aPE after preinc. with LO-CD4-a	99
OKT4FITC only	100
OKT4FITC after preinc. with LO-CD4-b	99.6
Leu3aPE only	100
Leu3aPE after preinc. with LO-CD4-b	99

---

From Fig. 4 and Table 5 it is apparent that preincubation with either LO-CD4-a or LO-CD4-b does not reduce the Relative Fluorescence Intensity for either OKT4FITC or Leu3aPE. Thus, LO-CD4-a and LO-CD4-b do not compete for either of the epitopes for which OKT4 and Leu3a are specific.

## EXAMPLE 6

### BINDING OF MONOCLONAL ANTIBODIES LO-CD4-a AND LO-CD4-b TO CD4 POSITIVE DEPLETED PBLs

This Example demonstrates that monoclonal antibodies LO-CD4-a and LO-CD4-b bind to the same lymphocyte subpopulation as OKT4.

Lymphocytes were isolated from peripheral blood by a single step 1.077 density Ficoll-Hypaque separation (PBLs).

Goat anti-rat monoclonal antibody was obtained from Jackson Research Laboratories. Mouse monoclonal antibody OKT4 (CD4) and OKT8 (CD8) were purchased from Ortho Diagnostics. Mouse monoclonal antibody Leu3 (CD4) was purchased from Becton-Dickinson. Goat anti-rat phycoerythrin conjugate (GARPE), monoclonal antibody OKT4FITC, OKT8FITC and Leu3aPE were prepared according to conventional methods. Sheep anti-mouse coated magnetic microparticles (SAM Beads) were obtained from DYNAL INC.

The PBL concentration was adjusted to  $1 \times 10^7$  cells/ml with AB (see Example 5).  $2 \times 10^7$  cells were incubated with 200  $\mu$ l (100  $\mu$ g/ml) OKT4 for 30 min. at 4°C with occasional agitation. The cells were washed by resuspending in 2 ml of AB and then centrifuged at 400xg for 5 min. Washing and centrifugation was repeated two more times. The cells were resuspended in 1 ml of AB and incubated with SAM Beads (40:1 beads per cell) for 30 min. at 4°C with agitating every 5 min. A magnetic field was then applied for 1 min. and

nonadherent cells were removed. The nonadherent cells were washed twice with AB and the concentration adjusted to  $1 \times 10^7$  cells/ml with AB.

The CD4 depleted cell population was then immunofluorescently stained for OKT4, Leu3a, OKT8, LO-CD4-a and LO-CD4-b by conventional methods, and analyzed on a Becton Dickinson FACScan flow cytometer.

Alternatively, undepleted cells were adjusted to  $1 \times 10^7$  cells/ml with AB, washed as described in Example 5 and immunofluorescently stained for OKT4, Leu3a, OKT8, LO-CD4-a and LO-CD4-b by conventional methods, and then analyzed on a Becton Dickinson FACScan flow cytometer.

The data are shown in Figure 5.

In Fig. 5, the X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) and the Y-axis in all panels represents counts full scale.

Figs. 5(A) to 5(H) show the data for undepleted PBLs and Figs. 5(I) to 5(P) show the data for CD4 depleted PBLs.

Figs. 5(A) and 5(B) are controls showing green and red autofluorescence, respectively, for PBLs in the absence of added antibody conjugate. Figs. 5(C) and 5(D) show binding of monoclonal antibodies LO-CD4-a and LO-CD4-b, respectively, to undepleted PBLs detected by GARPE. Fig. 5(E) is a control showing immunofluorescence of PBLs after incubation with GARPE. Figs. 5(F), 5(G) and 5(H) show the fluorescence intensity of undepleted PBLs after incubation with Leu3aPE, OKT4FITC and OKT8FITC, respectively.

Figs. 5(I) and 5(P) parallel Figs. 5(A) to 5(H), except that depleted PBLs were used in place of undepleted PBLs.

The data in Figure 5 is summarized in Table 6 below.

TABLE 6

BINDING OF MONOCLONAL ANTIBODIES LO-CD4-a AND LO-CD4-b TO UNDEPLETED AND DEPLETED PBLs

	% PBLs Labeled
<u>Undepleted PBLs</u>	
OKT4 positive	44
Leu3a positive	39
OKT8 positive	31
LO-CD4-a positive	41
LO-CD4-b positive	42
<u>CD4 Depleted PBLs</u>	
OKT4 positive	2
Leu3a positive	5
OKT8 positive	58
LO-CD4-a positive	7
LO-CD4-b positive	8

From Fig. 5 and Table 6 it is apparent that depletion of CD4 positive lymphocytes from PBLs was nearly complete with only 2% OKT4 positive cells and 5% Leu3a remaining. Also, both LO-CD4-a and LO-CD4-b positive lymphocytes were significantly reduced in the depleted PBL population. This data indicates that both LO-CD4-a and LO-CD4-b rat monoclonal antibodies are indeed specific for the same lymphocyte subpopulation as OKT4.

#### EXAMPLE 7

##### TWO COLOR FLUORESCENCE PBL BINDING ASSAY

This Example shows that monoclonal antibodies LO-CD4-a and LO-CD4-b bind exclusively to CD4 positive lymphocytes.

Lymphocytes were isolated from peripheral blood by a single step 1.077 density Ficoll-Hypaque separation (PBLs).

Mouse Leu3A (CD4) was purchased from Becton-Dickenson. Mouse-anti-rat monoclonal antibody was obtained from Jackson Immunoresearch Laboratories. Leu3aPE was purchased from Bectin-Dickenson and MARFITC was purchased from Jackson Immunoresearch Laboratories.

The PBL concentration was adjusted to  $1 \times 10^7$  cells/ml in AB (see Example 5). 100  $\mu$ l of cell suspension was incubated with 10  $\mu$ l (100  $\mu$ g/ml) of Leu3aPE for 30 min. at 4°C with occasional agitation. The cells were washed by resuspending in 2 ml of AB and then centrifuged at 400xg for 5 min. Washing and



centrifugation was repeated two more times. Cells were resuspended in 100  $\mu$ l of AB. Monoclonal antibodies LO-CD4-a and LO-CD4-b were diluted to 100  $\mu$ g/ml in AB. The washed cells were resuspended in 100  $\mu$ l AB and incubated with 10  $\mu$ l of diluted antibody for 30 min. at 4°C with occasional agitation. The cells were washed twice with AB and resuspended in 100  $\mu$ l of AB. MARFITC was then diluted 1:10 (v:v) (100  $\mu$ g/ml) in AB. 10  $\mu$ l of diluted MARFITC was then added to the 100  $\mu$ l of washed cells and incubated for 20 min. at 4°C with occasional agitation. Alternatively, before adding MARFITC, the cells ( $1 \times 10^7$  in 100  $\mu$ l AB) were incubated with Leu3aPE 10 $\mu$ l (100  $\mu$ g/ml) and washed, as described above. The incubation mixture was centrifuged and washed twice with AB as described above and fixed in 0.8 ml 1% formalin in PBS. The cells were analyzed on a Becton Dickinson FACScan flow cytometer.

Dot plot analyses of the flow cytometry data of red fluorescence vs. green fluorescence was then carried out. The results were shown in Fig. 6, where the X-axis of each plot represents green fluorescence (Fluorescence 1) and the Y-axis of each plot represents red fluorescence (Fluorescence 2).

In the dot plot analysis, those cells which remain unbound by either Leu3a or the LO-CD-4 rat monoclonal antibodies are represented in the third quadrant (see Figs. 6(A) and 6(E)). These cells have the least amount of fluorescent character. Cells bound by Leu3aPE are represented in the first quadrant as having red fluorescent character and those cells bound by either rat anti-CD4 monoclonal are represented in the

fourth quadrant as having green fluorescent character. The particular cell population that binds both the Leu3a and the rat anti-CD4 monoclonals are represented in quadrant 2 having both green and red fluorescent character. If either LoCD4 rat monoclonal exclusively binds CD4 positive cells, quadrants 2 and 3 will be the only quadrants occupied. If either quadrants 1 and/or 4 are occupied as well as quadrant 2, this indicates that the immunoglobulin is not exclusively specific for CD4.

Figs. 6(A) to 6(D) show the data for monoclonal antibody LO-CD4-b, and Figs. 6(E) to 6(I) show the data for monoclonal antibody LO-CD4-a.

Fig. 6(A) is a control showing the dot plot for red and green autofluorescence of PBLs in the absence of added antibody conjugate. Fig. 6(B) shows the dot plot for fluorescence of PBLs incubated with monoclonal antibody Leu3aPE. Fig. 6(C) shows the dot plot for fluorescence of PBLs incubated with MARFITC after preincubation with monoclonal antibody LO-CD4-b. Fig. 6(D) shows the dot plot for fluorescence of PBLs incubated with monoclonal Leu3PE and then MARFITC after preincubation with LO-CD4-b.

Figs. 6(E) to 6(H) parallel Figs. 6(A) to 6(D), except that monoclonal antibody LO-CD4-a was used instead of monoclonal antibody LO-CD4-b. Fig. 6(I) shows the dot plot for fluorescence of PBLs incubated with MARFITC after preincubation with Leu3a.

The data from Figs. 6(A) to 6(D) and from Figs. 6(E) to 6(I) is summarized in Tables 7 and 8, respectively, below.

**TABLE 7**

**LO-CD4-b TWO COLOR ASSAY**

---

	Quad 1 (red)	Quad 2 (green)	Quad 3 (auto)	Quad 4 (red & green)
Leu3a +	48.3%	0.5%	51.0%	0.2%
LO-CD4-b +	0.0%	0.2%	52.4%	47.6%
Two Color	1.2%	47.1%	51.5%	0.2%

---

**TABLE 8**

**LO-CD4-a TWO COLOR ASSAY**

---

	Quad 1 (red)	Quad 2 (green)	Quad 3 (auto)	Quad 4 (red & green)
Leu3a +	41.2%	0.1%	58.7%	0.0%
LO-CD4-a +	0.1%	0.2%	58.1%	41.6%
Two Color	1.8%	40.4%	57.6%	0.0%

---

The data from this Example indicates that both LO-CD4-a and LO-CD4-b are specific for only CD4 positive PBLs. Greater than 96% of Leu3a positive lymphocytes became double labelled by either LO-CD4-a or LO-CD4-b. No other PBL population was double labelled by LO-CD4-a or LO-CD4-b.

### EXAMPLE 8

#### INDIRECT BINDING ASSAY OF MONOCLONAL ANTIBODIES LO-CD4-a AND LO-CD4-b TO PERIPHERAL BLOOD LYMPHOCYTES

This Example establishes binding patterns of monoclonal antibodies LO-CD4-a and LO-CD4-b to peripheral blood lymphocytes (PBLs).

The lymphocyte population was obtained from venous peripheral blood by a single step 1.077 density Ficoll-Hypaque separation.

Monoclonal antibodies OKT4 (CD4) and OKT8 (CD8) were purchased from Ortho Diagnostics and Leu3a (CD4) was purchased from Becton Dickinson. Goat anti-rat phycoerythrin conjugate (GARPE) and goat anti-mouse phycoerythrin conjugate (GAMPE) were obtained from Jackson Research Laboratories.

The PBL concentration was adjusted to  $1 \times 10^7$  cells/ml in AB (see Example 5). Rat monoclonal antibodies LO-CD4-a and LO-CD4-b and mouse monoclonal antibodies OKT4, OKT8 and Leu3a were used as received from the vendor. 10  $\mu$ l of the monoclonal antibodies was added to 100  $\mu$ l of the cell suspension and

incubated for 30 min. at 4°C with occasional agitation. The cells were washed by resuspending in 2 ml of AB and then centrifuged at 400 xg for 5 min. Washing and centrifugation was repeated two more times. Cells were resuspended in 100 µl of AB. The conjugate GARPE or GAMPE was diluted 1:10 (v:v) (100 µg/ml) in AB. 10 µl of the appropriate diluted conjugate was added to the 100 µl of washed cells and incubated for 20 min. at 4°C with occasional agitation. The cells were washed twice as described above and fixed in 0.8 ml 1% formalin in PBS. The cells were analyzed on a Becton Dickinson FACScan flow cytometer.

The data are shown in Figure 7.

In Fig. 7, the X-axis in all panels represents green fluorescence (Fluorescence 1) or red fluorescence (Fluorescence 2) and the Y-axis in all panels represents counts full scale. Figs. 7(A) and 7(B) are controls showing green and red autofluorescence, respectively, for PBLs in the absence of added antibody conjugate. Fig. 7(C) is a control showing the fluorescence for PBLs incubated only with GARPE. Fig. 7(D) shows the fluorescence intensity of PBLs incubated with OKT8 as detected by GAMPE. Figs. 7(E) and 7(F) show the fluorescence intensity of PBLs incubated with OKT4 and Leu3a, respectively, as detected by GAMPE. Figs. 7(G) and 7(H) show the fluorescence intensity of PBLs incubated with LO-CD4-a and LO-CD4-b, respectively, as detected by GARPE.

The data in Fig. 7 are summarized in Table 9 below.

TABLE 9  
 INDIRECT BINDING ASSAY OF LD-CD4-a AND  
 LD-CD4-b TO PBLs

Ab	% PBLs Bound
GARPE	0
OKT4	44
Leu3a	39
LO-CD4-a	42
LO-CD4-b	42

From the results in Fig. 7 and Table 9, it is evident that LO-CD4-a and LO-CD4-b are not "pan lymphocyte" antibodies because both bind less than 50% of a PBL population.

## EXAMPLE 9

### FUNCTIONAL ANALYSIS OF LO-CD4-a AND LO-CD4-b

In order to find out whether the antigens defined by LO-CD4-a and LO-CD4-b have any functional significance in immune response, the effect of these monoclonal antibodies on lymphocyte proliferation induced by lectins, antigens or OKT3 was studied.

For all experiments, cells were cultured at 37°C in the presence of 5% CO<sub>2</sub> in 96-well plates containing 200 µl RPMI-1640 medium supplemented with 20% human AB serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 mM Hepes. 10<sup>5</sup> peripheral blood mononuclear cells (PBMN) were cultured with 2 µg/ml PHA, 20 µg/ml ConA, 0.5 µg/ml T3 or 10 µg/ml pokeweed mitogen (PWM) or 20 mU/ml tetanus toxoid (TT) in the absence or the presence of rat monoclonal antibody serum diluted at 1/500. The PBMNs were isolated as described in Example 1. For the allogeneic mixed lymphocyte reaction, 10<sup>5</sup> PBMN from two individual donors were cultured together in the presence or absence of rat monoclonal antibody. 3H-thymidine (3H-TdR, 6.5 Ci/mmol, New England Nuclear) was added to each well (5 µCi/well) 3 days after stimulation with ConA, PHA, PWM, or T3, or 5 days after stimulation with TT or in MLR and incubation was continued overnight. The cells were harvested with a cell harvester (SKATRON) and 3H-TdR incorporation was measured in a scintillation counter.

The results are shown in Table 10 below.

TABLE 10

EFFECT OF MONOCLONAL ANTIBODIES LO-CD4-a AND  
LO-CD4-b ON LYMPHOCYTE PROLIFERATION  
INDUCED BY LECTINS, OKT3 OR ANTIGENS

Monoclonal Antibody	<u>Percentage of Control</u>		
	PHA	ConA	PWM
Diluent Control	100	100	100
LO-CD4-a	106±4	88±2	90±6
LO-CD4-b	116±6	59±16	72±1

Monoclonal Antibody	<u>Percentage of Control</u>		
	T3	TT	MLR
Diluent Control	100	100	100
LO-CD4-a	92±9	43±4	67±13
LO-CD4-b	73±9	32±5	34±9

Results are expressed as mean ± standard deviation.



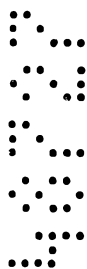
From Table 10, it is apparent that the effects of monoclonal antibodies LO-CD4-a and LO-CD4-b on lymphocyte proliferation are quantitatively different, LO-CD4-b always having stronger inhibitive effect than LO-CD4-a. The monoclonal antibodies interfered strongly with proliferation of lymphocytes induced by the soluble antigen tetanus toxoid (TT) and by cell-bound antigen (MLR): About 70% inhibition by LO-CD4-b and 40-60% inhibition by LO-CD4-a was observed. Inhibition of ConA, PWM and T3-induced proliferation was weak (30-40%, by LO-CD4-b, and was not significant by LO-CD4-a. Neither monoclonal antibody had a suppressive effect on PHA-induced proliferation.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Cell lines were deposited with the European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 21 January 1989 as set out below:

Cell Line                    LO-CD4-a  
Accession No.:            ECACC 89012101

Cell Line                    LO-CD4-b  
Accession No.:            ECACC 89012102



The claims defining the invention are as follows:

1. A method of producing rat monoclonal antibodies that bind to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b, comprising the steps of:
- (1) immunizing a rat or rat immunocompetent cells in vitro with an immunogen comprising CD4 antigen;
  - (2) fusing immunized cells from said rat or immunized rat immunocompetent cells with immunocytoma cells;
  - (3) selecting hybridoma cells that produce antibody that binds to the human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b;
  - (4) culturing the selected hybridoma cells; and
  - (5) recovering said antibody.
2. The method of Claim 1, wherein said immunogen is a T-cell line that expresses CD4 antigen or purified T lymphocytes.
3. The method of Claim 1 or Claim 2 wherein said immunocytoma cells are rat or mouse immunocytoma cells.
4. A method of producing rat monoclonal antibodies that bind to human CD4 antigen epitope substantially as hereinbefore described with reference to any one of the Examples.
5. A hybridoma that produces a rat monoclonal antibody that binds to human CD4 antigen epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.
6. The hybridoma of Claim 5, wherein binding to human CD4 antigen by murine monoclonal antibodies OKT4 and Leu3a is not inhibited by the binding of said rat monoclonal antibody to the antigen.
7. A hybridoma having the identifying characteristics of hybridoma LO-CD4-a having ECACC Deposit No. 89012101.
8. A hybridoma having the identifying characteristics of hybridoma LO-CD4-b having ECACC Deposit No. 8912102.
9. A hybridoma that produces a rat monoclonal antibody that binds to human CD4 antigen epitope substantially as hereinbefore described with reference to any one of the Examples.
10. A rat monoclonal antibody that binds to human CD4 antigen



epitope that in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b.

11. The rat monoclonal antibody of Claim 10, wherein binding to human CD4 antigen by murine monoclonal antibodies OKT4 and Leu3a is not  
5 inhibited by the binding of said rat monoclonal antibody to the antigen.

12. A rat monoclonal antibody having the identifying characteristics of monoclonal antibody LO-CD4-a produced by hybridoma LO-CD4-a having ECACC Deposit No. 89012101.

13. A rat monoclonal antibody having the identifying  
10 characteristics of monoclonal antibody LO-CD4-b produced by hybridoma LO-CD4-b having ECACC Deposit No. 89012102.

14. A binding protein having the identifying characteristics of monoclonal antibody LO-CD4-a produced by hybridoma LO-CD4-a having ECACC Deposit No. 89012101.

15. A binding protein having the identifying characteristics of monoclonal antibody LO-CD4-b produced by hybridoma LO-CD4-b having ECACC Deposit No. 89012102.

16. A method of producing rat monoclonal antibodies that bind to a human T-cell lymphocyte antigen epitope that in turn binds to a rat  
20 monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b comprising the steps of:

(a) immunizing a rat or rat immunocompetent cells in vitro with an immunogenic amount of an antigen comprising T-cell lymphocyte markers;

(2) fusing immunized cells from said rat or immunized rat  
25 immunocompetent cells with immunocytoma cells;

(3) selecting hybridoma cells that produce antibody that binds to the human T-cell lymphocyte antigen epitope which in turn binds to a rat monoclonal antibody produced by a reference hybridoma LO-CD4-a or LO-CD4-b;

30 (4) culturing the selected hybridoma cells; and

(5) recovering said antibody.

17. A rat monoclonal antibody produced by the method of Claim 16, wherein said monoclonal antibody binds to a human T-cell lymphocyte antigen epitope which in turn binds to a rat monoclonal antibody produced  
35 by a reference hybridoma LO-CD4-a or LO-CD4-b.



18. A method of producing rat monoclonal antibodies that bind to a human T-cell lymphocyte antigen epitope substantially as hereinbefore described with reference to any one of the Examples.

19. An in vivo therapeutic method for the treatment of immune disorders, which method comprises administering to a patient an amount effective for the treatment of immune disorders of a rat monoclonal antibody according to any one of Claims 10 to 13 or 17.

20. An in vitro diagnostic method for the diagnosis of an immune disorder in a patient, which method comprises contacting a rat monoclonal antibody produced according to any one of Claims 1, 2, 3, 4, 16 or 18 with a suitable test sample and assaying for the CD4 antigen.

21. A rat monoclonal antibody being the product of the method of any one of Claims 1, 2, 3, 4, 16, or 18.

DATED this TWENTY-SEVENTH day of JANUARY 1993

Abbott Laboratories

Patent Attorneys for the Applicant  
SPRUSON & FERGUSON

20 4 90 4877

PERIPHERAL BLOOD LYMPHOCYTES

PHA-ACTIVATED LYMPHOCYTES

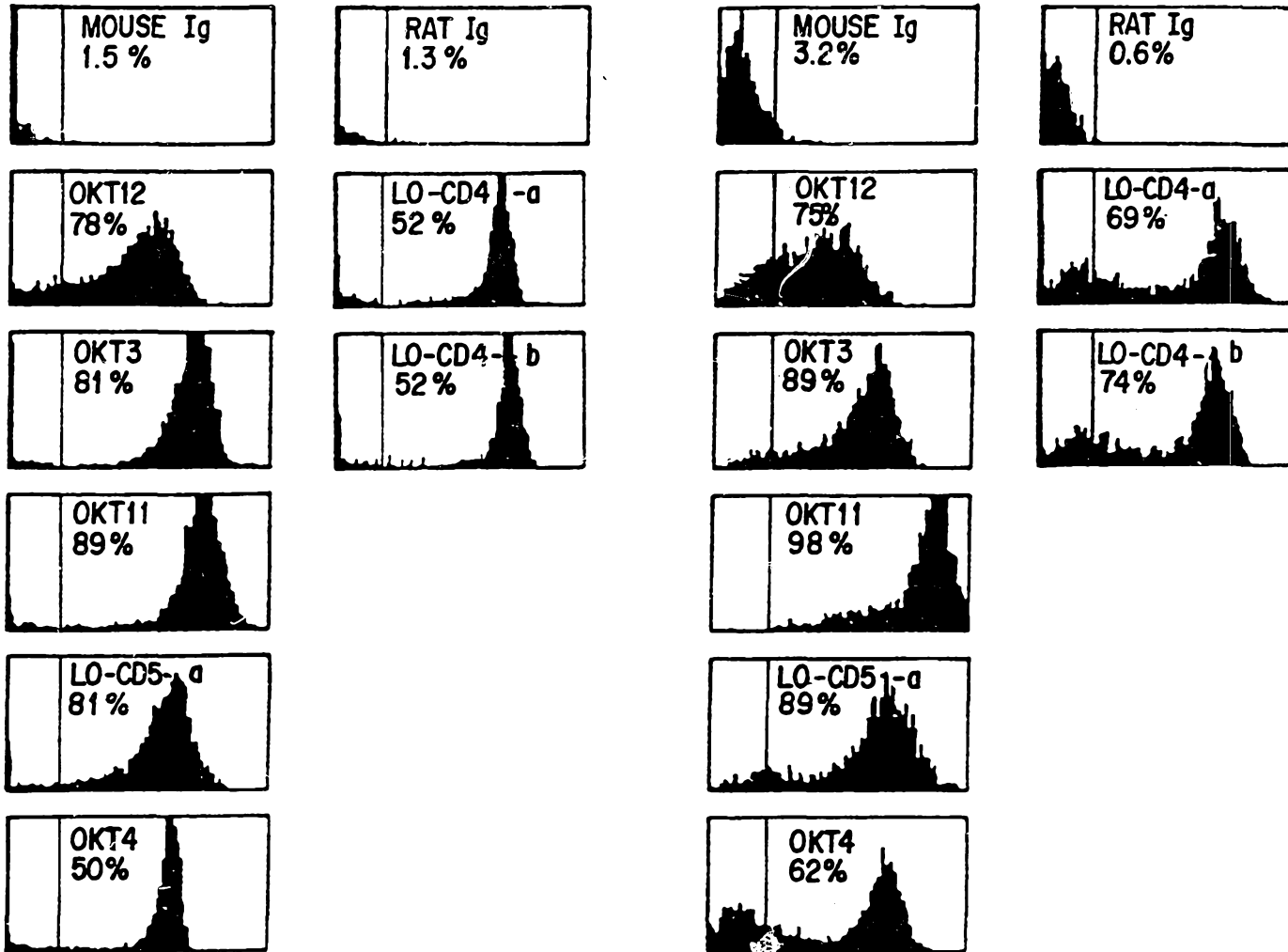


FIG. 1(A)

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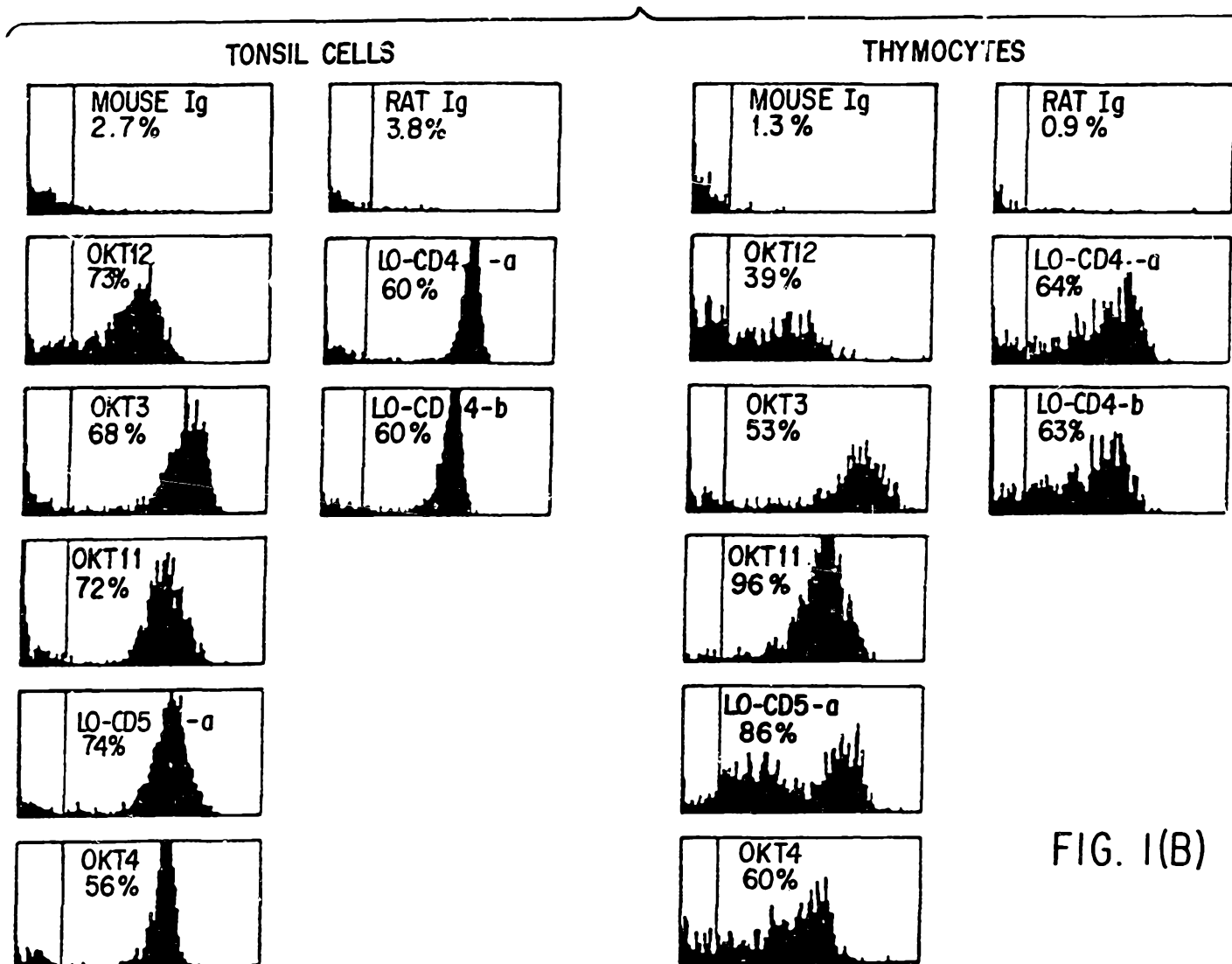


FIG. 1(B)

20 4 30

5 5 5

FIG. 2

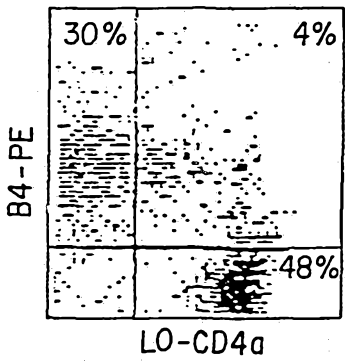
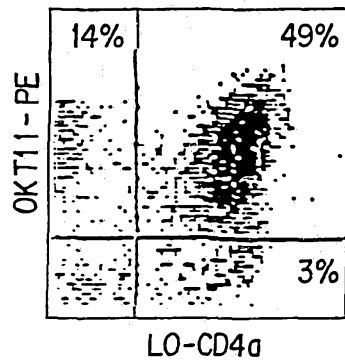
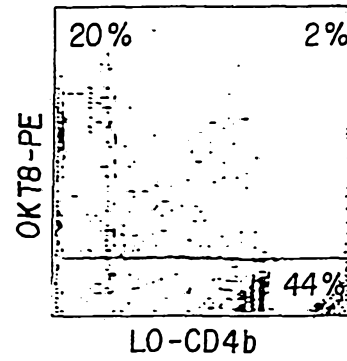
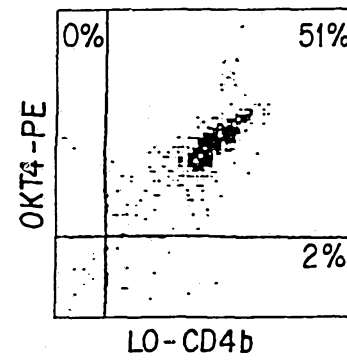
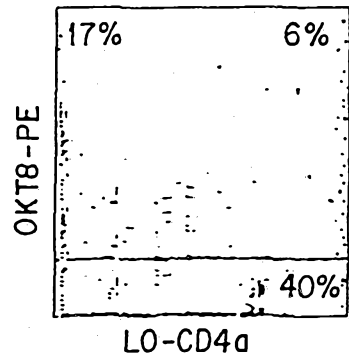
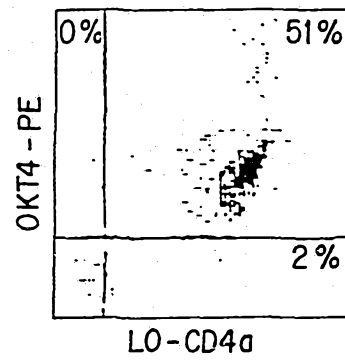
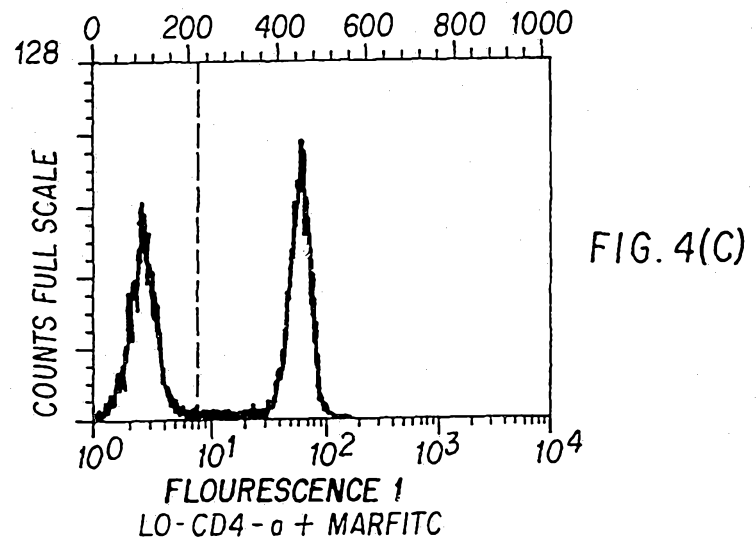
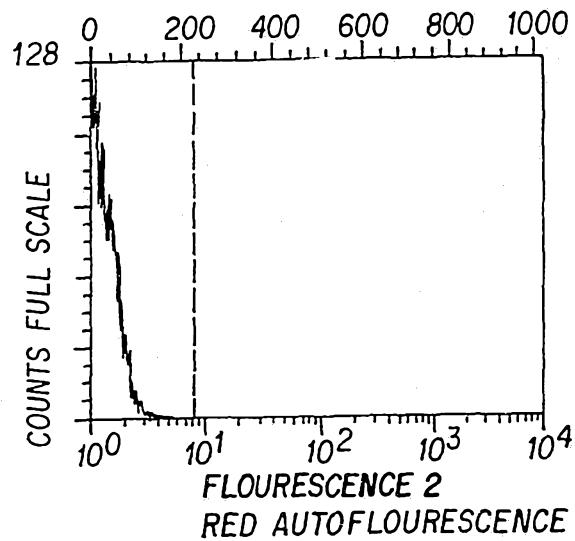
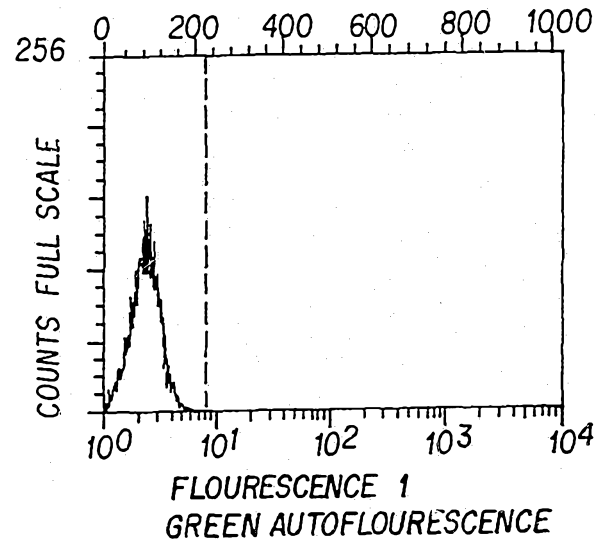


FIG. 3

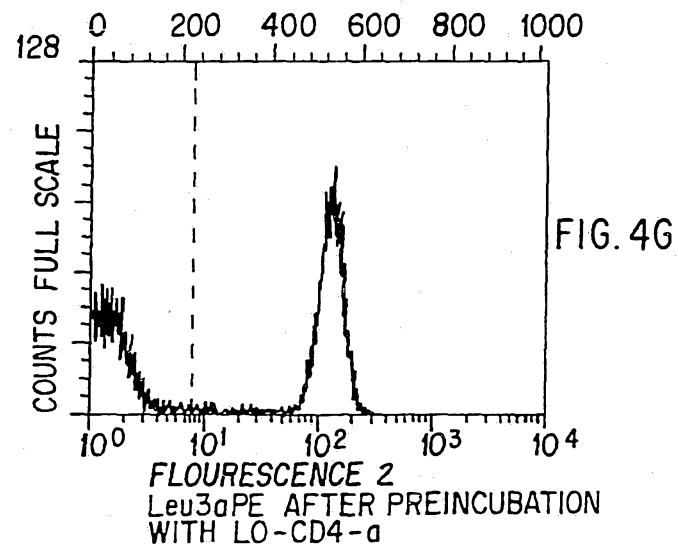
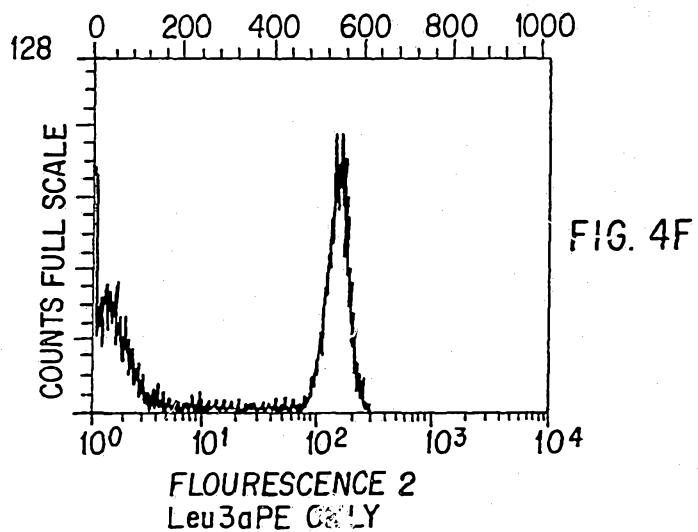
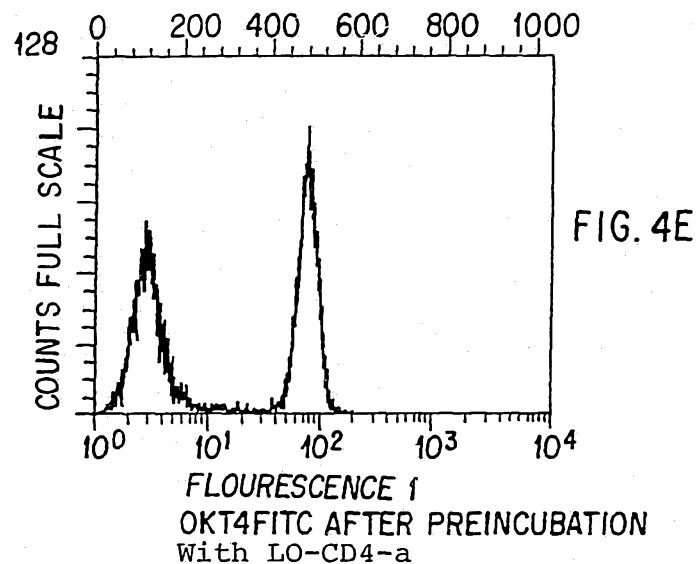
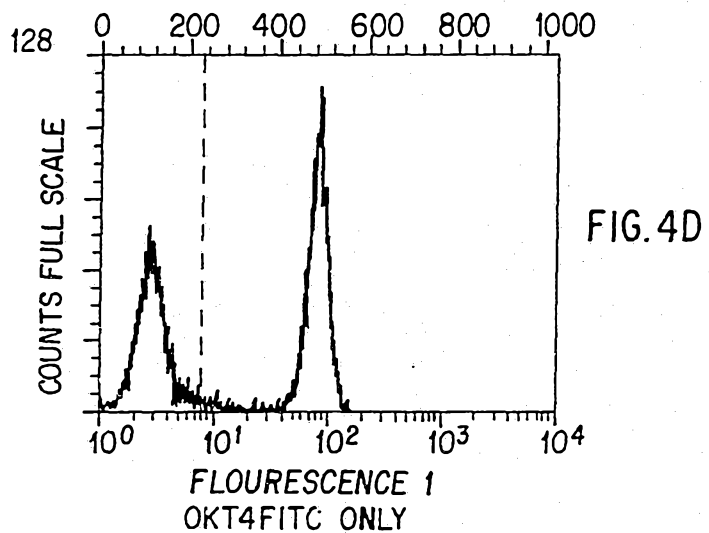




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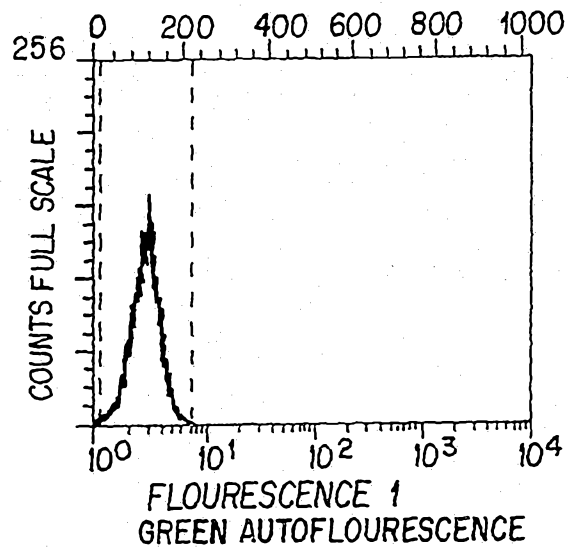


FIG. 4(H)

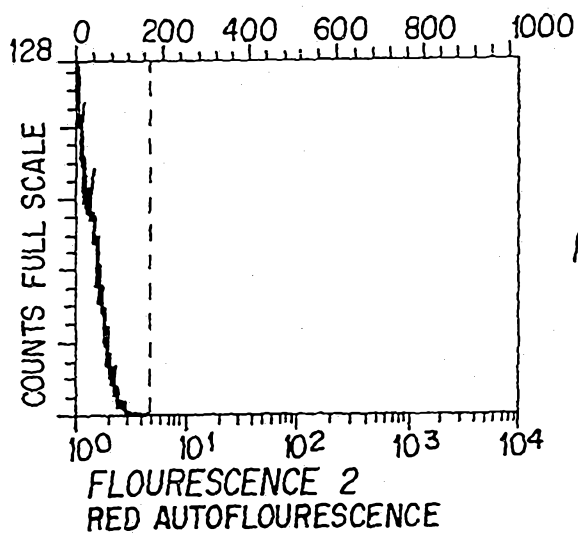


FIG. 4(I)

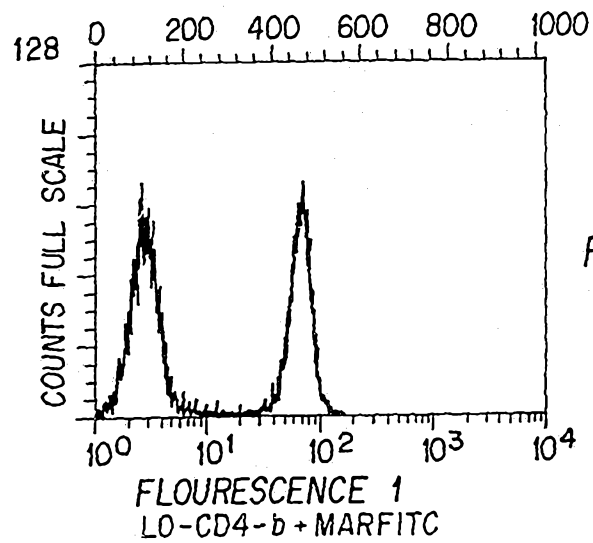


FIG. 4(J)

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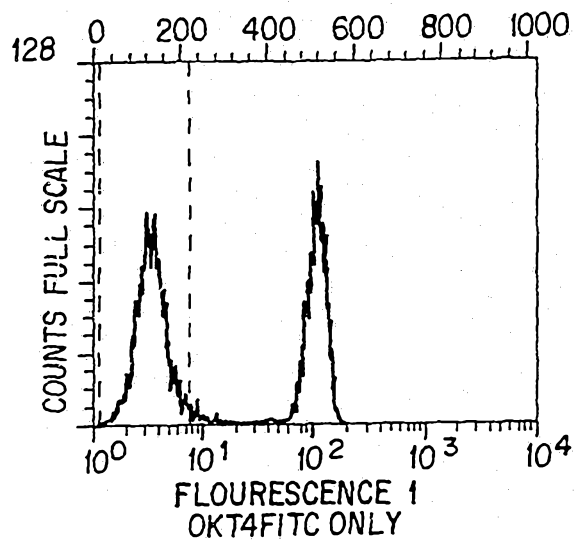


FIG. 4(K)

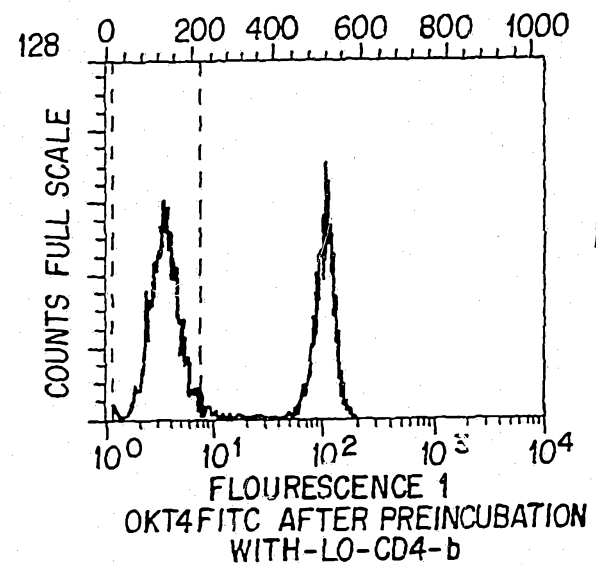


FIG. 4(L)

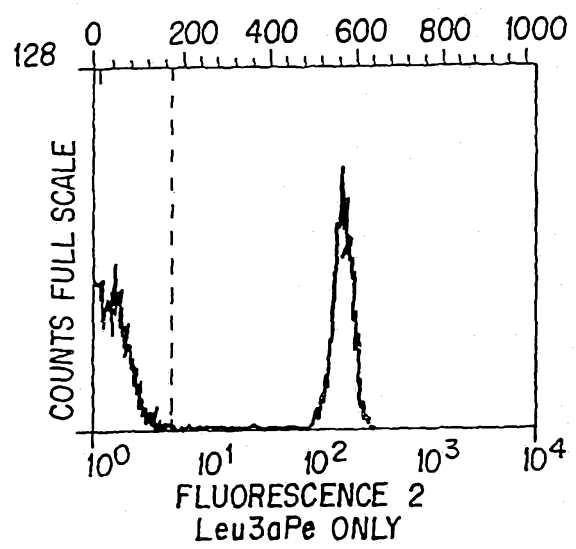


FIG. 4(M)

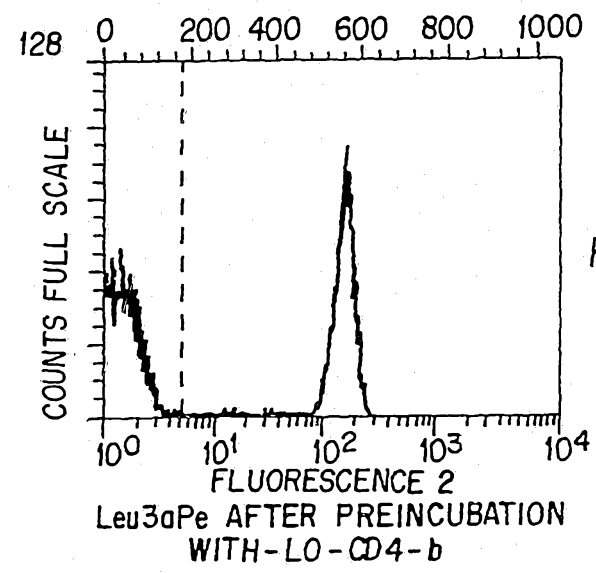


FIG. 4(N)

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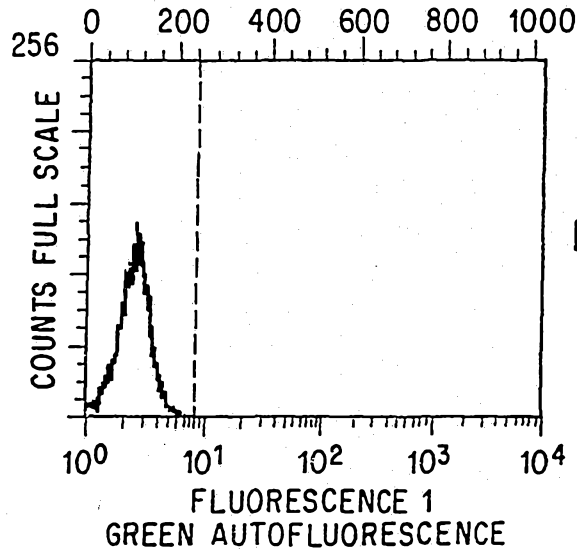


FIG. 5(A)

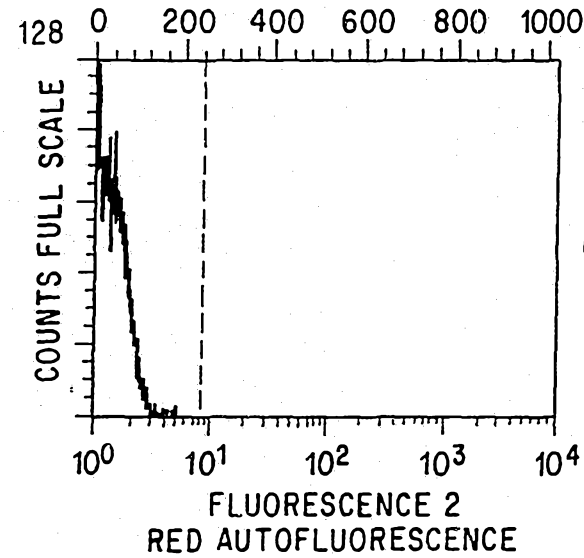


FIG. 5(B)

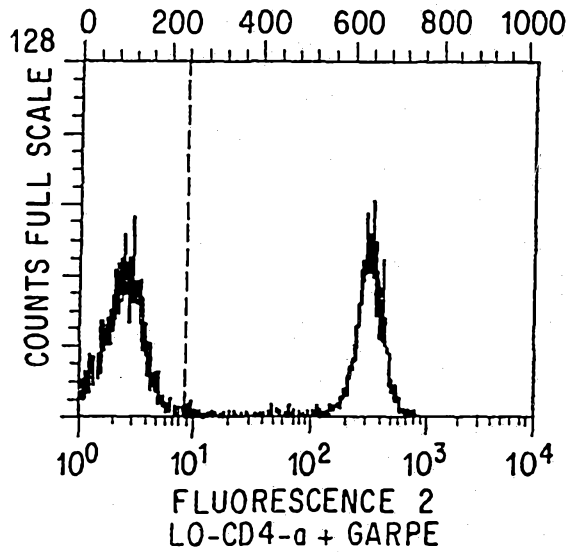


FIG. 5(C)

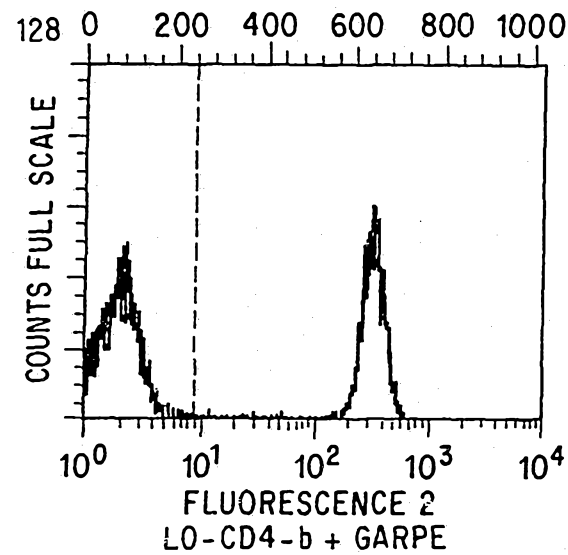


FIG. 5(D)

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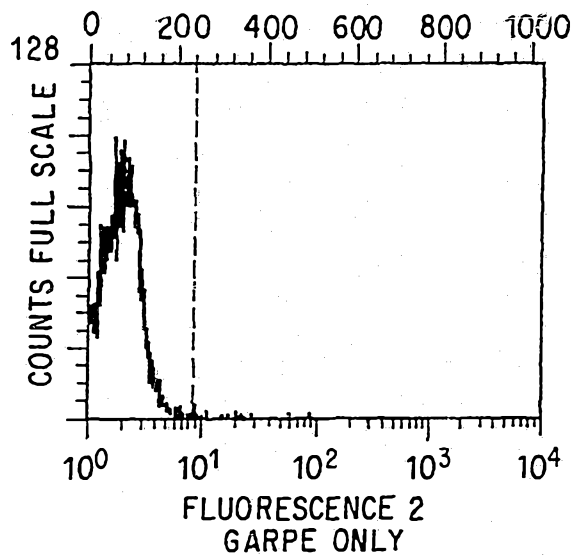


FIG. 5(E)

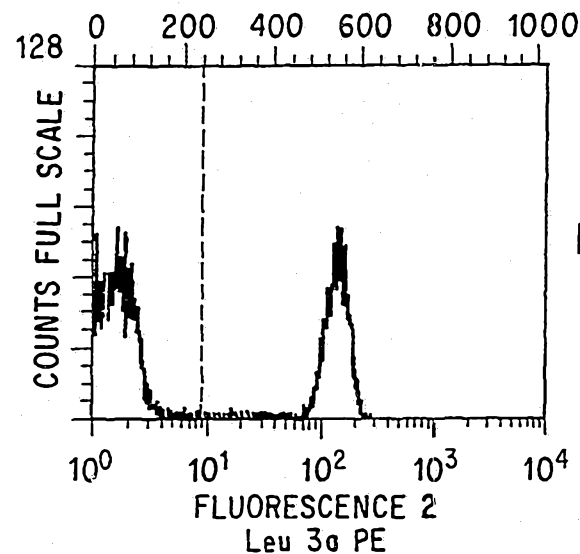


FIG. 5(F)

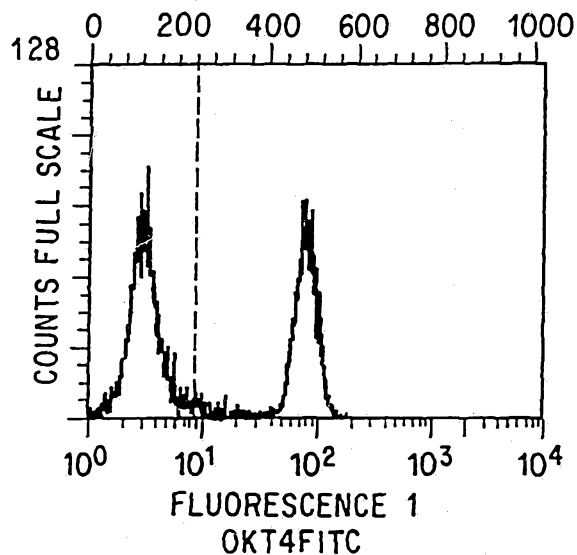


FIG. 5(G)

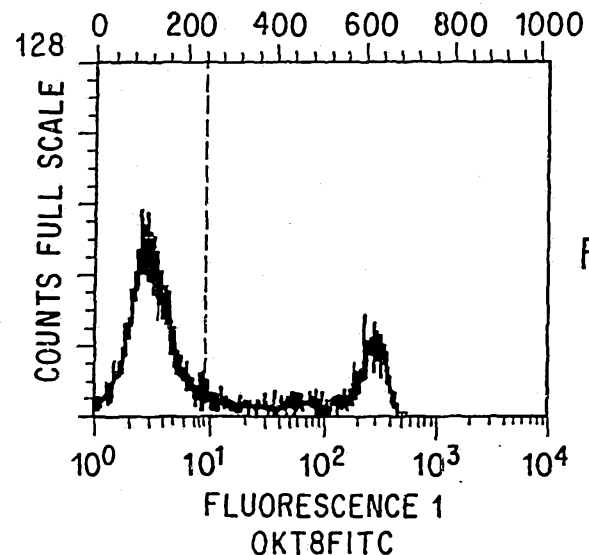


FIG. 5(H)

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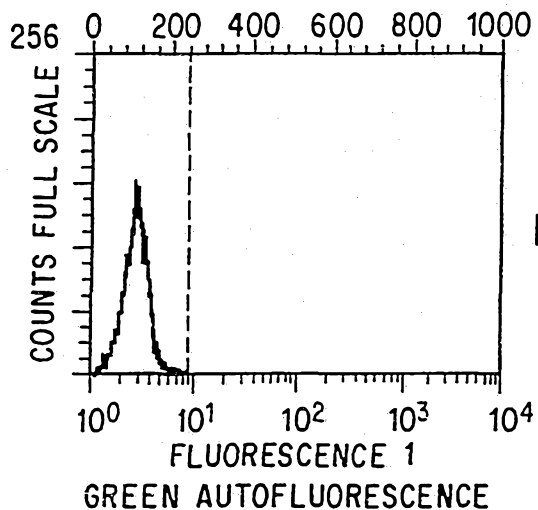


FIG. 5(I)

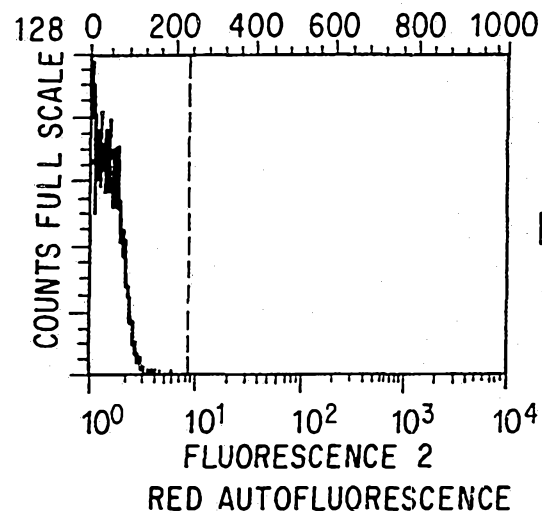


FIG. 5(J)

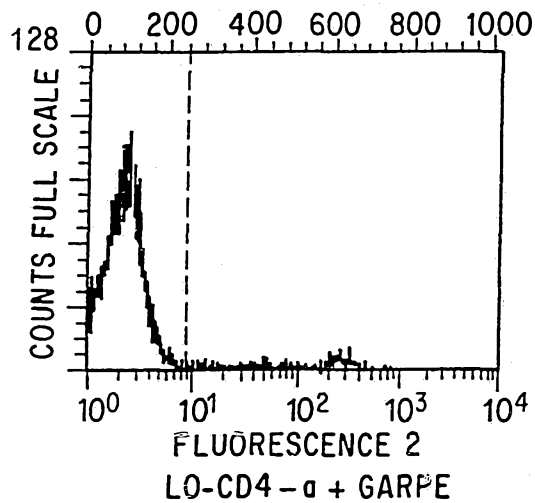


FIG. 5(K)

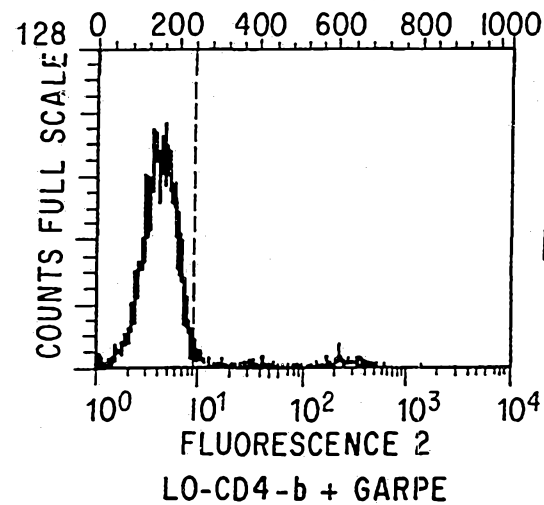


FIG. 5(L)

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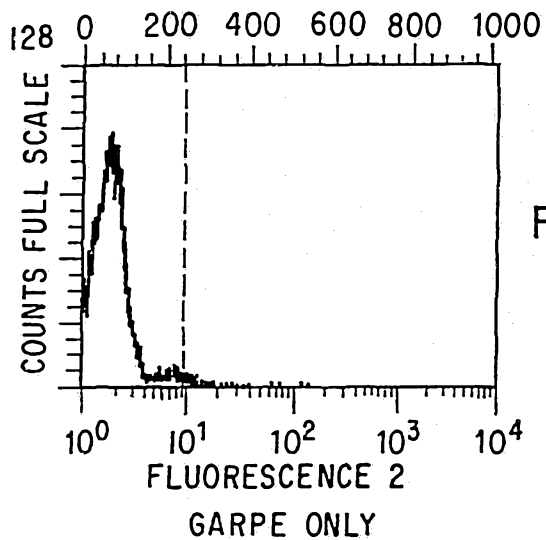


FIG. 5(M)

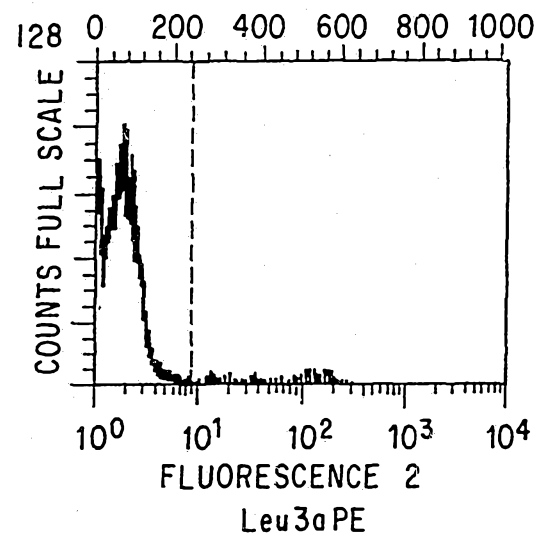


FIG. 5(N)

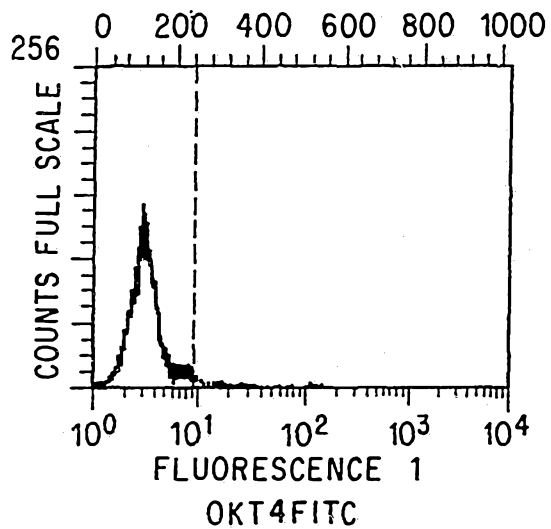


FIG. 5(O)

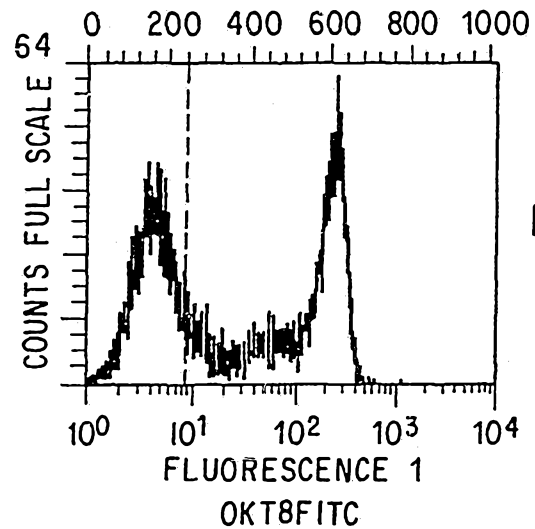


FIG. 5(P)



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FIG.6(A)

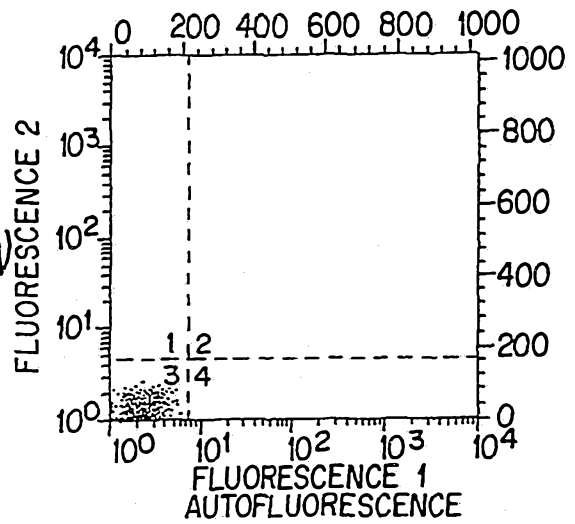


FIG.6(B)

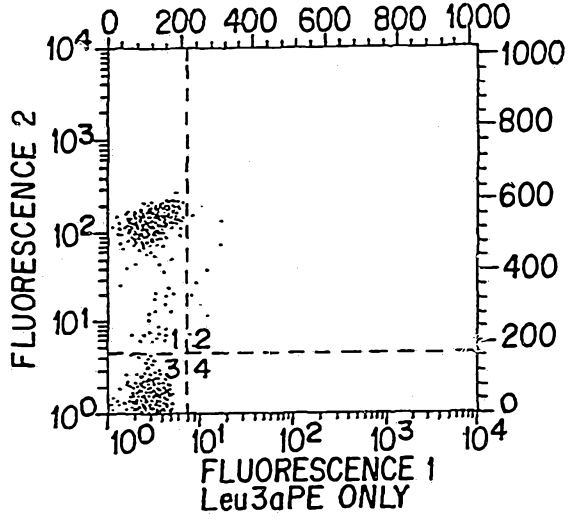


FIG.6(C)

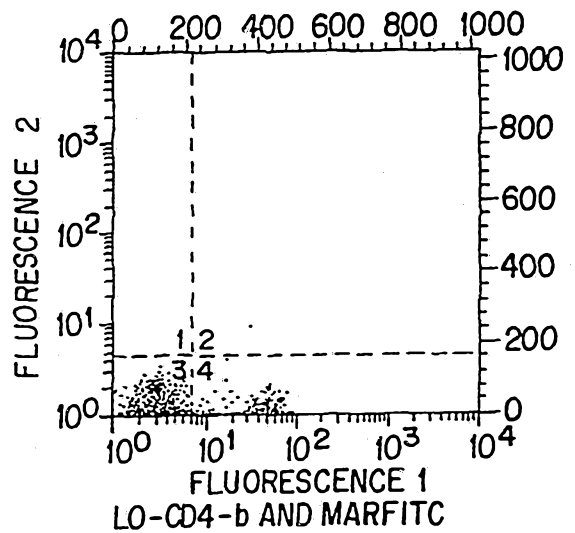
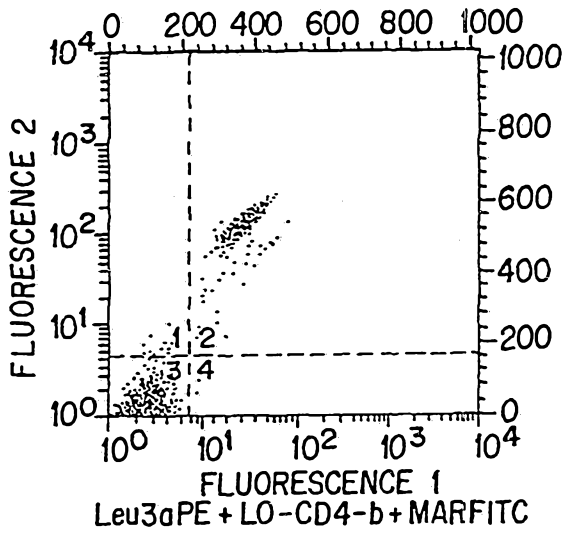


FIG.6(D)



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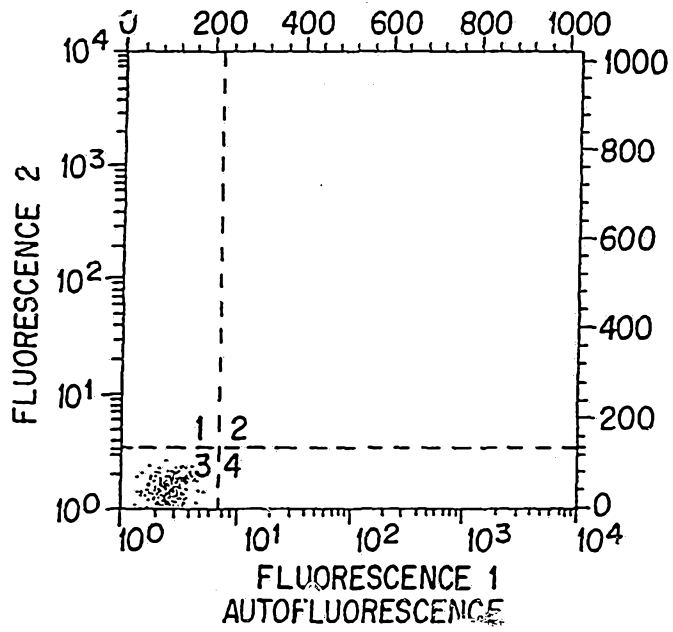


FIG. 6(E)

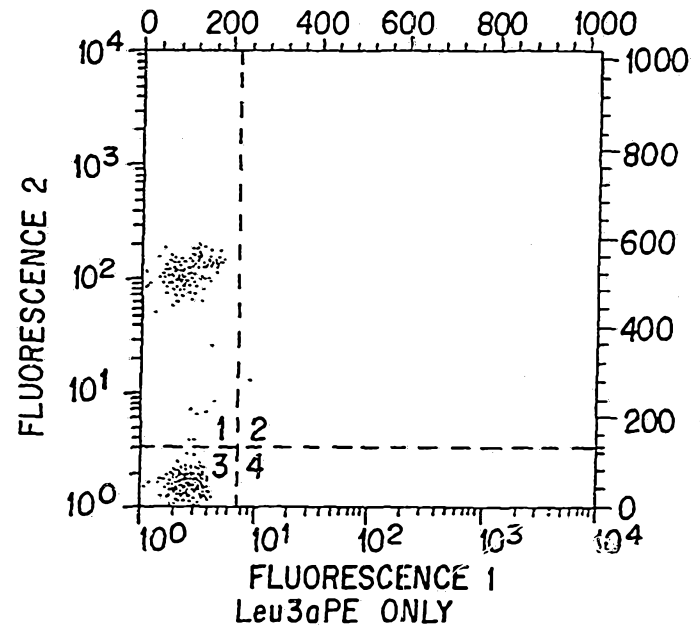


FIG. 6(F)

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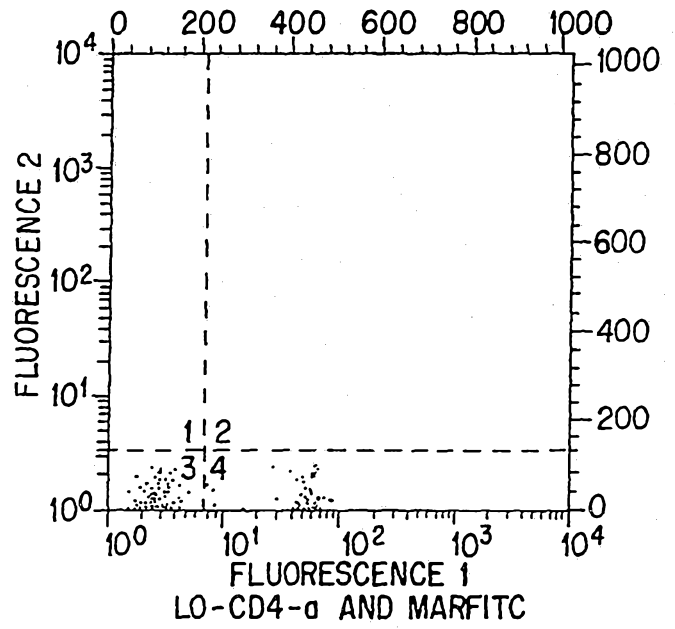


FIG. 6(G)

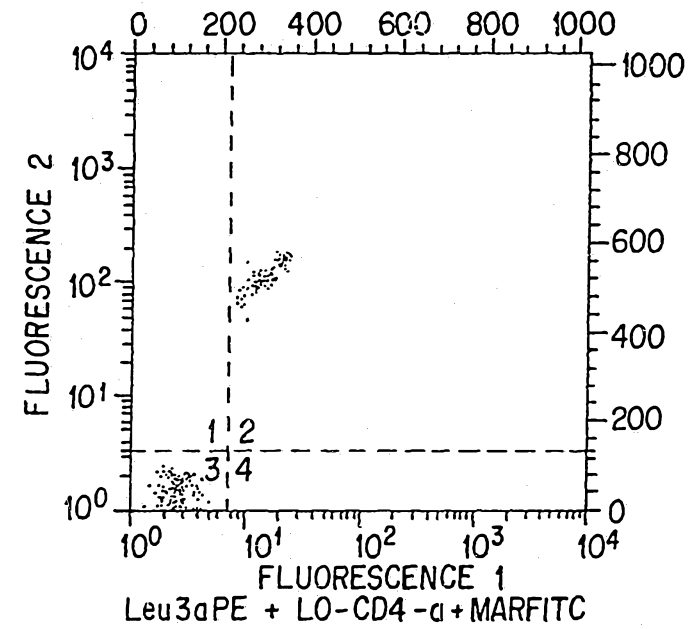


FIG. 6(H)

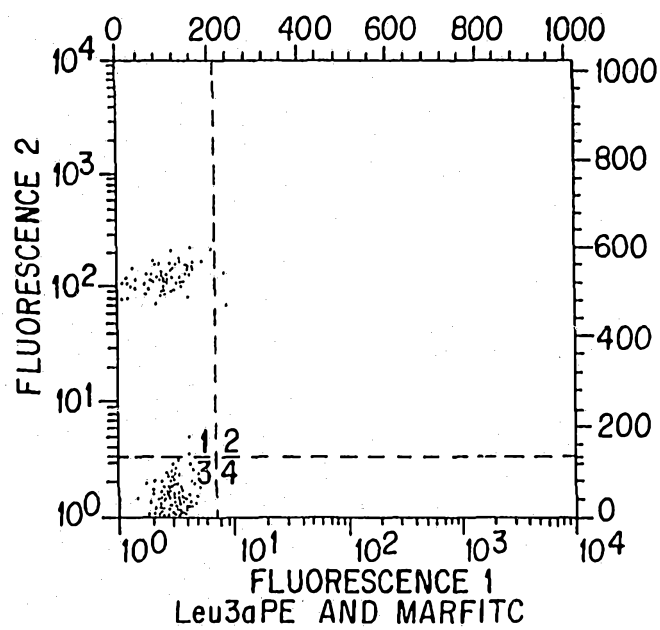
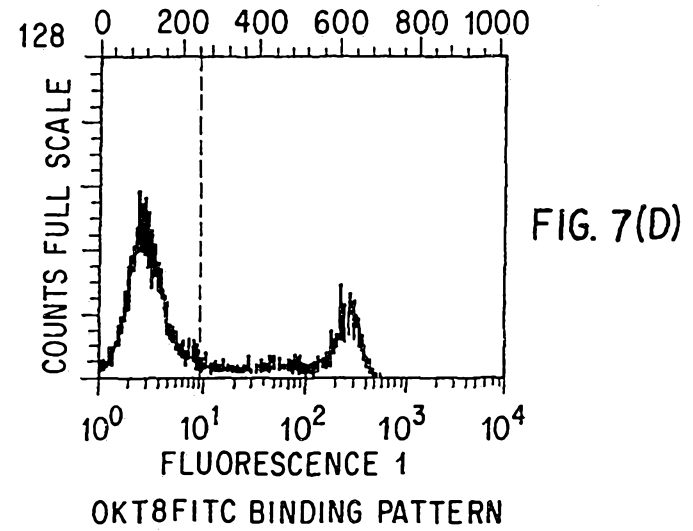
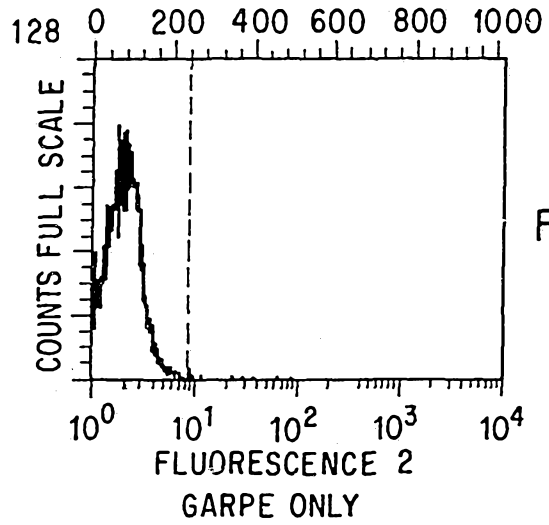
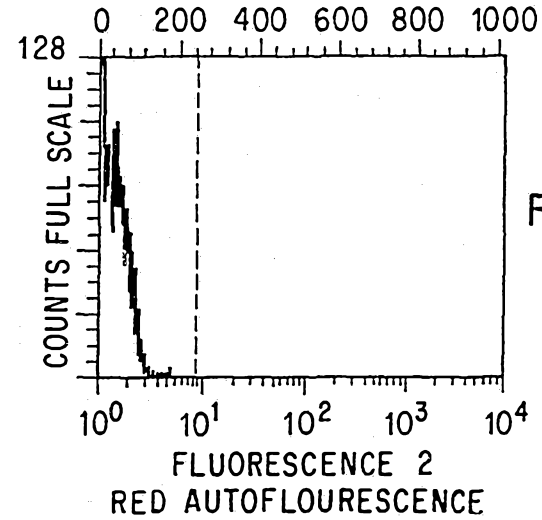
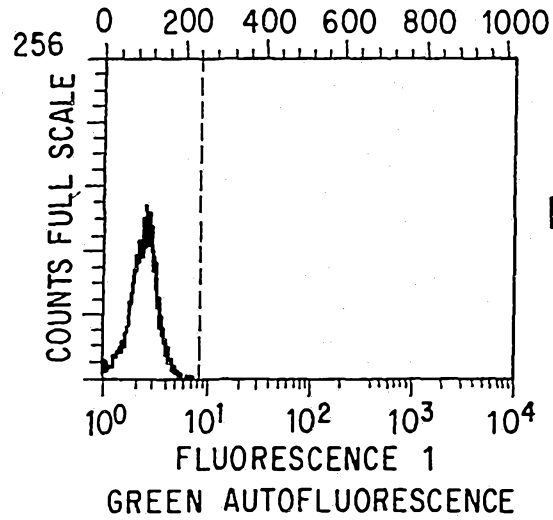


FIG. 6(I)

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