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(54) Title: METHODS FOR MEASUREMENT OF LYMPHOCYTE FUNCTION		
(57) Abstract		
<p>A method for measuring the responses of sets or subsets of lymphocytes to mitogens or antigens in a sample includes incubating a population of cells with a mitogen or antigen, separating the desired subset of cells by means of the interaction of a specific binding reagent that is attached to the solid phase with a cell surface determinant that is present on the cell subset of interest, lysing the separated cells, and measuring an intracellular component that is increased if the cells have responded to the stimulus. The method provides a convenient, simple, and reliable method for measuring immune function in a variety of conditions.</p>		

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METHODS FOR MEASUREMENT OF LYMPHOCYTE FUNCTION

DESCRIPTION

BACKGROUND OF THE INVENTION

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Field of the Invention

The invention relates to methods for measuring the function of lymphocytes and their responses to mitogens or specific antigens. The methods are suitable for measurement of the responses of T lymphocytes when they are a subpopulation of cells, and also for measuring the function of specific subsets of T lymphocytes, each subpopulation or subset of a subpopulation having characteristic determinants on their cell surface. The invention also relates to test kits used in performing such methods. The methods of the invention facilitate screening of complex biological fluids, such as whole blood, by means of incubating a sample of the fluid with a mitogen or antigen, separating the selected subset of interest, e.g., via affinity separation, and detecting the presence of an internal cellular component, advantageously ATP, that is increased as a result of the response.

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Background Description

The immune system is central to control of infectious diseases and cancer. Lymphocytes, a class of white blood cells, are critical cell types that are responsible for the activities of the immune system. Lymphocytes are divided into two major categories, T lymphocytes and B lymphocytes. Overall assessment of the function of the immune systems and, in particular,

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lymphocytes is important in assessment of immunodeficiency caused by genetic factors, infectious disease such as (HIV), drugs following transplantation, stress, aging, or nutritional deprivation.

Lymphocytes express receptors on the cell surface that bind with specific antigens or epitopes. Exposure to the antigen results in expansion in the population of the lymphocytes that are reactive to that antigen. Measurement of the response of the immune system to a specific antigen can be useful in diagnosis of infectious disease, hypersensitivity to certain agents, exposure to immunologically reactive drugs, or response to vaccination.

The function of B lymphocytes or their response to specific antigen can be assessed by measuring the level of specific antibody in bodily fluids such as blood, saliva or urine. The function of T lymphocytes or their response to specific antigens is more difficult to measure. Measurement of the functions of T lymphocytes or T cells is complicated by a number of factors. First, there are several different subsets of T cells with different functions. These subsets have been classified in part by the expression of characteristic cell surface markers and in part by a variety of functional assays including measurement of cytokines. Second, T cells respond to antigens only when they are presented by other cells in the context of major histocompatibility antigens on the surface of the presenting cell. Third, many of the functions of T cells depend on cell-cell contact with effector cells or the functions are fairly localized. Current methods for measuring immune function are tedious, time consuming, and poorly adapted to the clinical laboratory setting.

Methods that are currently used for measurement of immune function include: methods based on counting the number of T cells or different subsets; methods based on measuring the proliferation of lymphocytes, methods based on measurement of cytotoxic activity or secretion of cytokines, and methods used in vivo, such as skin tests and adoptive transfer. These methods are described in detail in the literature (see for example Groeneveld et al., Journal of the International Federation of Clinical

Chemistry, 6: 84-94; 1994; Clough and Roth, JAVMA 206:1208-1216, 1995).

The methods most commonly used in the clinical laboratories are based on counting the number of T cells or subsets. A variety of techniques have been described including immunofluorescence microscopy, immunocytochemistry, enzyme immunoassay, and flow cytometry. Flow cytometry, in particular, is widely used in clinical laboratory settings. Flow cytometry is particularly useful in measurement of subsets of interest within a complex population of cells. For example, U.S. Pat. No. 4,727,020 to Recktenwald describes the use of two fluorescent channels to detect cells in a subpopulation specifically labeled with two different immunofluorescent agents. U.S. Pat. No. 4,284,412 to Hansen, et al. describes the use of fluorescence channels to detect forward and right angle light scatter of cells of different subpopulations in blood. Major disadvantages of flow cytometry are that it requires complex and expensive equipment, each sample must be run and analyzed individually and the results require interpretation and are frequently not repeatable. These disadvantages are particularly acute in a clinical laboratory which must process multiple patient specimens daily and where the need for consistent and reliable results is extremely important.

U.S. patents 5,385,822 to Melnicoff et. al. and 5,374,531 to Jensen disclose alternative methods to flow cytometry for counting the number of lymphocytes or of a subset of lymphocytes within a mixed population of cells. The methods described in these patents involve coupling a detectable reporter substance to the bio-membrane or incorporating the reporter substance into the cell, then separating the subset or population of interest and detecting the reporter substance. These methods utilize affinity separation to isolate populations of interest from a complex mixture of cells. This technique offers improvements over flow cytometry but it is still based on cell counting techniques.

The major difficulty with all cell counting techniques is that they do not measure the function of specific cells or their responses to specific antigens or mitogens. Cells that respond to mitogens or antigens have unique cell surface markers found only on the responding cells. Methods for counting the number of cells exhibiting these markers have been described but these methods are relatively insensitive due to the fact that the responding cells are generally a small fraction of the total population. These methods are also tedious and subject to poor reproducibility.

Direct measurement of responses of lymphocytes have included lymphoproliferation assays, cytotoxicity assays, and measurement of cytokines. In general, these methods require separation of white cells from the original sample followed by incubation with antigen or mitogen. Measurement of the function of specific subsets of lymphocytes requires extensive manipulations prior to the assay. The requirement for antigen presenting cells then means that additional cells have to be added back to the culture. Lymphoproliferation assays are based on division of responding cells and are typically performed using radioactive isotopes. Because they evaluate the division of a small population of cells and require tissue culture, the assays take 3-10 days and are subject to significant variability based on the specific technique and the reagents used in the assay. Cytotoxic tests also require significant cell manipulation and are similarly highly variable depending on the specific conditions used. Cytokine assays can also be performed, but require many steps and separation of subsets of interest prior to stimulating the cells. U.S. Patent 5,344,755 to McMichael describes a modification of the cytotoxic assay based on initial immunomagnetic separation of T lymphocytes, but this method still requires extensive manipulation of effector cells. U.S. Patent No. 5,344,755 provides an example of use of cytokine measurements to assess immune status in HIV positive patients but is tedious and requires multiple steps. These methods have required separation of critical cell types, long incubation times, and in

some cases use of radioactive substances. For these reasons, these methods have not been suitable for clinical applications.

Affinity separation of cells using protein-coated magnetic particles or other types of solid supports such as polystyrene particles is known and is used as part of several of the methods cited above, see U.S. Patent Nos 5,374,531, 5,385,822, 5,344,755. Various methods for sorting biological populations via affinity separations on solid supports have been described in the patent literature and elsewhere. See, for example, U.S. Pat. Nos. 3,970,518, 4,710,472, 4,677,067, 4,666,595, 4,230,685, 4,219,411, 4,157,323; see also, E.T. Menz, et al., *Am. Biotech. Lab.* (1986); J.S. Kemshead et al., *Molec. Cell. Biochem.*, 67:11-18 (1985); T. Leivestag et al., *Tissue Antigens*, 28:46-52 (1986); and J.S. Bernan et al., *J. Immunol.*, 138: 2100-03 (1987). In performing such methods, a binding molecule (e.g., monoclonal antibody) is typically conjugated to the solid supports such as the magnetic particles or plastic beads, and added to a test sample under conditions causing binding to a characteristic determinant on the analyte of interest. The cells complexed with the solid support are then separated from the uncomplexed cells by exposure to a magnetic field or filtration or other method depending on the nature of the solid support. The use of this technology to separate certain subpopulations of lymphocytes from bone marrow cells prior to transplantation and to eliminate post-transplantation graft vs. host reaction, has also been reported. See A. Butturini et al., *Prog. Bone Marrow Transpl.* 4:13-22 (1987). Other reported uses of this technology include the separation of tumor cells (see: Kemshead et al., *B.J. Cancer* 54:771-78 (1986)) and the separation of lymphocyte subpopulations for subsequent functional evaluation.

The problems that arise when the lymphocytes are first separated by magnetic or other solid phase affinity techniques and then used for functional assays, are that the interaction of the lymphocyte with the binding molecule

can itself induce functional changes in the lymphocyte that may obscure later changes that are to be measured. In addition, the accessory cells required for response of the T cells may no longer be present especially if a specific subset of cells are isolated. In addition, isolated cells are removed from the native
5 environment and it is difficult to maintain the sterility of the sample required for further tissue culture.

SUMMARY OF THE INVENTION

The subject invention provides a convenient, reliable, and relatively rapid method for analyzing the function of various sets or subsets of
10 lymphocytes. In a preferred embodiment, the method of the invention involves incubating a population of cells with a mitogen or antigen, separating the desired subset of cells by means of the interaction of a specific binding reagent that is attached to the solid phase with a cell surface
15 determinant that is present on the cell subset of interest, lysing of the separated cells, and measuring an intracellular component that is increased if the cells have responded to the stimulus. However, other inducing agents besides mitogens and antigens may be used initially in the practice of this invention. Suitable inducing agents may include cytokines and growth
20 factors, allergens, allo-antigens, peptides or low molecular weight compounds conjugated to a protein carrier such as carbohydrates (polysaccharides), lipids and nucleic acids.

In an advantageous embodiment of the invention, the functional activity of a set or subset of lymphocytes which is distinguished by a characteristic cell surface determinant and which is contained within a mixed
25 cell population, is measured by: exposing the sample to a mitogen or antigen or other inducing agent, incubating the sample for a period of time, binding the set or subset of lymphocytes to a solid support through the interaction of

the cell surface determinant and a specific binding substance which is linked to the solid support, washing the cells to remove any unbound cells as well as potentially interfering substances in the media, lysing the cells, and detecting the ATP in the solution. The results obtained can be compared against a known standard. Alternatively, the sample can be divided into two or more parts with at least one of the parts being incubated without addition of any stimulant, while the second part is incubated with addition of an antigen or mitogen or other inducing agent.

One aspect of the invention is the determination of the response of lymphocytes to a mitogen. In this case, the response of a set of lymphocytes to a mitogen is a general measure of immune function. This application is of particular significance in the measurement of the effects of immunosuppressive drugs or agents. In this case, the responsiveness of an entire set of lymphocytes such as the T lymphocytes can be determined. Alternatively, the effect of a virus such as HIV can be assessed by evaluating the response to mitogens of a subset of T lymphocytes that express the CD4 cell surface determinant.

Another aspect of the invention is the determination of the response of lymphocytes to antigens that might include infectious agents, drugs, chemicals, autoantigens, or tumor antigens. This aspect of the invention is particularly important in monitoring the exposure of an individual to an infectious disease or an agent or to diagnosis of hypersensitivity, autoimmune disease or cancer. This aspect of the invention is also useful in monitoring vaccine efficacy and in assessment of immunotoxicity of chemicals, drugs, and industrial compounds.

Another aspect of the invention is that the response of T lymphocytes from various subsets including functional or differentiation based subsets to a mitogen or antigen can be assessed based on the expression of different determinants specific for a functional, differentiation, or activation marker on the cell surface. In this aspect of the invention, an antigen is added to a

sample and incubated for a period of time. Following the incubation, the cell subset of interest is isolated by binding the cells to a solid support through a determinant on the cell surface. The cells are washed and lysed, and the level of an intracellular component that increases as a result of the activation is measured.

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The method of the invention is highly sensitive due to the measurement of an intracellular component whose level increases rapidly following lymphocyte exposure to mitogen or antigen, for example ATP, other metabolic intermediates such as NADP, or proteins involved in cell cycle regulation such as PCNA. In an advantageous embodiment, the level of ATP is measured utilizing the bioluminescent reaction of luciferin:luciferase. Bioluminescent measurement of ATP is a highly sensitive measure.

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A further aspect of the invention is that the total time required for the method is generally 6 - 72 hours and typically 18-24 hours. The relatively short time period is an advantage in comparison to the current methods which requires 3-10 days to complete.

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In one embodiment of the invention, test kits are provided for performing the methods of the invention. Test kits typically contain the antigen or mitogen (or other inducing agent), the solid state with appropriate binding substance, the reagents required for detection of ATP levels, appropriate diluent and wash solutions, standards or instructions for preparing the same, and optionally, other accessories such as test tubes, magnetic separators, washers, and transfer pipettes, which are useful in carrying out the methods of the invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be

better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:

Figure 1 is a bar graph showing the relative light units elicited from lymphocytes stimulated with various mitogens and antigens as discussed in Example 2; and

Figure 2 is a three-panel line graph showing the PHA stimulation of T lymphocytes separated by CD4, CD69, or CD3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides sensitive and efficient methods for measuring the responsiveness of a set or subset of lymphocytes (e.g., T cells or B cells) distinguished by some characteristic determinant expressed on the cell surface within a mixed cell population. In particular, the invention provides a method for measuring the response of lymphocytes to inducing agents including mitogens or antigens, as well as cytokines and growth factors, allergens, allo-antigens, peptides or low molecular weight compounds conjugated to protein carrier such as carbohydrates (polysaccharides), lipids, and nucleic acids.

The current invention circumvents the problems of the prior art by separating the T cells from the sample after the cells are exposed to the antigen and in a time frame in which the interaction of the lymphocyte with the binding molecule on the beads will not affect the results.

A second distinguishing aspect of the invention involves the measurement of the levels of ATP in the cells following the separation of the subset of interest. It is well known that ATP levels are indicative of metabolic activity. See, for example, Kangas et al, Med. Biol. 62:338-43 (1984) and Lundin et al. Meth. Enzymol. 133 :27-42. Measurement of ATP

levels have been used in studies of chemotherapeutic drugs and other agents of cell lines and have been used to monitor increases in biomass and cell number. ATP levels can be measured very sensitively using the bioluminescent reaction of firefly luciferase with luciferin. See, for example, 5 Leach and Webster, Meth. Enzymol. 137:51-70, (1986). A number of methods for assessing ATP levels in bacteria or in somatic cells have been reported. See, for example, U.S. Patent Nos. 3,690,832; 5,283,179; 4,144,134; 4,283,490; and 4,303,752 each of which is hereby incorporated by reference. What has not been appreciated in the prior art is that as a result 10 of the response of T cells to an inducing agent such as a mitogen or antigen, the metabolic activity of the cell that responds increases significantly and that this increase is reflected in significant increases in the level of ATP levels. Further, small changes in ATP levels or changes in ATP levels in a small number of cells can be measured due to the sensitivity of the 15 luciferin:luciferase system.

The invention addresses a major existing need for improved methods to measure the responsiveness of T lymphocytes to mitogens and antigens, i.e. T cell function. These methods exhibit sensitivity comparable to or greater than methods heretofore available. These methods also permit the 20 user to analyze multiple samples in a relatively brief time, and eliminate the need for expensive equipment and highly skilled personnel to perform the method, and do not use radioactive materials.

The methods of the invention can be used as an adjunct to or a replacement for methods and tests performed in the clinical laboratories to 25 count the number of lymphocytes in different subpopulations. The methods described herein incorporate the required sensitivity of assays that take much longer time with the shortened time frame of cell counting assays. The utilization of standards and the provision of test kits based on these methods incorporating all the necessary reagents results in increased reproducibility 30 and consistency required for tests used in the clinical laboratory.

The methods of the invention have several advantages over current methods. The time required for response is significantly less than for other assays. The provision of all the materials required for the assay in a test kit and the simplicity of the nature of the measurement results in consistency in results from lab to lab. Further, multiple samples can be run simultaneously. The methods are simple, rapid, sensitive, and applicable to the clinical laboratory setting.

Inducing Agents

In the context of this invention, inducing agents are substances that interact with lymphocytes such that the result of the interaction is a change in the state of the cell. In particular, "inducing agents" refer to substances that cause resting lymphocytes to become activated and that can also induce functional activity in the cell. In general, inducing agents fall into two classes: (i) mitogens are inducing agents that interact with all the lymphocytes of a particular subset and induce activation followed by proliferation in the responding cells and (ii) antigens that interact through specific receptors on limited subpopulations of cells. However, other substances may also be used as inducing agents including cytokines and growth factors, allergens, allo-antigens, peptides or low molecular weight compounds conjugated to proteins carrier such as carbohydrates (polysaccharides), lipids and nucleic acids.

Mitogens for different populations of lymphocytes are known and include lectins, antibodies directed against certain lymphocyte cell surface receptors such as CD3 for T Lymphocytes or CD2 for B Lymphocytes, growth factors and lymphokines, phorbol esters, and other biochemical substances that are known to those versed in the art. Advantageous mitogens include phytohemagglutinin (PHA), Con A, and monoclonal antibody to CD3.

Antigens react with a smaller subset of lymphocytes through specific receptors on the cell surface. Each lymphocyte has on its cell surface a receptor for a specific antigen or molecule. For B lymphocytes, the cell surface receptor is antibody that is membrane bound. For T lymphocytes, the cell surface receptor is the T cell receptor with recognized antigen that is presented in the context of major histocompatibility molecules on the surface of another cell. The response of the immune system to specific foreign invaders is based on the recognition of antigens by the receptors on the cell surface of these cells and the resultant functional activation that occurs as a result of this interaction. In general, the antigens that are bound to these cell surface receptors are small parts of larger molecules and can include parts of infectious agents, such as viruses, bacteria, fungi, and the like, drugs, organic chemicals, and inorganic chemicals such as silicone, metals such as beryllium, and proteins such as tumor cell proteins, or proteins derived from implanted or transplanted organs. Advantageous antigens include gp 120 protein or peptides from gp 120 of HIV virus envelope glycoprotein, outer surface proteins from bacteria such as OSPA, B or C from *Borrelia burgdorferi*, SiO₂, disrupted inactivated Q fever cells, PPD, or tetanus toxoid.

Target Lymphocytes

The cell subsets of interest are present in test samples or specimens of varying origin including biological fluids such as whole blood, urine, stool, saliva, cerebrospinal fluid, amniotic fluids, tissue extracts, lavage fluids, tumor biopsies, transplant biopsies or they can be from culture. Cells of interest are of human or animal origin. Samples also include specimens from various biological origins that have been partially purified by density gradient centrifugation or other separation methods that are used to isolate partially purified samples of cells.

The invention can also be used with long term cell lines such as

MDLT-4, HL-60, TF-1, NFS60 and L-929. These cells as well as those from biological fluids may be derived from frozen stocks. In an advantageous embodiment, the use of frozen lymphocytes provides a mechanism for transporting clinical or experimental specimens without degradation.

5 In analyzing a sample containing a cell subset of interest according to the method of the invention, the cell population suspended in its natural biological fluid or in a suitable biological or synthetic medium, is initially exposed to the mitogen or specific antigen or other inducing agent of interest. It is a particular aspect of the invention that the exposure occurs for a relatively short period of time. Depending on the nature of the antigen or mitogen being examined, the time period of exposure is 6 - 72 hours or longer, but is usually of 24 hour duration.

10 Of particular interest in diagnostic, therapeutic, and research purposes are measurement of the responses of T lymphocytes and lymphocyte subsets, including major functional subsets such as T helper cells and suppressor/cytotoxic cells to mitogens or to specific antigens. Quantitation of the responsiveness of a specific subset of T lymphocytes is important in certain physiological conditions. For example, individuals infected with human immunodeficiency virus lose responsiveness in the CD4 cell population to both mitogens and antigens prior to loss of activity in other cell subsets. Likewise, responsiveness of subclasses of T cells to mitogens is important in monitoring individuals that are potentially immunosuppressed due to chronic stress, chemotherapy, or drug treatment. Quantitation of the responsiveness of a subset of lymphocytes to specific antigens is important in measuring the individual's exposure to an infectious agents or to a drug or compound or to determine a hypersensitivity reaction to a drug or chemical.

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30 In addition to the major functional subsets other cells subsets of interest include cells at different stages of differentiation or cells at different time points after initial interaction with a mitogen or antigen.

Characteristic Determinants

The functional subsets of interest are distinguished by expression of characteristic cell surface determinants. In addition, cells from the same subset but at different stages of differentiation are distinguished by
5 expression of characteristic determinants on the cell surface. Cells with different functional activity or at different times after the initial interaction with a mitogen or antigen can also express different cell surface determinants.

In this invention, characteristic determinant denotes an element that
10 identifies or determines the nature of something. When used in reference to the methods of the invention, "determinant" means a molecule expressed on the cell surface that characterizes the cell in some fashion. Cell-associated determinants include, for example, components of the cell membrane, such as membrane bound proteins or glycoproteins or lipids or glycolipids and
15 including cell surface antigens of either host cell or viral origin, histocompatibility antigens, or membrane receptors.

A determinant is the portion of the cell that interacts with a specific binding substance. Cells are separated by means of the specific interactions between determinants on the cell surface and specific binding substances that
20 are attached to solid phases. This process is referred to herein as "affinity separation". Specific binding substances that may interact with cell surface determinants include antibodies capable of recognizing the determinants.

The invention can be used as a method for measuring any
25 immunologically active cells which are distinguished by some characteristic surface determinant. For example, the invention may be used for measuring cancer cells, thymocytes, dendritic cells, Langerhans cells, NK cells, monocytes, myeloid cells, platelets, eosinophils, basophils, megakaryocytes, granulocytes, macrophages, Reed Sternberg cells, precursor B cells, pan myeloid, hematopoietic precursor cells, endothelial cells, plasma cells, pan

leucocytes, proliferating cells, pan B including progenitors, Burkett's lymphoma cells, circulating and interdigitating reticular dendritic cells, neutrophils, mucosa associated T lymphocytes and choriocarcinoma cells.

Binding Substances

5 Determination of the presence or quantity of cell subsets according to the methods of the invention is accomplished by the selective interaction between cells of the subset of interest and a specific binding substance. The specific binding substance used in the practice of this invention must exhibit selective recognition for the characteristic cellular determinant. In analyzing
10 a mixed cell population for a sub-population and/or subset having a characteristic cell surface antigen, for example, the specific binding substance can be the complementary antibody that immunospecifically recognizes the antigen of interest. Based on such selective recognition, the specific binding substance is capable of selective interaction and binding with the subset of
15 interest to form complexes or aggregates which are physically or chemically separate from the test medium and other components therein which are not of interest. In one advantageous embodiment, blood specimens containing T lymphocytes and monocytes bearing the surface antigen CD4 are exposed to a specific binding substance comprising a CD4 monoclonal antibody.

20 The term "antibodies" as used herein includes monoclonal or polyclonal immunoglobulins and immunoreactive immunoglobulin fragments. Other types of specific binding substances include lectins, hormones, cytokines, receptor ligands, etc.

25 Monoclonal antibodies to particular cell surface determinants are of particular importance in this embodiment of the invention. For example, lymphocytes, which comprise a sub-population of whole blood, can be selected by a monoclonal antibody which is directed against a leukocyte surface antigen. The CD45 antigen is uniformly expressed on all

lymphocytes; however, the CD45 antigen is also expressed on monocytes. Therefore, if selective binding of lymphocytes is desired, it is necessary to select a CD45 monoclonal antibody which binds to significantly more binding sites per cell on lymphocytes than monocytes or that binds with higher strength to lymphocytes than to monocytes.

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In a particularly advantageous embodiment, it is desirable to separate only T helper lymphocytes within a sample of whole blood. This is accomplished as described above, using monoclonal antibodies directed against the T cell surface antigens, such as CD4 or is accomplished using a combination of antibodies that react primarily with T helper lymphocytes. In another embodiment of the invention, lymphocytes that have been activated by exposure to specific antigen are separated by utilizing antibodies directed against antigens that are expressed only following activation on the cell surface. These antibodies react with one or a combination of the following cell surface antigens, CD25, CD69, CD71, CD45RO, or MHC Class II antigens. Using these antigens for separation results in a significant amplification of the signal from the assay since only the cells that express these markers have responded to the signal from antigen or mitogen.

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Specific binding substances are conveniently affixed to a solid phase or insoluble fluid phase to facilitate separation from the test medium. A variety of solid support materials can be used e.g., polystyrene, nylon or agarose beads, and are well known to those skilled in the art. In a particularly advantageous embodiment of the invention, the specific binding substance is affixed to a plurality of magnetic beads, which comprise ferromagnetic, paramagnetic or diamagnetic material. Techniques for attaching the specific binding substance to the beads are known to those skilled in the art. Suitable techniques include cross-linking, covalent binding or physical absorption. Alternatively, a non-solid phase, primary specific binding substance is used in conjunction with a second or auxiliary specific binding substance which is capable of interacting selectively with the primary specific binding substance,

and which is affixed to a solid phase. Representative primary and auxiliary specific binding substances useful for this purpose are: soluble murine antibody/Protein A affixed to a solid phase; soluble murine antibody/anti-mouse immunoglobulin raised in another species and affixed to a solid phase; and biotinylated antibody/avidin affixed to a solid phase.

In the case where the sample being tested is derived from culture or is separated by density gradient separation prior to separation on a solid support, a simple separation procedure is sufficient to separate the subset of lymphocytes of interest from the rest of the cell population. In the case of more complex samples such as whole blood, it is necessary at times to wash the complex to remove cells that are trapped or bound nonspecifically. A variety of solutions (e.g., 0.15M ammonium chloride, 1.0 M potassium carbonate, 0.1 M EDTA, pH 7.2) that specifically lyse red blood cells, platelets, or other potential contaminants are known to those versed in the art. In addition, solutions that contain other substances such as proteins, sugars, or salts or that are of specific pH values can be useful in reducing the nonspecific binding of other cell types or in eliminating or lysing cell types that are separate from those of interest (e.g., solution of Hank's buffered saline containing 10% of FCS is particularly useful). Upon separation and following washing, if necessary, the media is removed from the complex.

Lysis

Following separation of the cells of interest, the separated cell population is lysed by the addition of a solution containing substances that can lyse lymphocytes. A variety of such solutions exist and are well known to those in the art. These solutions include distilled water, solutions containing detergents such as Triton-X or NP-40, and buffered solution such as HEPES containing 0.1 M benzalkonium chloride, pH 7.4. It is important that the material and the solution chosen do not interfere with the system for measuring ATP, do not contain ATP, and do not degrade ATP.

It is a significant feature of the invention that the time from the exposure of the sample until the time that the cells are lysed is minimal, usually less than 2 hours preferably less than 1 hour. This is significant because interaction of lymphocytes with many antibodies against cell surface antigens can result in a response. This has been a significant problem in determining the function of specific cell types as the isolation of the cell type can induce cell activation.

Measurement of ATP Levels

Following lysis of the cells, the level of ATP in the solution is measured. In an advantageous embodiment, the ATP is measured by the addition of a solution containing firefly luciferase and luciferin in the presence of magnesium ions. ATP can also be measured by other means including immunochemical or biochemical reaction systems.

The measurement of an intrinsic component of the cell is important because it eliminates an additional step as well as the intrinsic variability of any labeling process. Increased ATP has been used as a marker of increased cell mass, but has been relatively insensitive because all the cells in the population exhibit a baseline level of ATP. The subject invention works because the measurement of ATP is made following separation of the cell population of interest.

Kits

According to another aspect of the invention, the different reagents, together with the various accessories used in practicing the methods of the invention, including media for dilutions, solid supports for immobilizing cells, lysis reagents, antigens or mitogens, and wash buffers, one or more standards, or instructions for the preparation thereof are conveniently packaged in a test kit. The reagents included in the test kit may take various

forms and are packaged dry together with appropriate diluents or may be supplied in ready to use form.

In accordance with the methods of the present invention, kits for evaluating the T cells responses are envisioned. In particular, the invention includes a kit containing antigens or mitogens or other inducing agents either
5 in liquid or lyophilized form, paramagnetic beads coupled with an antibody for isolation of the predetermined subset of cells, cell culture media for dilution of samples, wash buffer for washing complexes, and associated reagents for performing the assay.

10 The following examples are provided to describe the invention in further detail. These examples are intended to illustrate specific applications of the methods of the invention and should in no way be construed as limiting the invention.

EXAMPLES

15 Example 1: Detection of Mitogen Responsiveness of CD4 positive T Lymphocytes Useful for Measuring Immune Function in HIV infection, in Response to Stress, or Following Chemotherapy.

Peripheral blood mononuclear cells were isolated from source leukocytes (Gulf Coast Blood Bank) originally obtained from the peripheral
20 blood of normal individuals by gradient centrifugation over Ficoll-Hypaque. Cells from the buffy layer were rinsed by centrifugation in RPMI containing 10% fetal calf serum (FCS) and were adjusted to a cell density of approximately 1×10^5 cells/ml with RPMI containing 5% fetal calf serum (FCS). One aliquot of cells was cultured unstimulated while a second aliquot
25 of cells was stimulated with phytohemagglutinin (PHA), a T cell mitogen at a concentration of 1 ug/ml for 24 hours. The aliquots were diluted 1:20 with

RPMI 1640 containing 10% FBS. Antibody to CD4 at a concentration of 2 $\mu\text{g/ml}$ was added to all cultures. Cells were incubated at room temperature for 30 minutes, then 100 μl of paramagnetic beads coated with goat anti-mouse antibody (obtained from Advanced Magnetics) was added. The cell suspension was gently mixed and incubated for thirty minutes at room temperature. The cells and beads were re-suspended, then placed next to a permanent magnet that was placed such that the magnetic field was in a direction perpendicular to gravity. After the beads formed a dense pellet, the media was aspirated and the magnet removed. Additional buffer was added and the cells and beads were re-suspended. This process was repeated several times. After fully removing the media, the cells in the bead pellet were lysed with a detergent solution identified as somatic cell lysis reagent (Sigma) and the tube is placed into the luminometer. One hundred microliters of a mixture containing luciferin, luciferase, and Mg^{2+} in a solution containing 0.25 mM hepes, 0.1mM DTT, and 0.5% BSA was injected into the tube and the counts were determined.

Negative controls consisting of lysis buffer only or cells that are incubated with nonspecific beads, i.e., beads coated with a different isotype from that of the primary antibody were also tested. Finally, an ATP standard was run in each assay to confirm that the luciferin:luciferase reaction was occurring at appropriate levels. Table 1 below shows the results obtained.

TABLE 1: Response of PBMC separated using CD4 Antibody and Goat anti-Mouse Paramagnetic Beads.

Treatment	Relative Light Units
Blank	1,265
ATP Control	420,653
Unstimulated	8,768
PHA Stimulated	38,903
Uncoated Beads	1,050

Example 2: Measurement of the Response of CD4 positive T lymphocytes to Mitogens, Cytokines and to a Toxin Antigen.

Peripheral blood mononuclear cells were isolated from several different individuals by gradient centrifugation, washed, and cultured in RPMI 1640 containing 5% FCS. The cells were aliquoted and left either unstimulated or stimulated with PHA, CD3 antibody, tetanus toxoid, or influenza viral antigen. All samples were not incubated with the same antigens. Following a 48 hour incubation the cells were diluted with RPMI 1640, incubated with mouse monoclonal antibody to CD4 antigen for 30 minutes at room temperature, then incubated with goat antibody to mouse IgG conjugated to paramagnetic beads (Advanced Magnetics, Inc.) for 30 minutes at room temperature. Following separation using a permanent magnet and washing of the sample, the cells were lysed using somatic cell lysing reagent (Sigma Chemical). Luciferin/luciferase reagent (Sigma Chemical) was diluted 1:10 and 100 μ l was added. Relative light units were determined. Figure 1 shows the response of different patient samples to these mitogens and antigens and demonstrates the ability of the methods of the invention to detect responses to mitogens and antigens in individual patient samples.

Example 3: Comparison of Results Obtained after PHA Stimulation of T lymphocytes Separated by CD4, CD69, or CD3.

This Example examines the ability of the assay to be performed using different cell surface markers. Peripheral mononuclear cells were either unstimulated or stimulated with PHA (1%) for 24, 48, 72, or 96 hours. Aliquots of the unstimulated cells or the PHA stimulated cells were incubated with mouse monoclonal antibody to CD3, the T cell receptor, CD4, or CD69 for 30 minutes at room temperature. All aliquots were then incubated with paramagnetic beads onto which had been adsorbed goat anti-mouse IgG for 30 minutes at room temperature. The beads and cell complexes were separated using a permanent magnet and washed three times with RPMI

containing 10% FCS . The cells were lysed with a 0.5% NP-40 solution. Luciferin: luciferase mixture (Sigma Chemical Company) was diluted 1:10 and 100 μ l added to each tube. Figure 2 shows the results of these assays. In all cases, the relative light units from the stimulated cells were greater than those from the unstimulated cells. This demonstrates the use of different cell surface markers to evaluate the response of lymphocytes from different subsets.

Example 4: Measurement of the Response of T Lymphocytes to a Bacterial Antigen.

Balb/c mice were immunized with a total of 100 μ g of Q fever antigen prepared from Nine mile strain (Integrated Diagnostics, Inc.) emulsified in complete Freund's adjuvant. Prebleeds were taken from the orbital sinus of 10 mice. Additional bleeds were obtained at 7 days post inoculation. Approximately 100-200 μ l of blood were obtained from each mouse.

Peripheral blood mononuclear cells were prepared by density gradient centrifugation. White cell layer was removed and washed with RPMI-1640 containing 10% FCS. Approximately 100 μ l of cells were placed in each tube. The cell pellets were cultured either without any other additions or in the presence of Q fever antigen (10 μ g/ml or 1 μ g/ml). After 24 hours, the cultures were diluted 1:10 with RPMI-1640 containing 10% FCS and 100 μ l of super paramagnetic beads coated with antibody to mouse CD4 (Advanced Magnetics, Inc.) were added. The beads and cells were incubated at room temperature for 30 minutes. The beads, together with any complexed cells, were separated using a permanent magnetic and washed 3 times in RPMI containing 10% FCS. The cells were lysed by adding 100 μ l of Somatic Cell Lysing Reagent (Sigma Chemical Co.). After an additional 10 minutes at room temperature, 100 μ l of a luciferase:luciferin reaction mixture (Sigma Chemical Co.) was injected and the light output determined by a luminometer.

In the prebleeds from the mice, samples incubated with Q fever antigen gave signals that were identical to those incubated without Q fever antigen. The following table shows the results from samples obtained at 7 days following injection of the antigen. There was a dose dependent response of T cells detected.

Table 2: T cell response to Q fever antigen in mouse injected with antigen.

	Total RLU	Index
Control	8447	-
Q Antigen 10 μ g/ml	22346	2.6
Q Antigen 1 μ g/ml	16566	2.0

Example 5: Measurement of the Response of T Lymphocytes Using Whole Blood as a Sample.

Blood samples were obtained from normal donors and collected in heparin as an anti-coagulant. Aliquots of the blood (100 μ l) were diluted 1:5 with RPMI 1640. Replicates received either no addition, PHA at 1%, or IL-2 at 100U/ml final concentration. Samples were incubated overnight at 37°C. The following day, 100 μ l of the samples were removed, incubated with mouse monoclonal antibody to CD4 antigen for 30 minutes at 37°C. Paramagnetic beads coated with goat anti-mouse IgG were added to each sample (100 μ l) and incubated for 30 minutes at 37°C. The bead:cell complexes were isolated from the samples using a permanent magnetic and the complexes were washed three times with PBS containing 10% FCS and 0.1% BSA. Cells were lysed using distilled water. Luciferin:luciferase was added and the relative light units determined. The following table shows the results.

TABLE 3: Measurement of T cell response to mitogens in a whole blood sample.

Treatment	Relative Light Units
Control	458,451
PHA	5,836,682
IL-2	2,632,415

Example 6: Measurement of the Responses of CD8 Positive T Lymphocytes to a Viral Antigen.

Peripheral blood mononuclear cells were isolated from heparinized whole blood from normal blood donors by gradient density centrifugation. Cells were washed in RPMI containing 10% FCS and re-suspended in this media at a density of 1×10^5 cells/ml and aliquoted. Replicates received either no additions or different levels of influenza A antigen or PHA. After 24 hours, a mouse monoclonal antibody to CD8 was added and the cells were incubated for 30 minutes at room temperature. Paramagnetic beads coupled to goat antibody to mouse IgG were added and the complexes were separated by placing the cultures next to a permanent magnet. Complexes were washed three times with PBS containing 10% FCS then the cells were lysed by adding 0.05% Triton X 100 in PBS. ATP levels were determined using luciferin:luciferase. All individuals showed responses to PHA with stimulation indexes of >10 , while the range of stimulation indexes to influenza viral antigens ranges from 1.2 - 4.0.

Example 7: Measurement of the Response of T Lymphocytes to Recall Antigens Compared with Flow Cytometry.

Blood was collected from a normal donor in a collection tube containing heparin. Tetanus toxoid at 10 ng/ml; Candida albicans at 20 ng/ml; Influenza A at a dilution of 1:4; Human transferrin at 10 ng/ml or no

additive was added 500 μ l of blood and incubated at 37°C for 24-48 hours. One hundred microliter samples were removed and incubated with 20 μ l of paramagnetic beads coated with mouse monoclonal antibody to CD4 antigen (Perseptive BioSystems) for 30 minutes at room temperature. The bead:cell complexes were removed from the samples using a permanent magnet, and then washed three times using RPMI 1640 media containing 5% FCS. Cells were lysed by adding 15 μ l of Somatic Cell Lysing Reagent (Sigma Chemical Co.), followed by 150 μ l of a luciferase:luciferase reaction mixture (Sigma Chemical Co.). Light output was determined by a luminometer, and Stimulation Indices calculated.

Samples for Flow Cytometry were prepared by incubating 50 μ l of whole blood from each recall antigen sample with 10 μ l of mouse monoclonal antibody to CD4 conjugated to FITC (Dako Corp.). Cells were counted on a flow cytometer.

The following tables show that measurable T cell activation to recall antigens is achievable with the method described after 24 hours or 48 hours exposure. No increase in cell numbers were noted in parallel flow cytometry experiments. This occurs presumably because the assay method is sensitive enough to detect an increase in metabolic activity as evidenced by an increase in ATP levels before cell proliferation levels become significant enough to be measurable by flow cytometry. Human transferrin is a natural material not recognized as foreign, and therefore elicits no immune response. It is used as a negative control in the assay.

Table 4: Measurement of the Response of T Lymphocytes to Recall Antigens after 24 hours:

	Control	PHA	Tetanus Toxoid	Candida	Influ- enza	Trans- ferrin
Samp. 1	0.403	8.05	1.498	1.526	2.055	0.489
Samp. 2	0.491	7.14	1.321	1.517	1.957	0.498

Samp. 3	0.446		1.364	1.619	1.822	0.485
SI		13.48	3.18	3.56	4.51	1.12

Table 5: Measurement of the Response of T Lymphocytes to Recall Antigens after 48 hours:

	Control	PHA	Tetanus Toxoid	Candida	Influ- enza	Trans- ferin
5 Samp. 1	0.736	4.35	2.51	2.574	3.202	0.708
Samp. 2	0.742	4.58	2.92	2.754	3.021	0.6
Samp. 3	0.762	5.49	2.855	3.29	3.6	0.573
SI		4.89	3.76	3.79	4.43	0.85

10 It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications of changes in light thereof will be suggested to persons skilled in the art and are to be included within the purview of this application and the scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for measuring the function or response of a selected subset of lymphocytes in a test sample containing a mixed population of cells types, to a mitogen or antigen comprising:
 - a) incubating said sample with said mitogen or said antigen; and
 - b) detecting the level of ATP in said selected subset of lymphocytes.

2. A method as in claim 1 wherein step b) comprises:
 - i) contacting said sample with a solid support having a specific binding substance, said binding substance being specific for at least one characteristic determinant of said subset of lymphocytes, resulting in the formation of a complex of cells and solid support;
 - ii) separating said complex from the remainder of said sample;
 - iii) adding to said complex a solution to lyse any cells in said complex;and
 - iv) detecting the level of ATP in the lysis product of step iii).

3. A method according to claim 1, wherein said mitogen is a T lymphocyte mitogen.

4. A method according to claim 1, wherein said antigen is an infectious organism.

5. A method as in claim 4 where said infectious organism is a virus or bacteria or a subcomponent thereof.

6. A method according to claim 1, wherein said antigen is a protein or peptide.
7. A method according to claim 1, wherein said subset of lymphocytes is selected from the group consisting of T lymphocytes, helper T lymphocytes, TH1 lymphocytes, TH2 lymphocytes, natural killer T lymphocytes, cytotoxic T lymphocytes, and suppressor T lymphocytes.
8. A method according to claim 2, wherein said determinant of T cells is a functional marker, a marker of a particular differentiation stage, or an activation marker.
9. A method according to claim 2, wherein said solid support comprises magnetic or paramagnetic material.
10. A method as in claim 9 wherein in step ii) said complex is separated by magnetic separation.
11. A method according to claim 2, wherein said solid support comprises polystyrene.
12. A method according to claim 2, wherein step iv) is conducting a bioluminescent reaction using luciferase and luciferin.
13. A method according to claim 2, wherein the said specific binding substance is an antibody.
14. A method according to claim 2, wherein said specific binding substance is a cytokine.

15. A method according to claim 2, wherein step i) comprises (a) contacting said sample with a first binding substance of a binding pair which specifically binds to at least one cell surface determinant which is common to said subset of lymphocytes and, b) contacting the product of step a) with a solid support having a second binding substance of said binding pair which specifically binds said first binding substance.
- 5
16. A method according to claim 15 wherein said binding pair is comprised of two antibodies, a first antibody recognizing said cell surface determinant and a second antibody recognizing said first antibody.
- 10
17. A method according to claim 15 wherein said binding pair comprises biotin and avidin.
18. A method according to claim 1 wherein a standard sample is also subjected to step b) and the level of ATP of said test sample is compared to said standard sample.
- 15
19. A method according to claim 18 wherein said standard sample is liposomes containing ATP .
20. A method according to claim 1 wherein the total time required for performing all steps is 6-72 hours.
21. A method according to claim 1 wherein said subset of lymphocytes are B lymphocytes.
- 20
22. The method according to claim 1 further comprising the step of comparing the level of ATP in said sample with a standard level of ATP.

23. A method for measuring the function or response of a selected subset of lymphocytes in a test sample containing a mixed population of cells types, to a mitogen or antigen comprising:

a) dividing said test sample into two or more portions;

5 b) incubating at least one of said portions with said mitogen or antigen, and incubating at least one of said portions without said mitogen or antigen;

10 c) contacting each portion with a solid support having a specific binding substance, said binding substance being specific for at least one characteristic determinant of said subset of lymphocytes, resulting in the formation of a complex of cells and solid support for each portion;

d) separating said complex from the remainder of said sample for each portion;

e) washing said complex for each portion;

15 f) adding to each complex a solution that will lyse any cells in said complex;

g) measuring the level of ATP in each of the lysis products of step f); and

20 h) comparing the results of step g) for each of said portions that have been exposed to said mitogen or antigen with each of said portions that have not been exposed to said mitogen or antigen.

24. The method according to claim 23 wherein washing step e) is performed with a solution that lyses red blood cells.

25 The method according to claim 23 wherein washing step e) is performed with a solution that lyses platelets.

26. A method according to claim 23 wherein said characteristic determinant is selected from the group consisting of CD69, CD25, CD26, CD 27, CD28,

MHC Class II antigens, and CD71.

27. A method for determining the response of T lymphocytes within a test sample containing a mixed populations of cells to an antigen or mitogen comprising:

- 5 a) dividing said test sample into two or more portions;
- b) contacting at least one of said portions of said test sample with said mitogen or antigen, and not contacting at least one of said portions of said test sample with said mitogen or antigen;
- c) contacting each portion with a solid support having a specific
10 binding substance, said binding substance being specific for at least one determinant on the surface of said T lymphocytes, resulting in the formation of a complex of cells and solid support;
- d) separating said complex from the remainder of said sample for each
portion;
- 15 e) washing said complex for each portion;
- f) adding to each complex a solution that will lyse any cells in said complex;
- g) measuring the level of ATP in each of the lysis products of step f),
and
- 20 h) comparing the results of step g) for each of said portions that have been exposed to said mitogen or antigen with each of said portions that have not been exposed to said mitogen or antigen.

28. A method according to claim 27 wherein the concentration of said
25 determinant increases as a result of the response of said T lymphocytes to said antigen or mitogen.

29. A test kit for determining the response of a set or subset of a sub-
population of cells in a test sample to mitogen or antigen, said test kit

comprising:

- a) a vial containing said antigen or mitogen; and
- b) means for measuring ATP level in said set or subset.

5 30. A test kit as in claim 29 wherein said means for measuring ATP level comprises magnetic beads coated with antibody specific for said set or subset of cells, and a vial containing luciferase and luciferin.

31. A test kit as in claim 30 further comprising a vial containing culture media for dilution of said test sample, and a vial containing wash solution for washing a complex of said beads with said set or subset of cells.

10 32. A method for analyzing biological samples, comprising the steps of:
combining a biological sample containing lymphocytes with an inducing agent;
separating a selected subset of lymphocytes from said biological sample;
15 lysing lymphocytes in said selected subset to release intracellular constituents; and
detecting the level of a selected intracellular constituent released from said lymphocytes in said selected subset.

33. The method of claim 32 wherein said intracellular constituent is ATP.

20 34. The method of claim 33 wherein said inducing agent is selected from the group consisting of mitogens, antigens, cytokines, growth factors, allergens, allo-antigens, peptides, and low molecular compounds conjugated to a protein carrier.

35. The method of claim 32 wherein said separating step includes binding lymphocytes in said selected subset to a binding substance specific for a characteristic determinant on surfaces of said lymphocytes in said selected subset.

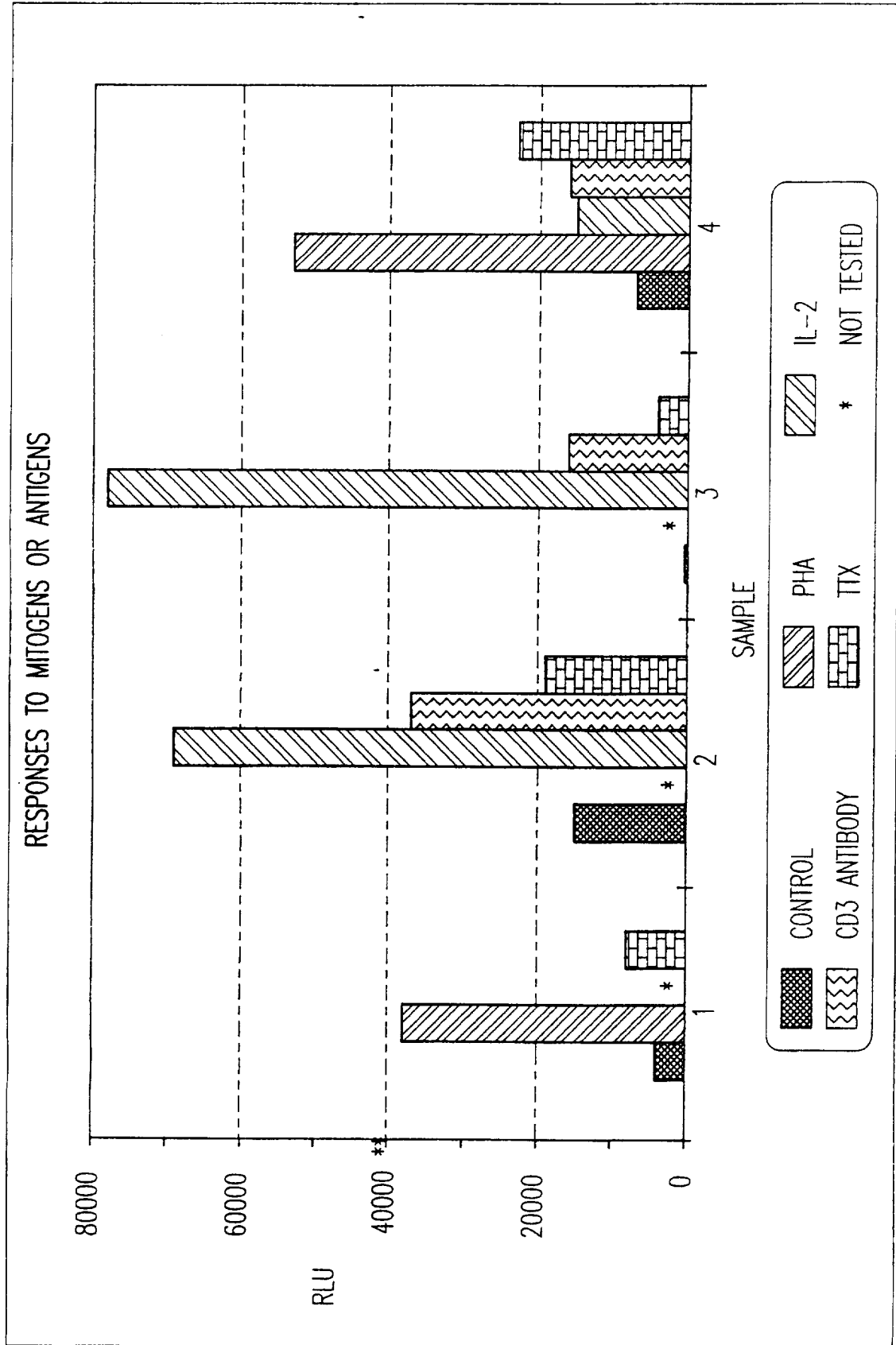


FIG.1

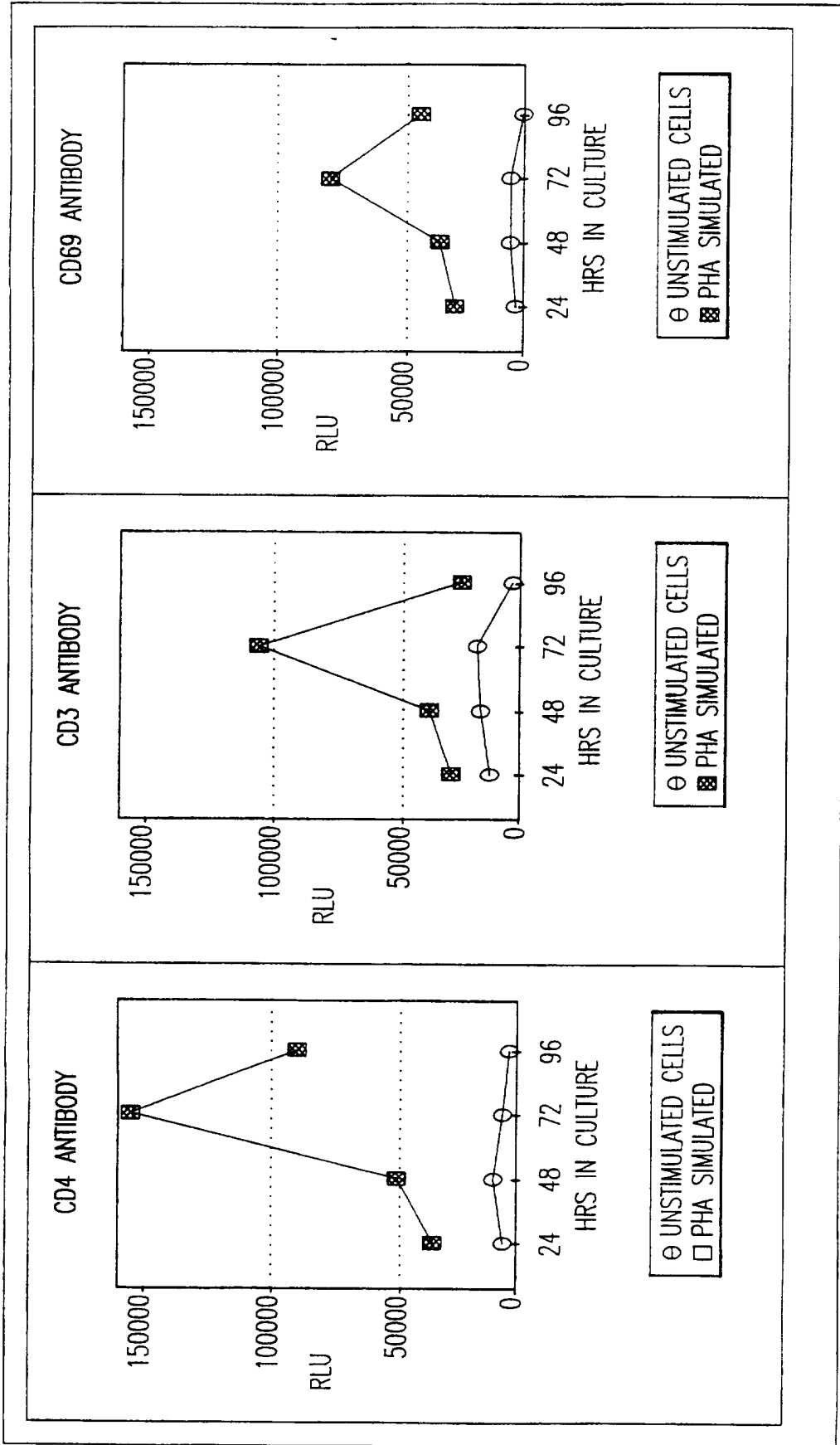


FIG.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04735

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/70, 1/24; G01N 33/53, 33/553

US CL : 435/5, 7.24, 7.9, 30; 436/526, 548

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.24, 7.9, 30; 436/526, 548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, BIOSIS, SCISEARCH, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	ISHIZAKA. A. Evaluation of the Proliferative Response of Lymphocytes by Measurement of Intracellular ATP. Journal of immun. meth. March 1984. Vol. 72. pages 127-132, especially pages 127-128.	1, 3, 7, 18, 20-22 ----- 2, 4-6, 8-17, 19, 23-35
Y	US 5,385,822 A (MELNICOFF et al.) 31 January 1995, column 2, lines 40-68, column 16, lines 40-65, column 22, lines 20-35.	2, 4-6, 8-17, 19, 23-35
Y	US 4,778,750 A (GOTTLIEB et al.) 18 October 1988, column 2, lines 40-68.	2, 4-6, 8-17, 19, 23, 35

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(a) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

&

document member of the same patent family

Date of the actual completion of the international search

07 MAY 1997

Date of mailing of the international search report

03 JUN 1997

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04735**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.