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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 3/12, 15/00, C12N 15/00
C12Q 1/02, 1/68, G01N 33/543
G01N 33/564, 33/567

A1

(11) International Publication Number: WO 94/03478

(43) International Publication Date: 17 February 1994 (17.02.94)

(21) International Application Number: PCT/US93/07218

(22) International Filing Date: 30 July 1993 (30.07.93)

(30) Priority data:
07/923,143 31 July 1992 (31.07.92) US
07/987,472 4 December 1992 (04.12.92) US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: SENSITIVE ASSAY FOR ANTIGENS AND ANTIBODIES AND ITS USE TO IDENTIFY RHEUMATOID ARTHRITIS ANTIGEN

(57) Abstract

A sensitive assay method for determination of an antigen or a specific binding component therefor is disclosed. The method utilizes a population of T-cells corresponding to the antigen along with histocompatible antigen-presenting cells to detect a complex formed between antigen and its specific binding partner. Proliferation of the T-cells is an index of the amount of complex. The ability of corresponding T-cells and their antigen-presenting cells to recognize antigen although the antigen is bound in a complex permits the design of immunoassay-type systems for antigens and their specific binding partners when the nature of these components is not completely known. In addition, the assay method permits retrieval of antibodies specific for antigens capable of stimulating T-cell proliferation in the presence of APC, such as autoantigens. This assay is applied to identify autoantigens. For example, an immunoglobulin factor is present in the synoval fluid or plasma of rheumatoid arthritis patients, which immunoglobulin or its heavy chain along is capable of stimulating T-cell proliferation. Inhibition of the activity of this immunoglobulin or heavy chain using a tolerizing or binding partner strategy interferes with the course of the disease; presence of this immunoglobulin in the body fluids of the subject indicates the presence of the disease.

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SENSITIVE ASSAY FOR ANTIGENS AND ANTIBODIES AND ITS USE TO IDENTIFY RHEUMATOID ARTHRITIS ANTIGEN

Technical Field

The invention generally relates to analysis of substances and binding partners therefor. More specifically the invention can relate to analysis of substances that are either antigens or antibodies immunoreactive with them. High sensitivity is conferred on the assay by utilizing T-cell proliferation as the method for detection, and the assay can be used to identify antibodies specific for antigens that activate T-cell populations. In one important aspect, the invention relates to diagnosis and treatment of rheumatoid arthritis, and the use of an immunoglobulin component or the heavy chain thereof derived from the biological fluid of a subject, which component is capable of effecting the activation of the subject's own T-cells.

Background Art

Immunoassay procedures are well known in the art and commonly used for the detection of analytes that may be antigens or antibodies. The specificity of these assays depends on the immunoreactivity of the analyte with respect to its specific immunoreactive binding partner (*i.e.*, immunospecific binding partner), such that formation of a complex will result only if the analyte is present to react with the immunospecific binding partner. The presence of the complex is then detected by a variety of methods. The complex is generally labeled either by providing the immunospecific binding partner in labeled form, or more commonly by addition of a secondary reagent capable of binding the complex which is labeled with, for

example, a radioisotope, a fluorescent moiety or an enzyme.

Generally, the complex formed between the analyte and the specifically immunoreactive binding partner must be separated from the remaining components of the sample prior to detection.* Thus, in one typical format, the reagent or sample to be tested is first immobilized on a solid support such as a microtiter plate and then treated with a solution containing the specifically immunoreactive binding partner. After washing away irrelevant components, the presence or absence of the analyte on the solid support is detected by the presence or absence of the complex formed between the analyte and its partner. However, other means of separating the complex from the irrelevant components are also known, such as effecting precipitation of the complex and recovery of either the precipitated complex or the supernatant for measurement of label.

T-cell Activation Assay

The methods for detecting the formation of complexes known in the art are satisfactory in a large number of instances where the nature and characteristics of the analyte and its corresponding partner are known. This knowledge makes possible the design of suitable detection strategies and manipulation of the components to formulate a satisfactory assay. However, these methods are not satisfactory to detect analytes that are less well defined. The present invention provides a method

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* In some formats, not germane to the invention here, the formation of the complex can be detected directly in the context of the sample by taking advantage of the modification of some property of one of the components as it resides in the complex as compared to free in solution to detect complex formation.

for detection of analytes wherein the only property needed to be known is the ability of an immunoreactive binding partner to stimulate a T-cell or B-cell response in the immune system. The assay can be used for the identification of such antigens themselves, or for the identification of the antibodies that they are capable of generating.

The parent application, U.S. Serial No. 07/711,338, filed 6 June 1991, describes a method for screening diverse protein sequences using T-cell proliferation as a detection method. In the method described in the copending application, the diverse peptides to be tested for T-cell proliferation activity are produced recombinantly as a set of fusion proteins containing a fused binding portion for which an antibody is available. The use of protein A as the fused portion is illustrated; this permits the use of IgG as a binding reagent on a solid support. The IgG is able to capture the protein A portion of the fusion protein. The bound protein is subsequently processed by the antigen presenting cell (APC). In turn, the APC presents the peptide to a T-cell. Each fusion protein is tested individually or in pools for the ability to activate T-cells in the presence of APC.

A similar process for screening expression libraries for epitopes recognized by mouse T-cells was described by Villarreal-Ramos et al. in Eur J Immunol (1991) 21:2621-2624. The method described in the Villarreal-Ramos article differs from the method described in the copending patent application in that the fusion peptides utilize β-galactosidase extensions rather than protein A extensions as the portions bound by antibody, and that anti-βGal antibodies rather than IgG are used as binding reagents.

In the method described hereinbelow the relevant antigen is used both for specific binding partner recognition and for its ability to stimulate T-cell proliferation; there is no "extension" to accommodate complexation with antibody. Thus, the antigen appears able to stimulate T-cell proliferation despite its direct binding by the antibody. In addition, by using the antigen as the recognition component for the binding partner and as the stimulatory component for the assay, the assay can be adapted to detect the presence or absence in a sample of the specific binding partner associated with the antigen responsible for the T-cell proliferation. In the case where a specific binding partner of the antigen is available, the assay can be adapted to detect the presence or absence in a sample of the specific antigen that stimulates proliferation of a certain T-cell clone. This permits identification of, for example, monoclonal antibodies that bind to antigens which are in turn capable of T-cell activation, thus a nexus is established between T-cell populations specific for the antigen and the antibodies specific for the same antigen. Prior art methods do not permit such a nexus to be established.

Rheumatoid Arthritis T-cell Activating Antigen

Rheumatoid arthritis is a degenerative disease of the joints that is believed to be autoimmune in nature. Diagnosis and treatment of this disease offers a considerable challenge. The synovial fluid and sera of patients suffering from this disease have been analyzed to ascertain whether there are factors present that characterize such patents. A number of such factors have been identified.

Of particular importance are the anti-IgG antibodies discovered in the sera of patients with rheumatoid arthritis as early as the 1940s. These anti-IgG antibodies have been termed rheumatoid factors (RF) and the factors are present both in IgM and IgG forms.

Indeed, RF can be defined functionally as a human immunoglobulin which, in turn, binds the constant regions of human immunoglobulin. In a standard test, latex particles are coated with human IgG, and agglutination of the particles in the presence of a candidate factor is used as a criterion for the classification as RF.

Studies have been conducted of the genetics of these rheumatoid factors, and their expression by B-lymphocytes in the plasma or synovial fluid has also been studied. It has been thought that T-cells play a role in the generation of RF in rheumatoid arthritis; however, T-lymphocytes reactive with autologous IgG have not been detected in rheumatoid arthritis patients or in mice producing autoantibodies, leading to the conclusion that it is unlikely that the T-lymphocytes which trigger RF class switching and affect maturation recognize epitopes in the RF itself. It is considered more likely that the T-cells that facilitate IgM-RF to IgG-RF class switching react with antigen in an immune complex that is bound and processed by RF precursor B-lymphocytes (Carson, D.A. et. al., J Clin Invest (1991) 87:379-383). An extensive genetic analysis of the variable regions of these factors was reported by Olee, T. et.al., in J Exp Med (1992) 175:831-842.

An additional protein that seems to characterize rheumatoid arthritis plasma was named rheumatoid arthritis specific protein (RASP) and is described and claimed in U.S. 4,863,850. This protein was isolated from the plasma of a single patient and was shown to be a

protein unique to the rheumatoid arthritis condition by comparative two dimensional gel electrophoresis. The purified RASP binds to protein A; it is not clear whether it binds to immunoglobulins. RASP is itself an immunoglobulin and contains a heavy and light chain. According to the disclosure in this patent, RASP is capable of raising antibodies which recognize it, specifically, to the exclusion of IgG in general. However, apparently, it is problematic to remove all IgG from purified forms of RASP.

In a report published in 1987, Duke, O. et. al., Clin Exp Immunol (1987) 70:10-17, showed spontaneous proliferation of synovial fluid mononuclear cells to be a T-cell response inhibited by the addition of cyclosporin A or monoclonal anti-HLA-DR antibody and enhanced by the addition of IL-2; the enhancement by IL-2 was also inhibited by HLA-DR antibody. The authors conclude that an antigen presenting cell/T-cell interaction is important in this proliferation.

More recently, Devereux, D. et. al., Int Immunol (1991) 3:635-640 showed that of four polyclonal T-cell lines derived from four rheumatoid arthritis patients, two proliferated in a dose-dependent manner only to autologous synovial fluid in the presence of autologous or DR4Dw4 histocompatible antigen presenting cells. This proliferation could be inhibited by monomorphic anti-HLA-DR monoclonal antibody, but not by anti-DQ or anti-Class I antibodies. The authors concluded that the antigen specificity of T-cell lines cloned from these polyclonal starting cultures showed that they were reactive with a component of synovial fluid, suggesting the presence of an MHC Class II-restricted antigen in the rheumatoid synovial compartment that induces proliferation of *in vivo* activated T-cells.

It has now been found that the antigen responsible for this T-cell proliferation is itself an immunoglobulin and that this effect of the antigen can be effected by the heavy chain alone. An assay method for the presence of this factor in synovial fluid or other biological fluids has been developed.

Disclosure of the Invention

In one aspect, the invention is directed to an immunoassay-type method utilizing a detection system that depends on stimulation of T-cell proliferation by an antigen analyte. Alternatively, if the analyte is an antibody specific for antigen (which may also be an antibody), the detection system utilized by the method depends on the T-cell proliferation in response to the antigen for which it is immunospecific. The use of this detection system enhances the sensitivity of the assay and also permits identification of autoantigens and antibodies raised by, or specific to, these autoantigens wherein the nature of the autoantigen and/or of the antibody is unknown. Thus, autoantigens or immunoglobulins specifically reactive with an antigen can be detected according to the process of the invention.

Thus, in one aspect, the invention is directed to a method to detect a complex formed by an antigen capable of stimulating T-cell proliferation which method comprises contacting a specific binding complex (comprising the antigen and an immunologically reactive binding partner), with a T-cell population responsive to said antigen along with a composition containing histocompatible antigen-presenting cells (APCs), and therefore using the resulting T-cell proliferation as a measure of the complex.

The most convenient protocol for such an assay comprises coupling a specific binding partner for the antigen to a solid support, treating the coupled solid support with the antigen under conditions wherein a complex is formed between the specific binding partner and the antigen, washing away any components not bound on the solid support, and treating the solid support with a T-cell population and a histocompatible source of antigen-presenting cells. The proliferation of the T-cells is then measured by any convenient means such as, most commonly, by the uptake of labeled thymidine. An alternative method involves immortalization to generate a T-cell hybridoma which can be assayed for its production of cytokines, such as IL-2. The assay may be used to determine either the presence of the antigen or of its specific binding partner. Importantly, the assay also establishes a nexus between T-cells activated by an antigen and binding partners which specifically bind to the antigen.

The identification of the antigen responsible for the proliferation of T-cells in RA as an immunoglobulin, and specifically the heavy chain portion, leads to the development of methodologies for diagnosis and treatment of rheumatoid arthritis which take advantage of the nexus between the proliferative functionality and the immunoglobulin nature of the protein. The immunoglobulins or their heavy chain components of the biological fluid can be immobilized for convenient testing of their ability to effect proliferation of T-cells and the knowledge that this antigen is instrumental in T-cell proliferation permits design of therapeutic protocols based on responses that induce tolerance to this antigen. Furthermore, as a factor identified as crucial in the development of the disease, the

immunoglobulin of the invention or its heavy chain are suitable targets for therapy. Thus, antibodies to this immunoglobulin or its heavy chain coupled to a cytotoxic drug can be used to target the B-lymphocytes responsible for the production of this antigen.

Thus, the invention is also directed to a method to diagnose a subject for rheumatoid arthritis, which method comprises immobilizing onto a solid support the immunoglobulin fraction, or the heavy chain portion thereof, from a biological fluid derived from the subject to be tested. The solid support containing the immobilized immunoglobulin is then contacted with the T-cell containing synovial fluid from the same subject or a T-cell containing fraction thereof and with HLA-DR compatible antigen-presenting cells. The presence or absence of proliferation of the T-cells in this assay is then diagnostic of the presence or absence of the antigen and, in turn, of rheumatoid arthritis.

In another aspect, the invention is directed to a method to retard the onset or progression of rheumatoid arthritis in a subject comprising administering a subpopulation of immunoglobulins derived from the subject or their heavy chain components, or fragments thereof, which subpopulation has been shown to proliferate T-cells as described above. The subpopulation is administered in nonimmunogenic form so as to result in tolerance to these autoantigens influential in the condition.

In still another aspect, the invention is directed to a method to prevent or treat rheumatoid arthritis by administering a specific binding partner, such as an antibody or fragment thereof specifically reactive with the above T-cell stimulating antigen, which binding partner is coupled to a cytotoxic drug. The conjugate

then homes to B-lymphocytes producing the antigen and incapacitates them.

In still another aspect, the invention is directed to a method to purify an immunoglobulin in rheumatoid arthritic subjects that is capable of stimulating T-cell activation, which method comprises subjecting the biological fluid containing the antigen to purification steps such as protein A affinity chromatography, ion exchange chromatography, selective precipitation and the like and monitoring the progress of the purification by assessing the ability of the antigen to stimulate T-cell activation. The effectiveness of a treatment protocol for RA can also be monitored by assessing levels of T-cell stimulating antigen in the fluids of the subject.

Brief Description of the Drawings

Figure 1 is a graphical representation of the response of T-cells to KLH antigen coupled to solid support through antigen-antibody interaction.0

Figure 2 is a graphical representation of the response of T-cells from NOD mice as compared to BALB/c mice to captured insulinoma antigens.

Figures 3A-3C show the results of screening hybridomas for production of autoantigen-reactive antibodies.

Figure 4 is a graph showing the effect of autologous synovial fluid at various dilutions on several T-cell clones.

Figure 5 is a graph showing the dependence of thymidine incorporation into T-cells on the antigen concentration of synovial fluid used as a stimulus.

Figures 6A and 6B show the use of a T-cell proliferation assay to follow the purification of antigen by monitoring chromatographic eluate fractions.

Figure 7 is a graph that shows the dependence of T-cell proliferation on concentration of purified antigen.

Figures 8A and 8B are graphical representations on semilog plots of the response T-cells to autologous and allogenic synovial fluid. Figure 5A shows the response of T-cells to allogeneic synovial fluid; Figure 5B shows the response to autologous synovial fluid.

Figures 9A-9D are graphical representations of the ability of synovial fluid, as a function of concentration, to stimulate the production of various cytokines from cloned T-cells. Figure 9A shows the production of TNF-α; Figure 9B shows the production of IL-10; Figure 9C shows the production of IFN-γ; and Figure 9D shows the production of IL-4.

Modes of Carrying Out the Invention

The assay method of the invention results from the ability of an antigen to stimulate the proliferation of T-cells responsive to it even though the antigen is complexed with a specific binding agent. Conduction of the assay thus requires a T-cell population responsive to the antigen and a source of histocompatible antigen-presenting cells, as well as a means to detect the proliferation of the T-cell population supplied. Thus, the components of the detection system are the specific binding complex (comprising the antigen and a specific binding partner), a population of T-cells specifically responsive to the antigen, a histocompatible source of antigen-presenting cells, and a means for detecting T-cell proliferation.

The antigen-containing complex formed between the antigen and a specific binding partner for the antigen is detected by the assay. Thus, the assay may be designed

so that the analyte is either the antigen, its specific binding partner, or both the antigen and its specific binding partner.

As used herein, "antigen" may comprise a protein, a fragment of a protein, a polypeptide, an antibody or a fragment of an antibody. The "T-cell-stimulating antigen" or TCSA of the invention is an immunoglobulin which is capable of stimulating T-cell activation in autologous synovial fluid in the presence of HLA-DR compatible antigen presenting cells (APC). The heavy chain alone is also capable of this stimulation. Thus, either the TCSA per se or only the heavy chain thereof may be used for diagnosis and treatment of rheumatoid arthritis. While it is not known whether the TCSA of the invention is or is not identical with what was previously identified as rheumatoid factor (RF), in view of the fact that both are immunoglobulins, bind to protein A and bind the F_c portion of IgG (Benedek, T.G., Ann Int Med (1987) 106:304) it is possible that the TCSA of the invention is related to RF.

The nexus of an immunoglobulin factor or its heavy chain and the T-cell response was previously unknown. Indeed, the literature would suggest that the RF in RA patents is the outcome of an antigen-driven immune response indicated by the fact that the V gene of RF is hypermutated and that IgM-to-IgG class switch has occurred. This has been noted in the references cited above and by Randen, I. et al., J Immunol (1992) 148:3296.

A "binding partner" is most commonly an antibody but can also be an immunoreactive fragment thereof, or it can be a receptor or receptor ligand where the counterpart is the antigen, or it can be any other moiety which reacts and binds with the antigen, so long as the antigen is

associated with the T-cell population responsive thereto. The term "specific binding" expressly refers to the binding between a molecule(s) of interest and a binding partner therefor where the binding affinity between the binding partner and said molecule(s) of interest is substantially greater than a binding affinity between the partner and other molecules. Similarly, "immunologically reactive" and "immunoreactive" expressly refer to the binding between an antigen and an antibody where the binding affinity is substantially greater than any lesser binding affinity. As used herein, "antibody" expressly refers both to antibodies per se and to immunologically reactive fragments thereof where the antibody is used as a reagent in the assay.

While the complex might be formed in a variety of ways, including recovery of the complex as a precipitate, the most convenient strategy is utilization of a solid support to separate the complex from unbound components in the mixture. In this format, the specific binding partner to the antigen is first coupled to the support, thereafter the antigen is coupled to the support through specific binding to its partner. The specific binding partner can be used as the analyte in this format, since the antigen will bind to solid support only if the analyte has been coupled thereto. The antigen may also be the analyte in this assay. The treated support is then subjected to the detection method of the invention.

Even if the analyte is an antigen whose nature is unknown, an immunospecific binding partner may be generated by preparing immune serum to a crude mixture known to contain the analyte. This is particularly convenient since the immunized subject also provides a source of T-cells immunoresponsive to the same antigen.

The assay is useful generally for detecting the presence or absence of the antigen or its specific binding partner, and is particularly useful in a number of applications. For example, a method according to the present invention detects a complex comprising an antigen capable of stimulating T-cell proliferation in a corresponding T-cell population. The method comprises a step of contacting the complex formed from the antigen and a specific binding partner of the antigen with the corresponding T-cell population and with histocompatible antigen-processing cells. The method then comprises the step of detecting the proliferation of the T-cell population as a way to carry out a measure of the complex. Additionally, a method according to the present invention can comprise a step wherein the complex of the antigen and a specific binding partner for the antigen are evaluated and used as a measure of the antigen. Furthermore, a method according toe the present invention can comprise the step wherein the complex formed from the antigen and the specific binding partner for the antigen is evaluated and used as a measure of the specific binding partner fcr the antigen.

Thus, the system can be used to provide antibody reagents for the screening of expression libraries (libraries containing, *e.g.*, cDNAs) to recover DNAs encoding antigens, the nature of which may or may not be known in advance.

In one such application, the assay can be used to recover antibodies that will bind specifically to autoantigens involved in autoimmune responses. Such autoantigens can comprise autoantigens correlated with autoimmune responses such as, but not limited to, diabetes mellitus, or rheumatoid arthritis. In the case of rheumatoid arthritis, for example, the antigen may be

supplied as a crude extract of synovial tissue, cultured synoviocytes, cultured chondrocytes, or other cells producing such antigens. The T-cells and histocompatible antigen-presenting cells are obtained from synovial fluid and other tissues, and the antibodies are prepared from hybridomas or other immortalized forms of peripheral blood lymphocytes, or synovial joint fluid. In the assay, the antibodies from individual hybridoma supernatants, the antibodies are coupled to discrete portions of a test support. Thereafter, the bound antibodies are treated with the mixture of antigens. Those portions of the test support that result in T-cell proliferation when the T-cell/APC combination is added contain antibodies that specifically bind the antigen that is capable of the T-cell simulation. These antibodies from the hybridoma that supplied the antibodies to these portions of the test support may be used in screening expression libraries for these same antigens.

Accordingly, these antibodies may again be attached to a column, thereby allowing the bound antibodies to serve as an affinity column to isolate the antigen. Antigens are expressed by procedures known in the art for utilizing expression libraries. The antigens expressed by such systems are then placed in contact with the affinity column. The antigen of interest will be bound to the antibodies on the column. After the antigen is eluted by procedures known to those skilled in the art, the antigen may be further characterized. Accordingly, when expression libraries are screened for the antigens bound by the antibodies attached as an affinity column, the DNA from the member of the library that yielded the specific antigen can be cloned, recovered, and further characterized.

In a further example, hybridomas secreting antibodies specifically reactive with antigens capable of eliciting a T-cell response in potential diabetics can be identified. Accordingly, peripheral blood lymphocytes (PBLs) from human prediabetics or from NOD mice (an animal model for insulin-dependent diabetes mellitus (IDDM)) are immortalized as sources of candidate antibodies. Islet cells or cells from an insulinoma or can be used as a source of the antigen. T-cells derived from NOD mice or prediabetic humans may be used as the corresponding T-cell preparation in the assay. Again, the hybridoma supernatants are tested individually to identify a hybridoma that secrets an antibody which has a nexus with the T-cells, the proliferation of which T-cells is effected by antigen in the presence of APCs.

In general, the T-cell composition used in the assay is derived from the lymphocytes of a subject immunoresponsive to the antigen. The T-cell population may be either T-lymphocytes prepared directly from plasma, spleen, lymphoid organs or other T-cell sources, or suitable T-cell lines derived therefrom.

The T-cell population must "correspond" to the analyte. That is, the T-cell composition must contain a T-cell population that is dedicated to respond to the particular antigen which will be presented in the complex between the antigen and a specific binding partner therefor. Such compositions containing corresponding T-cell populations can be obtained by immunizing suitable subjects with the antigen used in the assay, or, when the antigen is an autoantigen, from autoimmune subjects. For example, in the case of assays for antibodies immunoreactive with autoantigens in rheumatoid arthritis as set forth above, suitable T-cell compositions containing "corresponding" populations can be obtained

from subjects having this condition; in the case of IDDM described above, T-cell compositions with "corresponding" populations can be obtained from NOD mice or prediabetic human subjects.

Antigen-presenting cells must be histocompatible with the T-cell population. This is automatic if the T-cells and APCs are obtained from the same source. However, they may also be prepared separately. In murine models the APCs are generally syngeneic spleen cells, purified B-cells or purified macrophages. Further, the APCs in murine models can be B-cell or macrophage cell lines which are transfected to be Class II MHC compatible, or B-cell or macrophage hybridomas which are Class II compatible. For human-related assays, immortalized B-cell lines from the same subject can be used, MHC Class II compatible cell lines, or compatible peripheral blood mononuclear cells can be used as APCs. For example, in the use of the assay to obtain antibodies immunoreactive with the autoantigens in rheumatoid arthritis, APC which are HLA-DR4Dw4-restricted can be used.

In conducting the assay, the complex between the antigen and a specific binding partner therefor can be contacted simultaneously with the T cells and APC, or may first be provided APC so that the antigen may be processed to a form suitable for presentation to the T-cells, and then the composition containing the corresponding T-cell population added afterward.

The proliferation of the T-cells may be detected by a variety of means. If radiolabeled thymidine uptake is used as a method to detect proliferation, cells are pulsed with about 1 μCi of tritiated thymidine and harvested a number of hours later, and the incorporated radioactivity is determined using scintillation counting.

In addition, IL-2 secretion can be used as a measure of T-cell activation; other methods include colorimetric assays and FACS analysis of the level of expression of the nuclear proliferation protein.

In a typical illustrative measurement, typical sources of APC and T-cells, such as PBLs, pancreatic or peripheral lymph nodes, or spleens are prepared as homogeneous cell suspensions in RPMI-1640 medium supplemented with 5% FCS, 10 units/ml pen/strep and 200 mm L-glutamine by separately layering the cell suspensions on Ficoll 400 cushions (Lymphoprep, Boehringer-Mannheim) and centrifuging for 30 min. at 300 x g. The interface containing mononuclear cells is recovered, washed and used in the assays at about 1-3 x 10^5 cells/ well. These suspensions are provided to the specific binding partner-coated wells which have been treated with antigen-containing compositions. The cultures are incubated for 72-86 hours, then pulsed with 1 μCi/well of tritiated thymidine and harvested 16 hours later. The incorporated radioactivity is determined using a β-plate scintillation counter.

Thus, in general, a typical protocol for the invention involves the steps of:

(1) coupling a specific binding partner for an antigen to a solid support (this partner may serve as the analyte or as a reagent);

(2) washing unbound partner from the support;

(3) treating the partner-treated solid support with a composition containing antigen;

(4) washing unbound components from the support;

(5) treating the coupled support with a composition comprising a "corresponding" T-cell population immunoresponsive to the antigen and with a

histocompatible population of antigen-presenting cells; and

(6) detecting the proliferation of the T-cells in the composition as a measure of the antigen coupled to the support.

In the use of the above method to identify an antibody specifically binding an antigen which simulates T-cell proliferation, the specific binding partner applied to each well will be the supernatant of an immortalized antibody-secreting B-cell, the antigen can be provided as such or, if the nature of the antigen is unknown, the antigen can be provided as a crude extract of a fluid or tissue known to contain the antigen, and the T-cell/APCs are those known to respond to the antigen.

Conversely, the assay could be used to test individual antigens for their ability to effect the proliferation of T-cells in the presence of APC using polyclonal serum as the source of the specific binding partner and testing individual antigens in each well. The polyclonal antiserum may be, for example, autoimmune serum, or sera raised in response to crude extracts known to contain the antigen.

Use of the Invention Assay for TCSA

The T-cell proliferation assay which shows the ability of the TCSA found in biological fluids to stimulate a T-cell response is most conveniently conducted by first immobilizing the candidate antigen to a solid support and then measuring the activation of T-cells placed in contact with the support along with a histocompatible source of antigen-presenting cells.

This antigen is able to stimulate the proliferation of T-cells responsive to it even though the antigen is complexed with a specific binding agent. As generally

stated above, conduct of the assay requires a T-cell population responsive to the antigen, i.e., T-cells derived, usually, from the same subject as that from whom the antigen is obtained, and a source of histocompatible antigen-presenting cells, as well as a means to detect the activation or proliferation of the T-cell population supplied. Thus, the components of the assay system are the specific binding complex of which the antigen is a member, a population of T-cells specifically responsive to the antigen, a histocompatible source of antigen-presenting cells, and a means for detecting T-cell activation.

The "binding partner" for the TCSA is most commonly an antibody but can also be an immunoreactive fragment thereof, or it can be any other moiety which reacts and binds with the antigen. As the TCSA is known to bind Protein A and human immunoglobulin, specifically to the F_c portion thereof, these materials are convenient specific binding moieties which permit the capture of the antigen from the biological fluid. The term "specific binding" expressly refers to the binding between a molecule(s) of interest and a binding partner therefor where the binding affinity between the binding partner and said molecule(s) of interest is substantially greater than a binding affinity between the partner and other molecules. Similarly, "immunologically reactive" and "immunoreactive" expressly refer to the binding between an antigen and an antibody where the binding affinity is sufficient to effect complexation needed for the purpose in question. Where the antibody is used as a reagent in an assay, "antibody" expressly refers both to antibodies per se and to immunologically reactive fragments thereof.

While the above-mentioned complex might be formed in a variety of ways, including recovery of the complex as a

precipitate, the most convenient strategy is utilization of a solid support to separate the complex from unbound components in the mixture. In this format, the specific binding partner to the TCSA is first coupled to the support, thereafter the TCSA is coupled to the support through specific binding to its partner. The treated support is then subjected to the assay.

In general, the T-cell composition used in the assay is derived from the lymphocytes of a subject immunoresponsive to the antigen, usually the same individual. The T-cell population may be either T-lymphocytes prepared directly from plasma, spleen, lymphoid organs or other T-cell sources, or suitable T-cell lines derived therefrom. While, in most cases, the T-cells of a particular subject will react only with an antigen derived from autologous synovial fluid or plasma, in a few instances there is cross-reactivity among human subjects. Thus, it appears that there are subsets of subjects with cross-reactive TCSA components; there are designated "cross reactive" subjects herein. When the T-cells are reactive with the TCSA, whether autologous or allogeneic, the T-cells are said to "correspond" to the TCSA being assessed.

Antigen-presenting cells must be histocompatible with the T-cell population. This is automatic if the T-cells and APCs are obtained from the same source. However, they may also be prepared separately. In murine models the APCs can be syngeneic spleen cells, purified B-cells or macrophage, or B-cell or macrophage cell lines which are transfected to be Class II MHC compatible. For humans, as is the case here, immortalized B-cell lines from the same subject can be used, or MHC Class II compatible peripheral blood mononuclear cells or cell lines can be used. In the use of the assay herein for

TCSA, APC which are HLA-DRB1*0401-restricted can be used.

In conducting the assay, the TCSA and a specific binding partner therefor can be contacted simultaneously with the T-cells and APC, or may first be provided APC so that the antigen may be processed to a form suitable for presentation to the T-cells, and then the composition containing the corresponding T-cell population added afterward.

The activation of the T-cells may be detected by a variety of means. Assays for proliferation may, for example, be used. If radiolabeled thymidine uptake is used as a method to detect proliferation, cells are pulsed with about 1 μCi of tritiated thymidine and harvested a number of hours later, and the incorporated radioactivity is determined using scintillation counting. In addition, cytokine secretion such as IL-2 secretion can be used as a measure of activation; other methods include colorimetric assays and FACS analysis of expression of the nuclear proliferation protein. As shown hereinbelow, for some T-cell clones derived from rheumatoid arthritis patients, the autologous antigen is able to stimulate the production of TNF-α, IL-10, IFN-γ, and IL-4.

In a typical illustrative measurement, sources of APC and T-cells, such as PBL, pancreatic or peripheral lymph nodes, or spleens are prepared as homogeneous cell suspensions in RPMI-1640 medium supplemented with 5% FCS, 10 units/ml pen/strep and 200 mm L-glutamine by separately layering the cell suspensions on Ficoll 400 cushions (Lymphoprep, Boehringer-Mannheim) and centrifuging for 30 min. at 200 x g. The interface containing mononuclear cells is recovered, washed and used in the assays at about 1-3 x 10^5 cells/ well. These

suspensions are provided to specific binding partner-coated wells, such as wells coated with human Ig or with Protein A, which have been treated with the TCSA compositions to be tested. The cultures are incubated for 72-86 hours, then pulsed with 1 μCi/well of tritiated thymidine and harvested 16 hours later. The incorporated radioactivity is determined using a β-plate scintillation counter.

Thus, in general, a typical protocol involves the steps of:

(1) coupling the specific binding partner for TCSA to a solid support;

(2) washing unbound partner from the support;

(3) treating the partner-treated solid support with a TCSA-containing fluid such as plasma or synovial fluid or a fraction thereof;

(4) washing unbound components from the support;

(5) treating the coupled support with a composition comprising a "corresponding" T-cell population immunoresponsive to the TCSA and with a histocompatible population of antigen-presenting cells; and

(6) detecting the activation of the T-cells in the composition as a measure of the antigen coupled to the support.

The TCSA of the invention may be purified from synovial fluid by a protocol which involves recovery of immunoglobulins using affinity chromatography (for example, antihuman Ig-coupled Sepharose or protein A-coupled support). Proteins diluted from the anti-IgG or protein A columns can then be further purified by SDS-PAGE under reducing and non-reducing conditions and molecular weights determined. Under reducing conditions, light and heavy chains are obtained which have molecular weights near 20 kd and 50 kd respectively.

The assay described above can be used to follow the progress of purification. The nature of suitable affinity columns as containing Protein A or Ig as affinity reagents is understood; however, any suitable separation method can be used in view of the availability of this assay procedure. The TCSA obtained in desired purity can then be used in the method of the invention to tolerize subjects to the antigen.

The assay described above can also be used to follow therapeutic protocols. The effectiveness of the protocol may be monitored by the effect on TCSA levels.

Diagnosis of Subjects for Rheumatoid Arthritis

The assay described above can conveniently be used as a method to assess a subject for rheumatoid arthritis since such patients are capable of autologous activation of their T-cells. In the use of this assay, the plasma or synovial fluid of the subject to be tested is used as the source of putative TCSA in the assay described above. Since, by virtue of the invention herein, it is understood that TCSA binds to human immunoglobulin or Protein A, suitable solid substrates such as microtiter wells, latex beads, or specifically designed assay plates covalently coupled to or adsorbed with Protein A or human immunoglobulin can be used. General methods for providing substrate surfaces with these or other proteins are well known in the art, and are conventional. Covalent attachment can be direct or through linkers that are commercially available. Techniques for adsorption are well known.

Thus, the physical design of the assay device can accommodate a wide variety of formats. The conduct of the assay is as described above, using as a test sample the appropriate biological fluid, typically synovial

fluid. Any measure of activation of the T-cell complement derived from the same subject as described above in the presence of compatible APC can be used.

The method of the invention is useful to ascertain those subjects whose symptomologies may be associated with rheumatoid arthritis, and suitable treatment method appropriate to this indication can then be employed. The treatment protocol can be monitored by the assay as well.

Methods to Effect Autoimmunotolerance

The TCSA associated with the rheumatoid arthritis condition of a subject, or only the heavy chain portion thereof, or a fragment thereof which is shown to effect T-cell activation in the assay described above can be used to tolerize the affected individual with respect to this condition. The individual may already show symptomology of the rheumatoid arthritis or may be known from family history or other analysis, such as genetic analysis, to be at risk.

Methods for tolerizing individuals by administering antigens responsible for an undesired immune response in nonimmunogenic form are known in the art. For example, PCT Application WO 88/10120 to Brigham and Women's Hospital describes and claims the treatment of autoimmune diseases by oral administration of autoantigens. Protocols for administration of such autoantigens are described in this application. The techniques described in this application are applicable as well to the TCSA and fragments thereof herein.

Thus, exposure of subjects to the TCSA, the heavy chain portion, or fragments thereof which comprise at least one T-cell epitope of the TCSA will tolerize or anergize appropriate T-cell populations such that they become unresponsive to the TCSA and do not participate in

stimulating an immune response upon such exposure. By "T-cell epitope" is meant the smallest unit of the TCSA that is recognized by the appropriate T-cell receptor.

Administration of the TCSA or appropriate fragment comprising at least one T-cell epitope also may modify the lymphokine secretion profile of the target T-cells as compared to exposure with the TCSA as it occurs naturally. Exposure to the TCSA or appropriate portion also influences T-cell populations which normally participate in the response to the natively occurring TCSA so that these are drawn away from the sites of normal exposure to the autoantigen -- e.g., from synovial fluid -- toward the sites of therapeutic administration of the immunotolerating TCSA. This redistribution of T-cell subpopulations may ameliorate or reduce the ability of the subject's immune system to stimulate the usual immune response in the joint.

Administration of the prophylactic or therapeutic compositions of the invention to an individual to be tolerized can be conducted using known techniques, as described above. The TCSA or portion thereof may be administered in combination with an appropriate diluent or carrier, but generally in the absence of adjuvant. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions and suitable carriers include polyethylene glycol and liposomes. In general, for purposes of inducing tolerance, the TCSA or portion thereof is administered in nonimmunogenic form without adjuvant. Administration is by injection preferably using subcutaneous or intramuscular, or by oral administration, inhalation, transdermal application or rectal administration. The compositions are administered to subjects at risk for or suffering from rheumatoid arthritis (for example, a subject diagnosed using the

invention method) at dosages and for a length of time effective to reduce susceptibility or symptoms of the rheumatoid arthritis. Effective amounts of the compositions will vary according to factors such as the severity of the condition; the age, sex and weight of the individual; and the ability of the particular TCSA or fragment chosen to elicit the immunotolerizing response.

In most instances, the TCSA or fragment is derived from the same individual being treated; however, as described herein, cross-reactivity of TCSA components among individuals is sometimes exhibited. These individuals are designated as "TCSA cross-reacting" individuals. Thus, the compositions may include the active ingredient derived from any TCSA cross-reacting individual, including the subject per se.

The TCSA or heavy chain portion thereof is preferably purified prior to administration; however, for autologous administration, it may be possible to use compositions of lesser purification, since there is no risk of immunogenic response to the subject's own fluids. Suitable purification methods, however, are desirable for providing subunits which are effective in the invention method. As stated above, the identification of these subunits may be accomplished using the assay method described herein.

Interference with TCSA Secretion

In an alternative approach to retarding the progress or onset of rheumatoid arthritis, the ability of the B-lymphocytes to secrete TCSA may be diminished or destroyed by administration of a specific binding partner for the TCSA preferably coupled to a cytotoxic molecule. The specific binding partner for use in this method must be immunospecific for the TCSA; it is not sufficient that

it merely be immunoreactive therewith. Thus, the specific binding partner must bind the B-lymphocytes secreting TCSA to the exclusion of other B-lymphocytes. If the binding partner is supplied in sufficient concentration it may be effective alone; however, it is preferred that it be coupled to a toxin to enhance its effectiveness in inactivating the target B-lymphocytes. any cytotoxic moiety may be used; preferred are such toxins as ricin, diphtheria and botulism toxins.

To prepare the specific binding partner for use in this aspect of the invention, the TCSA purified from the subject or a uniquely immunogenic portion thereof is used to immunize mammalian hosts such as mice, rabbits, sheep, and the like. The antibodies are obtained by standard purification methods from the antisera or monoclonal antibodies are prepared from the peripheral blood lymphocytes or spleen cells using standard immortalization techniques to obtain cell lines that are capable of secreting the desired monoclonal antibodies. Suitable such cell lines can be identified by screening with the TCSA or the subunit.

In addition, such antibodies may be manipulated by using recombinant methods to produce chimeric antibodies containing human constant regions or various other modified recombinant forms to reduce immunogenicity.

In addition to providing specific binding partners in the form of antibodies or their reactive fragments, the relevant antigen specificity of the TCSA can be used. For example, RF is known to bind the constant region of IgG; this antigen can thus target RF-producing B cells. Analogously, the TCSA's antigen can be used as a targeting moiety.

Cytotoxic moieties are coupled to the specific binding partner using conventional techniques appropriate

to the toxin. Protein toxins may be linked as fusion extensions or using covalent coupling through linkers and the like; nonprotein toxins are linked through their available functional groups.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Model System Using KLH as Antigen

Keyhole limpet hemocyanin (KLH) was used as a model antigen/analyte to illustrate the method of the invention.

First, the specific binding partner needed for the KLH to be analyzed and the T-cells corresponding to KLH were generated by preparing KLH-specific antiserum and lymphocytes. Female NOD mice, 5-8 weeks of age, were immunized subcutaneously with 50 μg of KLH emulsified in Complete Freund's Adjuvant on day 0. On day 7, the mice received a booster of 50 μg KLH in Incomplete Freund's Adjuvant at the same site of inoculation. Control mice were mock-immunized with PBS under the same conditions. On day 10, the mice were sacrificed and bled and the draining axillary, brachial and cervical lymph nodes were harvested. Sera were collected from the pooled, clotted blood and T-cells were partially purified from the pooled lymph nodes by passage over nylon wool columns.

For the assay, 96-well EIA plates (Costar) were coated with goat anti-mouse immunoglobulin overnight at 4°C and then blocked with PBS containing 10% FCS and washed. Sera from the KLH-immune and mock-immune mice were serially diluted and incubated on the plates for 2 hours at room temperature. After washing, KLH diluted in

PBS was added (1 μg/well) and incubated for 2 hours at room temperature. The plates were then washed.

The nylon wool-purified T-cells from KLH-immune or mock-immune mice, prepared as above, were added to the plates, along with irradiated spleen cells from naive NOD mice as a source of antigen-presenting cells. The plates were incubated at 37°C for three days and then pulsed with 1 μCi of labeled thymidine for 6 hours. The T-cells associated with the wells were harvested and counted in a Beta-Plate scintillation counter.

The results are shown in Figure 1, expressed as the mean CPM ±SD of triplicate wells. None of the combinations of 1) mock-immune sera + mock immune T-cells; 2) mock-immune sera + immune T-cells; or 3) immune sera + mock immune T-cells showed T-cell proliferation. However, when the plates were coated with antisera from immunized mice and T-cells from immunized mice were used in the assay, T-cell proliferation was pronounced. Detectable results were obtained even with a 1:243,000 serum dilution; at a serum dilution of 1:81,000, the difference from background was readily discernable.

Example 2

Detection of Autoantibodies in the Serum of NOD Mice

To prepare β-insulinoma cytoplasmic extracts and crude membranes for use in the assay, the β-insulinoma cell line B23720 was maintained in roller bottles with DMEM containing 15% horse serum and in 5% fetal calf serum. The cells were harvested, washed with PBS and then resuspended in hypotonic lysis buffer; 20 mM HEPES, pH 7.4, 1.5 Mm $MgCl_2$, 1 mM EDTA, and Dounce-homogenized. Nuclei and cell debris were pelleted by low-speed centrifugation and the supernatant then spun at 40,000 g. for 30 minutes. Pelleted membranes were resuspended in 20 mM HEPES, and both crude membranes and cytosolic extracts were stored at -70°C.

Extracts of the membranes were obtained by incubating the membranes in SDS at a final concentration of 0.1% at room temperature for 1 hour and centrifuging at 40,000 g. The supernatant extract was then used.

The assay was conducted using 96-well Costar plates (Tissue culture brand) which were first coated with rabbit anti-mouse antibodies (100 μg/ml) in 50 mM HEPES buffer, pH 9 (final volume 100 μl) overnight at 4°C. After washing, the wells were blocked with PBS, the wells were blocked with PBS containing 5% FCS for 4 hours to overnight at 4°C. Protein G purified antibodies were obtained from either naive NOD mice or from NOD mice subcutaneously immunized once 10 days beforehand with a 50 μg protein extract of insulinoma membranes, prepared as above. The protein G purified antibodies, were added to the plates and incubated for 1 hour at 4°C. The plates were then washed three times with PBS, and the insulinoma antigen was added. The antigen was a mixture of the membrane extract and cyotosolic extract at 2 μg cytosolic and 2 μg of membrane extract/well. The plates

were again incubated at 4°C for 1 hour and washed three times with PBS.

The antigen-presenting cells and T-cells from unprimed NOD mice were prepared for use in the assay either as whole spleen cells, or B- and macrophage-depleted spleen cells.

To obtain B- and macrophage-depleted spleen cells (*i.e.*, spleen cells enriched in T-cell content), a two-stage process was carried out. First, a single-cell suspension from untreated spleen was incubated in plastic dishes at 37°C for 2 hours. Microphages characteristically adhere to the plastic dishes. Then the nonadherent cells, depleted of macrophages, were then treated with goat antimouse Ig conjugated to magnetic beads (Dynal) and incubated at 4°C, rotating for 45 minutes. The B-cells attached to the magnetic beads via the cell surface Ig were separated by using a strong magnet; the cells in suspension, cells enriched in T-cell content, were collected and washed, then used in the assay.

Thereafter, either whole spleen cells or the T-enriched spleen cells were added to the wells, and T-cell proliferation was measured by H^3 labeled thymidine incorporation as described above. The results are shown in Figure 2.

These results indicate that both immunized NOD mice and unprimed NOD mice contain antibodies immunoreactive with the insulinoma antigen. As shown in the figure, the sera of Balb/C mice do not contain antibodies to the insulinoma antigen. Both unprimed NOD mice and NOD mice immunized with insulinoma membrane have sera containing antibody to the insulinoma antigen, antibody capable of capturing the polypeptide(s) which stimulates T-cell proliferation in the presence of APCs. There appears to

be no significant difference in results, whether the plates were initially coated with rabbit antimouse antibodies or whether the sera were directly coated to the wells. In both cases, the T-cell-enriched spleen suspension appeared to give somewhat greater response than whole spleen cells.

Example 3

Assay for Autoantigen Antibody Producing Hybridomas

To obtain hybridomas, spleen cells or lymph node cells were fused with Ag8 myeloma cells using standard procedures. The spleen cells or lymph node cells were obtained from, either, naive 42-59 day old NOD mice, or from NOD mice immunized with whole insulinoma cells.

For immunization, NOD mice were injected intraperitoneally with 1-2 x 10^6 live insulinoma cells. Twenty days postimmunization, the mice were sacrificed and the spleen cells or lymph node cells were used in the fusions. The unprimed NOD mice has been injected with one dose of Ribi adjuvant 4 days before recovering the spleen cells or lymph nodes for the fusions.

The hybridoma supernatants were then screened in the assay of the invention with T-cell lines generated against insulinoma antigen as follows:

To generate T-cell lines from unprimed mice, cells from the spleen or the lymph nodes from naive NOD female 30-40 day old mice were stimulated *in vitro* with insulinoma (B23720) antigen (whole cell extract, membranes, or cytosol) at a protein concentration of 10 μg/ml, or with irradiated insulinoma cells at 5,000 cells/ml in RPMI medium containing 1% NMS, pen/strep, glutamine and 1 μg/ml leuko-A.

The spleen or lymph cells were then incubated at 37°C, 5% CO_2 for 3-4 days, then washed and resuspended in

RPMI medium containing 10% FCS pen/strep, glutamine and 15% rat Con-A supernatant or 20 units /ml RMIL-2.

To generate T-cell lines from immunized mice, T-cell lines were obtained from NOD female 30-40 day old mice which had been injected with a single intra-footpad injection of insulinoma antigen membrane extracts (see Example 1), 50 μg/mouse with or without Ribi adjuvant. Nine to ten days postinjection, spleen or both popliteal and inguinal lymph nodes were removed and stimulated as described for the generation of T-cell lines from unprimed mice.

The assay was then conducted as follows. Ninety-six well plates were coated with goat antimouse Ig and incubated overnight at 4°C. The plates were washed with PBS and blocked with 5% FCS-PBS for 2-4 hours at room temperature or 4-16 hours at 4_0C. Hybridoma supernatants were added individually to the wells, and incubated overnight at 4°C. The plates were then washed with PBS.

The antigen was added as a mixture of 2 μg/well insulinoma cytosolic extracts and 2 μg/well SDS membrane extract. The plates were incubated for 2-4 hours at 4°C, washed with PBS, and a T-cell suspension, either from immunized mice or from unprimed mice, was added.

Irradiated (5,000 RADs) splenic APC were added at 5 x 10^5 cell/well with between 20,000 to 50,000 cell/well of the T-cell line. The plates were then incubated at 37°C for three days and pulsed with 1 μCi/well of tritiated thymidine. Sixteen hours later, the cells were harvested and the amount of incorporated thymidine was counted.

Figures 3A through 3C show the results of several fusions. The assay is successful in identifying hybridomas producing antibody specific to insulinoma antigen, antibodies that in this format are capable of capturing antigenic polypeptide.

Example 4

Verification of Autoantigen in Synovial Fluid

The synovial fluid of a rheumatoid arthritis patient was used as the source of a previously unidentified autoantigen measured by the T-cell activation assay described above. The autoantigen is believed to be an antibody, and thus reacts with anti-human Ig. Rabbit antibodies specific for human immunoglobulin H and L dimers were diluted in 50 mM carbonate buffer, pH 9, and coated onto a Nunc Maxisorb Microtiter plate. The plates were incubated overnight at 4°C, then washed, and then blocked by incubation for 1 hour with PBS containing 1% FBS. The synovial fluid to be tested was diluted in PBS and added to the wells. After incubation at room temperature for 4 hours, the synovial fluid was removed and the wells were washed three times with PBS.

DRB1*0401-matched antigen-presenting cells were irradiated, added at 5 x 10^4/well, and the plates were incubated overnight at 37°C to process the antigen. Various cloned T-cell lines were established using T-cell preparations from the same patient from which the synovial fluid was obtained. These T-cell lines were established as described by Devereux, D., at al, Internat Immunol (1991) 3:635-640. Cells from these T-cell lines were added at 10^4/well. Cells from these T-cell lines were added at 10^4/well, and the plates were incubated at 37°C for three days and then pulsed with 1 μCi/well of [^{3}H]-thymidine for 6 hours before harvest of the T-cells on glass filters using a semiautomatic sample harvester. The [^{3}H] incorporation was assessed by counting in a Beta Plate Scintillation Counter. The results are shown in Figure 4.

As shown, several of these cloned cell lines (arbitrarily numbered 1.25, 1.44, 3.1 and 3.27) were

stimulated to proliferate by the synovial fluid containing the antigen. This experiment demonstrates that there is an antigen present in the synovial fluid, possibly an antibody, which is capable of stimulating T-cell proliferation.

Example 5

TCR Typing of T-Cells That Recognize TCSA

Six synovial T-cell clones were obtained from a single rheumatoid arthritis patient and verified to recognize autologous synovial fluid by the method of Example 1. TCR V-region gene usage was then determined using PCR and commercially purchased specific V-region polymers which identify 22 Vβ and 18 Vα genes (Midland Reagents, TX). In each determination, 5′ and 3′ Cα primers were used as positive controls for PCR.

The results in Table 1 show that a variety of T-cell clones recognize the TCSA. In the table, N is a negative result indicating that the Vα or Vβ usage was not identified because Cα probes were positive.

Table 1. Vβ and Vα TCR usage by a panel of six rheumatoid arthritis synovial T-cell clones recognizing autologous synovial fluid. TCR V-region gene usage was determined using the PCR technique and commercially purchased specific V region primers, which identify 22 Vβ and 18 Vα genes (Midland Reagents, TX). In each determination, 5′ and 3′ Cα primers were used as positive controls for the PCR.

T-cell clone	Vβ gene	Vα gene
1.4	18	8
1.26	N*	N
3.27	N	6
3.39	8	1
3.43	9	N
3.47	3	2

* Negative result indicating that the Vα or Vβ usage was not identified by the noncompendious panel of probes used, because in all cases the Cα probes were positive.

As shown in the table, although all of the T-cell clones recognized the antigen and proliferated in response to it, the Vβ genes used were 18, 8, 9 and 3 and the Vα genes used were 8, 6, 1 and 2. Thus, a multiplicity of TCR are capable of responding to TCSA.

Example 6

Response of Autologous T-cells to TCSA

T-cell clones were generated from the synovial fluid of a 56 year old Caucasian male with a 7 year

history of RA as described by Devereux, D. et. al., Int Immunol (1991) 3:635. Assays were conducted as in Example 1. T-cells (2 x 10^4/cell) were added to 96-well microtiter plates containing 10^5 irradiated DRB1*0401 matched peripheral blood mononuclear cells and varying concentrations of autologous synovial fluid and plasma. After 72 hours at 37°C, 1 μCi of tritiated thymidine was added to each well and the plates were harvested 18 hours later. Background incorporation of labeled thymidine was less than 500 cpm.

As shown in Figure 5, synovial fluid provided the most marked response of either T-cell clone 1.26 (open circles) or T-cell clone 3.27 (closed circles). Plasma stimulated T-cell clone 1.26 (open triangles) and T-cell clone 3.27 (closed triangles) less effectively.

Example 7

Activity of TCSA light chains and heavy chains.

Immunoglobulin was purified from the synovial fluid of the patient of Example 1 using a protein A column according to the manufacturer's instructions (Pierce, Rockford, Illinois). The eluates from the protein A columns were applied to SDS-PAGE gels under non-reducing or reducing conditions. For reducing conditions the sample was first boiled for 5 minutes in the presence of 10 mM DTT. In total, 800μg of protein was loaded onto 11% polyacrylamide gels 0.1 cm x 11 cm x 6-8 cm. A vertical slice was stained with Coomassie blue and is shown at the left of each graph. The gels were cut into 10 equal slices with a razor blade and the slices were minced into small fragments and transferred to a tube containing 10 ml 0.05 M ammonium carbonate, 0.1% SDS, and the protein was allowed to diffuse out of the gel by incubating overnight at room temperature under gentle

rocking. The next day 9 ml of the supernatant was mixed with 36 ml methanol and the protein was precipitated by incubation at -20° overnight. The precipitate was centrifuged into a pellet at 500 g for 15 minutes and the supernatant discarded. The pellet was suspended in water and lyophilized.

The dry white powder was dissolved in 1 ml PBS and protein concentration was determined with the BCA assay (Pierce, Rockford IL). The recovery from the gel was always between 10 and 80%. The proliferation assay was performed as described in Example 1. The results are shown in Figures 6A and 6B.

Figure 6A shows the results for the non-reducing gels. High molecular weight fractions were capable of stimulating proliferation.

Figure 6B shows the results using reducing gels. The fractions representing the heavy chain portion of the immunoglobulin were themselves capable of stimulating the T-cell response.

Example 8

Response of Various T-Cell Lines to Protein A Purified TCSA.

Independent cell lines were obtained from four rheumatoid arthritis patients and designated IPCw 32, 113, 115 and 145. The response of each of these cell lines to the protein A eluate in the assay of Example 1 was determined and the results are shown in Figure 7. As shown, the cell lines vary in their response but all are capable of proliferation in response to TCSA.

Example 9

T-cells were prepared from a single patient (Patient 145) and tested in the assay of Example 1. A

polyclonal T-cell line was obtained from this patient, as described above, and used in the assays. The T-cells of this patient were used in assays versus autologous synovial fluid and versus allogeneic fluid derived from another patient (Patient 32). As shown in Figures 8A and 8B, response to autologous synovial fluid exceeded that of the response to allogeneic fluid; however, a significant response to the fluid of this different patient was obtained.

Example 10

Lymphokine Production

The ability of the TCSA of the invention to stimulate lymphokine production was also assessed. Samples of 10^6 T-cells derived from a rheumatoid arthritis patient were incubated for 24 hours with 10^6 irradiated PBMC expressing DRB1*0401 which had been prepulsed with varying concentrations of autologous synovial fluid. The supernatants were then harvested and tested in ELISA assays using various lymphokine-specific antibodies. Concentrations of the cytokines were determined (pg/ml).

The results of these determinations for various T-cell clones are shown in Figures 9A-9D. Figure 9A shows the effect of synovial fluid on TNF-α production; all cell lines show a monotonic increase in TNF-α as a result of contact with varying concentrations of synovial fluid. The effect on IFN-γ production was similar, as shown in Figure 9C. However, as shown in Figures 9B and 9D, while generally the presence of synovial fluid stimulated the production of IL-10 and IL-4, respectively, dependence on the concentration of synovial fluid was not uniform across cell lines. For production of IL-10, two of the three cell lines showed monotonic increases in IL-10 production, while a single cell line showed a decrease in

secretion at higher concentrations. This cell line, however, showed a monotonic increase in IL-4 production with increasing amounts of synovial fluid while the other two cell lines tested gave decreased secretion values at higher concentrations.

Claims

1. A method to detect a complex formed by an antigen capable of stimulating T-cell proliferation in a corresponding T-cell population, which method comprises:

contacting a complex formed from said antigen and a specific binding partner of said antigen with said corresponding T-cell population and with histocompatible antigen-presenting cells, and

detecting the proliferation of said T-cell population as a measure of the complex.

2. The method of claim 1 wherein the specific binding partner for the antigen comprises antibodies immunoreactive with said antigen, and/or

wherein the corresponding T-cell population is obtained by immunizing a subject with the antigen and recovering a composition comprising T-cells from the subject.

3. A method to determine the presence, absence or amount of, or to identify an antigen in a fluid sample said antigen having a specific binding partner therefor, which method comprises:

providing said specific binding partner coupled to a solid support;

treating the coupled support with said fluid sample suspected of containing said antigen to obtain support further coupled to any antigen in the sample;

washing the support;

treating the resulting antigen-coupled support with a corresponding T-cell population and histocompatible antigen-presenting cells; and

detecting the presence, absence or amount of T-cell proliferation as a measure of the antigen coupled to the support.

4. A method to determine the presence, absence or amount of or to identify a specific binding partner in a fluid sample for an antigen, which method comprises:

treating a solid support with said sample under conditions wherein any specific binding partner in the sample will be coupled to the support;

washing the support;

treating the support to which a specific binding partner is coupled with said antigen to obtain support coupled to antigen through said specific binding partner;

washing the support;

treating the resulting antigen-coupled support with a corresponding T-cell population and histocompatible antigen-presenting cells; and

detecting the presence, absence or amount of T-cell proliferation as a measure of antigen coupled to the support, and thereby as a measure of the specific binding partner absorbed to the solid support.

5. A method to screen a cDNA expression library for production of a desired antigen, which method comprises:

screening a cDNA expression library with a specific binding partner for the antigen identified by the method of clam 4.

6. A kit suitable for conducting the method of claim 3, which kit comprises solid support to which has been coupled a specific binding partner for said antigen;

a container containing T-cells corresponding to the antigen, and a container containing histocompatible antigen-presenting cells, wherein, optionally, said T-cells and said antigen-presenting cells are in the same container.

7. A kit suitable for conducting the method of claim 4 which method comprises a solid support, a container containing the antigen which binds to the specific binding partner; a container containing T-cells corresponding to the antigen; and a container containing histocompatible antigen-presenting cells, wherein, optionally, said T-cells and said antigen-presenting cells are in the same container.

8. The method according to claim 3 wherein the antigen is an autoantigen correlated with diabetes mellitus or correlated with rheumatoid arthritis.

9. An isolated autoantigen correlated with an autoimmune disease, said autoantigen isolated according to the method of claim 8.

10. The autoantigen of claim 9, wherein the autoimmune disease is diabetes mellitus or rheumatoid arthritis.

11. Antibodies specifically reactive to an antigen, said antibodies detected according to the method of claim 4.

12. An assay method to diagnose a subject for the presence or absence of rheumatoid arthritis (RA), which method comprises:

immobilizing a fraction of a biological fluid of said subject which fraction contains immunoglobulin or a fragment thereof, to a solid support;

contacting the solid support containing immobilized immunoglobulin with a population of T-cells derived from said subject along with compatible antigen presenting cells (APC); and

detecting the presence or absence of activation of said T-cells, wherein activation of said T-cells is an indication of the presence of RA.

13. The method of claim 12 wherein said immunoglobulin is immobilized to said solid support through antihuman Ig derivatized to said support or is immobilized to said support through protein A coupled to said support, and

wherein said activation is measured by measuring proliferation detected by thymidine uptake or wherein said activation is measured by measuring lymphokine production or wherein said activation is measured by detecting an increase in intracellular Ca^{+2} levels, and

wherein said T-cell population is derived from synovial fluid.

14. A method to retard the onset or progression of rheumatoid arthritis (RA) in a subject, which method comprises administering to a subject in need of such effect a composition of immunoglobulin or a fragment thereof derived from the biological fluid of said subject or a cross-reactive subject and capable of effecting activation of the T-cells of said subject,

in a form which is nonimmunogenic and effective in eliciting tolerance to said composition in said subject.

15. The method of claim 14 wherein said fragment comprises heavy chain free of light chain or wherein said fragment consists essentially of a T-cell epitope of said heavy chain.

16. The method of claim 15 wherein said composition includes said immunoglobulin or fragment in soluble form and/or wherein said composition is administered in the absence of adjuvant.

17. A method to retard the onset or progression of rheumatoid arthritis (RA), which method comprises administering to a subject in need of such effect an effective amount of a binding partner that is specific for an immunoglobulin fraction derived from said subject capable of effecting activation of the T-cells of said subject, wherein said binding partner is optionally coupled to a toxin.

18. A method to purify an antigen capable of activating T-cells from the biological fluid of an RA subject, which method comprises subjecting a fluid obtained from said subject to a series of procedures providing a multiplicity of fractions;

subjecting each fraction to an assay comprising immobilizing said fraction to a solid support capable of specifically binding immunoglobulin, followed by contacting the support with a population of T-cells derived from said subject along with compatible antigen presenting cells (APC); and

detecting the presence or absence of activation of said T-cells, and retaining or subjecting to further procedures those fractions shown to be capable of stimulating T-cell activation.

19. A method to monitor a subject for effectiveness of a treatment protocol for RA, which method comprises:

periodically subjecting the biological fluid of said subject during the course of said protocol to an assay comprising immobilizing said fraction to a solid support capable of specifically binding immunoglobulin, followed by contacting the support with a population of T-cells derived from said subject along with compatible antigen presenting cells (APC); and

detecting the presence or absence of activation of said T-cells,

and assessing a decrease in the level of T-cell activating activity in said fluid as a measure of the effectiveness of the protocol.

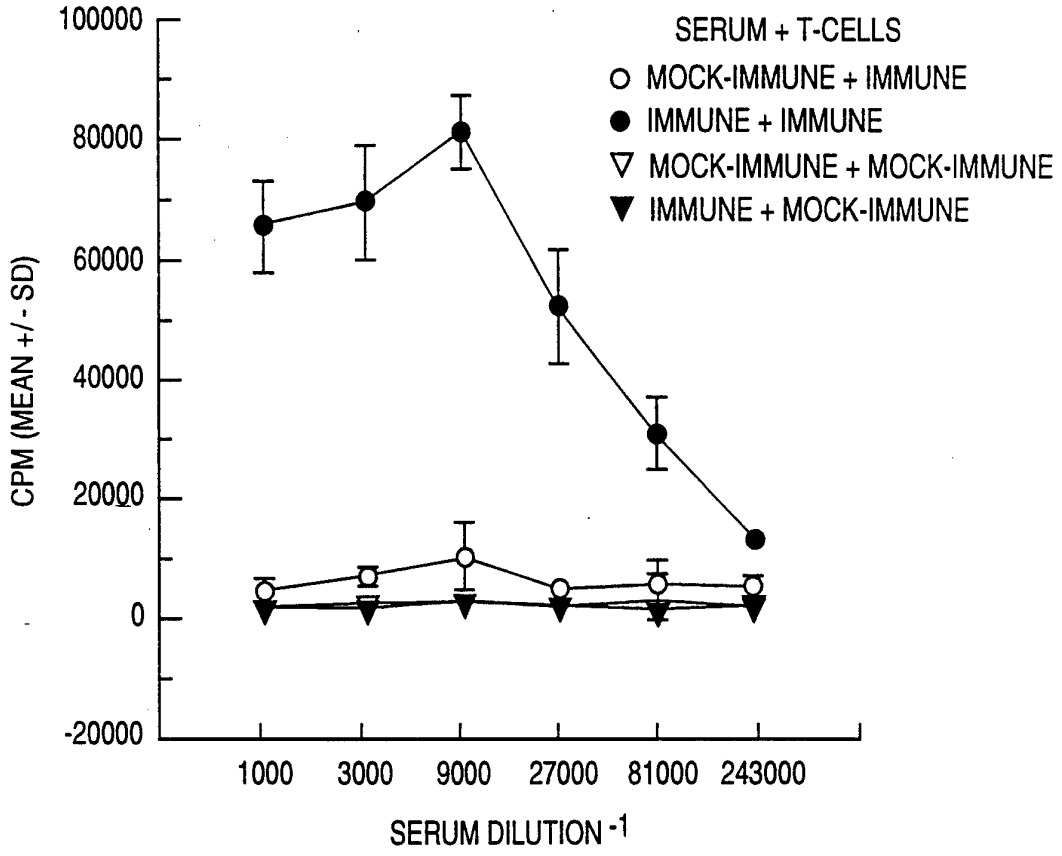
100000
80000
60000
40000
20000
0
-20000
CPM (MEAN +/- SD)
1000
3000
9000
27000
81000
243000
SERUM DILUTION -1
SERUM + T-CELLS
MOCK-IMMUNE + IMMUNE
IMMUNE + IMMUNE
MOCK-IMMUNE + MOCK-IMMUNE
IMMUNE + MOCK-IMMUNE

FIG. 1

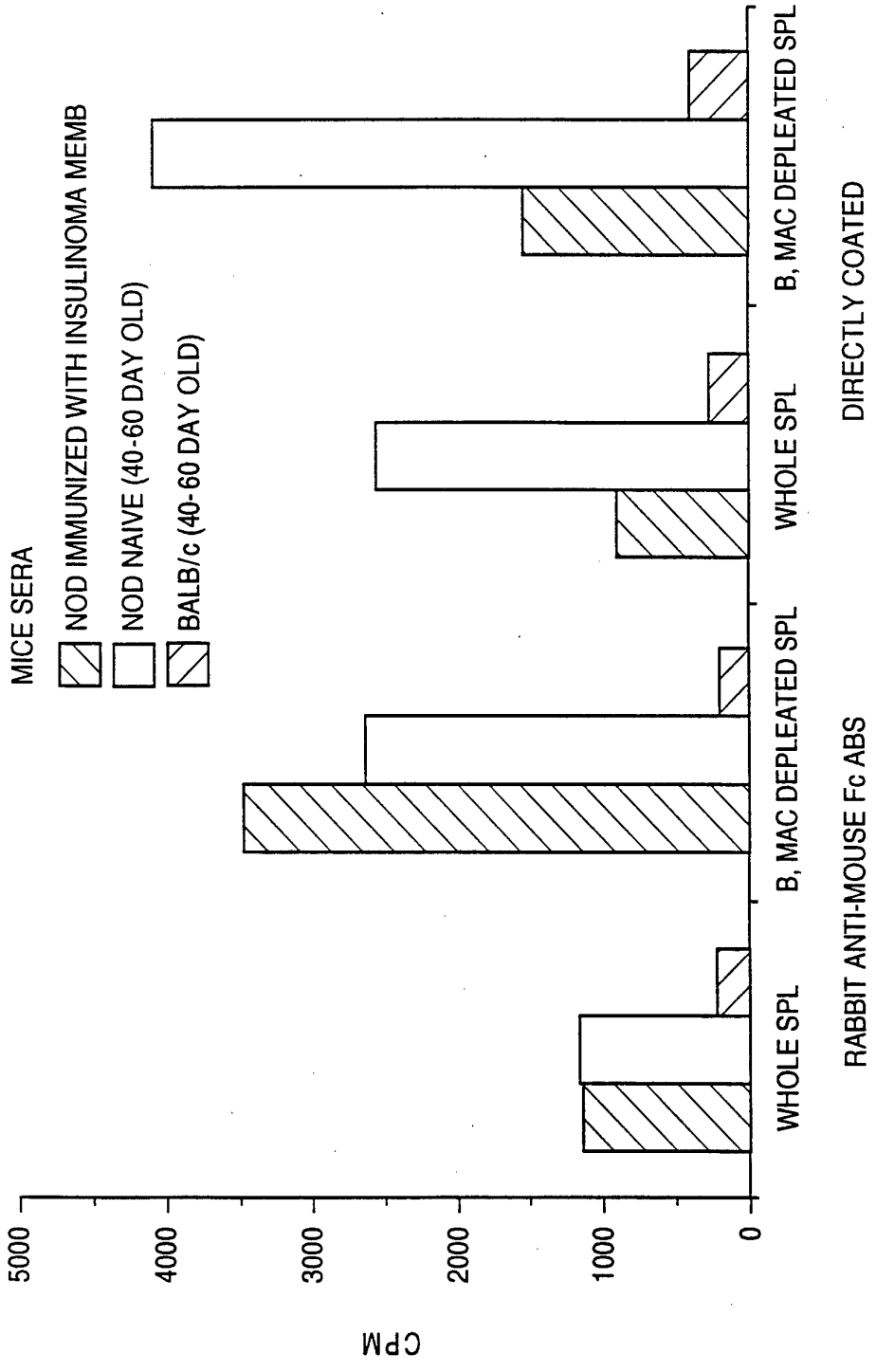
MICE SERA
NOD IMMUNIZED WITH INSULINOMA MEMB
NOD NAIVE (40-60 DAY OLD)
BALB/c (40-60 DAY OLD)
CPM
5000
4000
3000
2000
1000
0
WHOLE SPL
B, MAC DEPLEATED SPL
WHOLE SPL
B, MAC DEPLEATED SPL
RABBIT ANTI-MOUSE Fc ABS
DIRECTLY COATED

FIG. 2

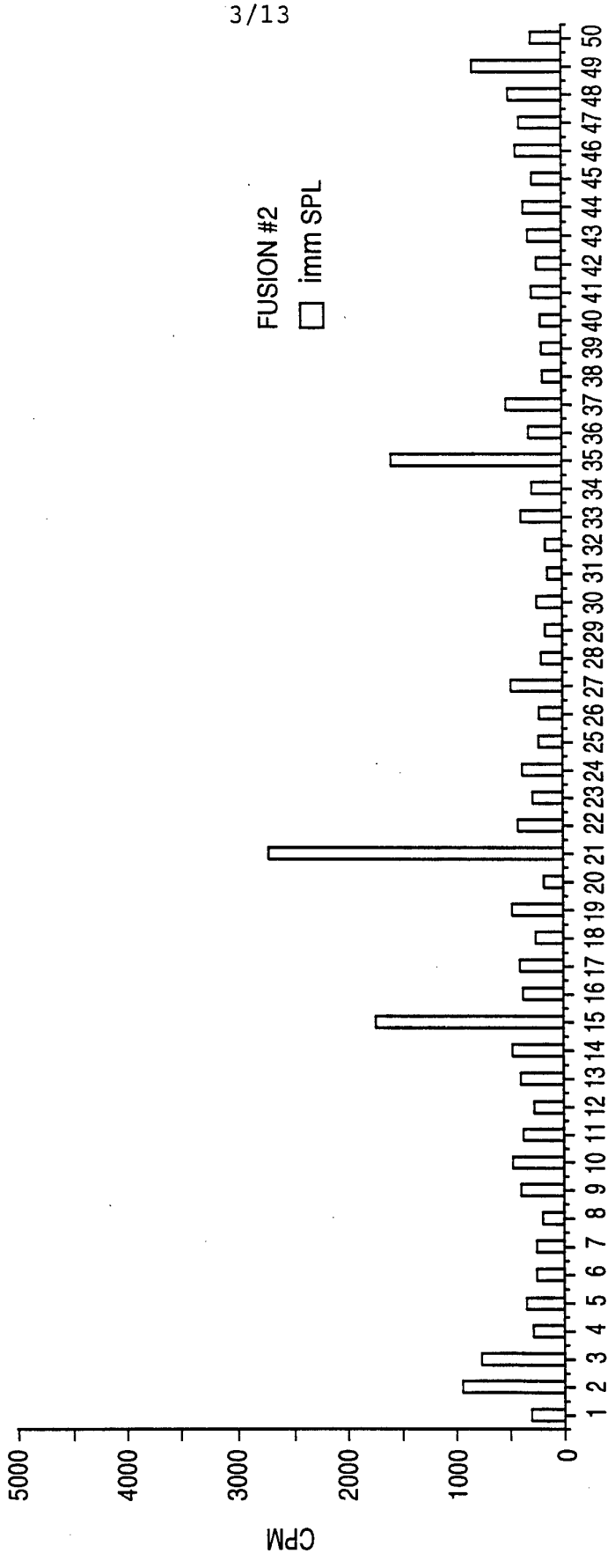
FUSION #2
imm SPL
CPM
5000
4000
3000
2000
1000
0
CLONE #
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

FIG. 3A

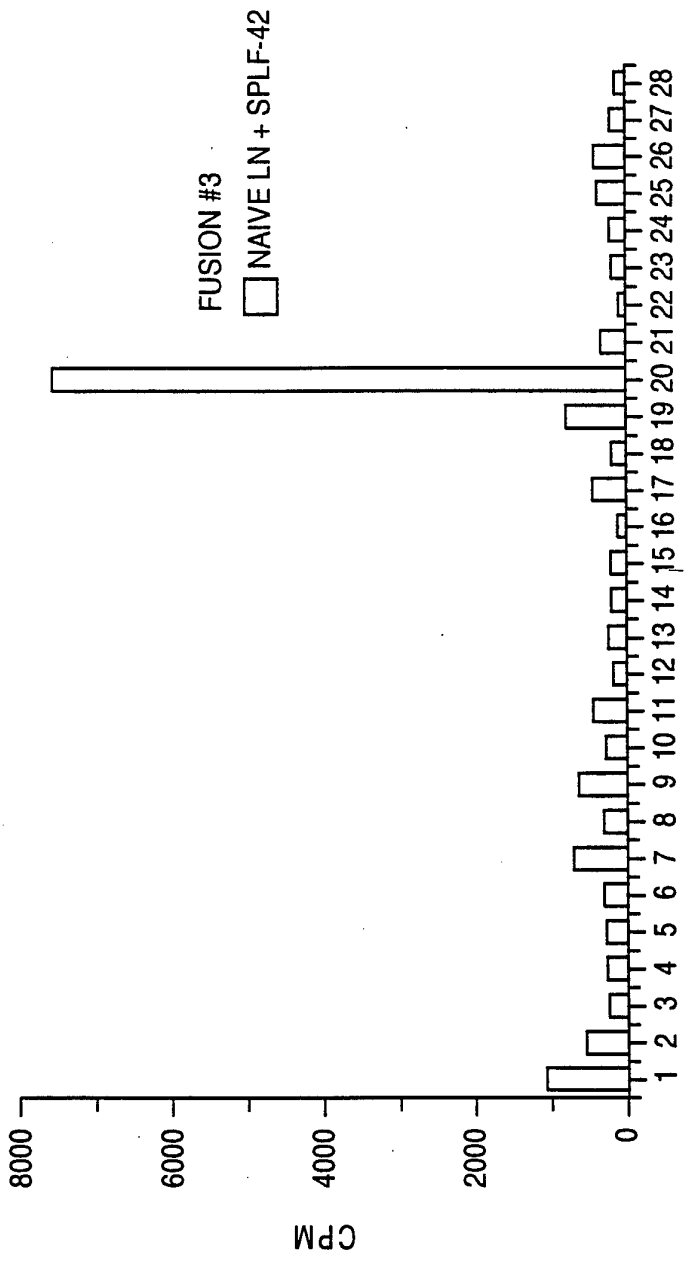
FUSION #3
NAIVE LN + SPLF-42
CPM
8000
6000
4000
2000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
CLONE #

FIG. 3B

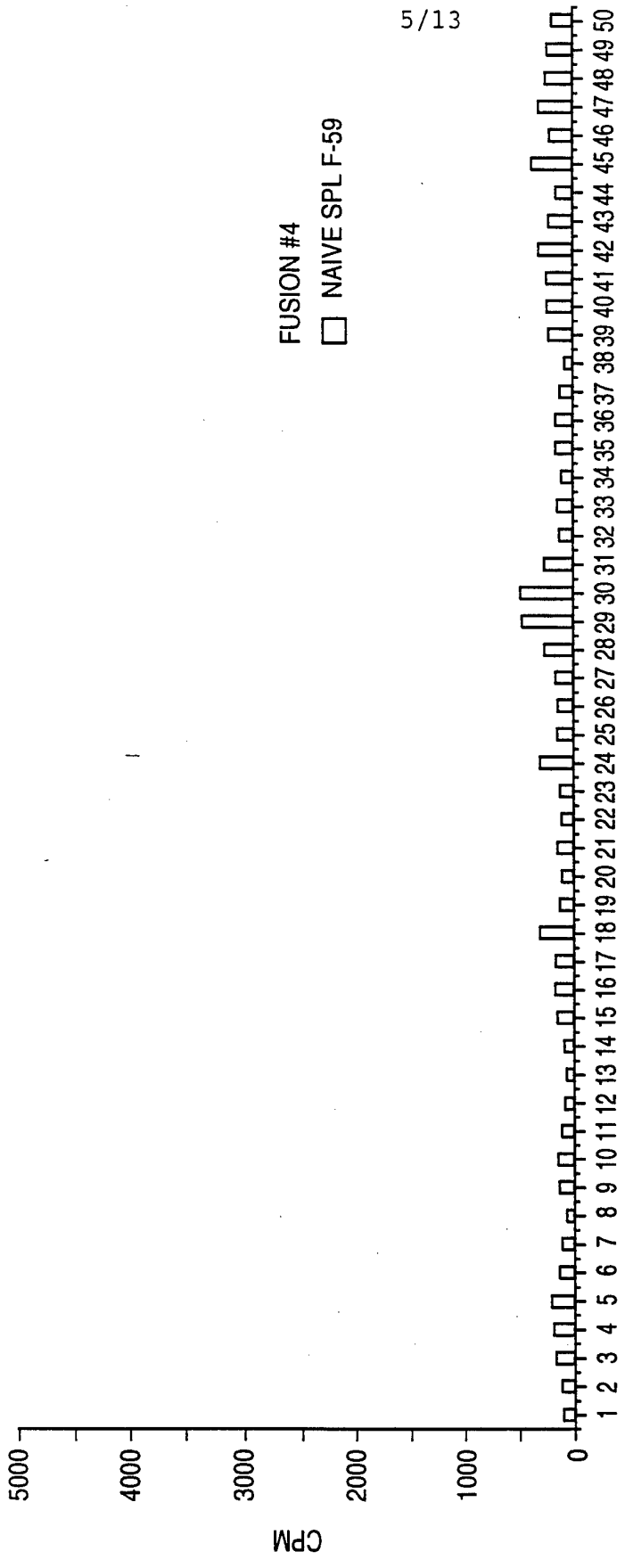
FUSION #4
NAIVE SPL F-59
CPM
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
CLONE #

FIG. 3C

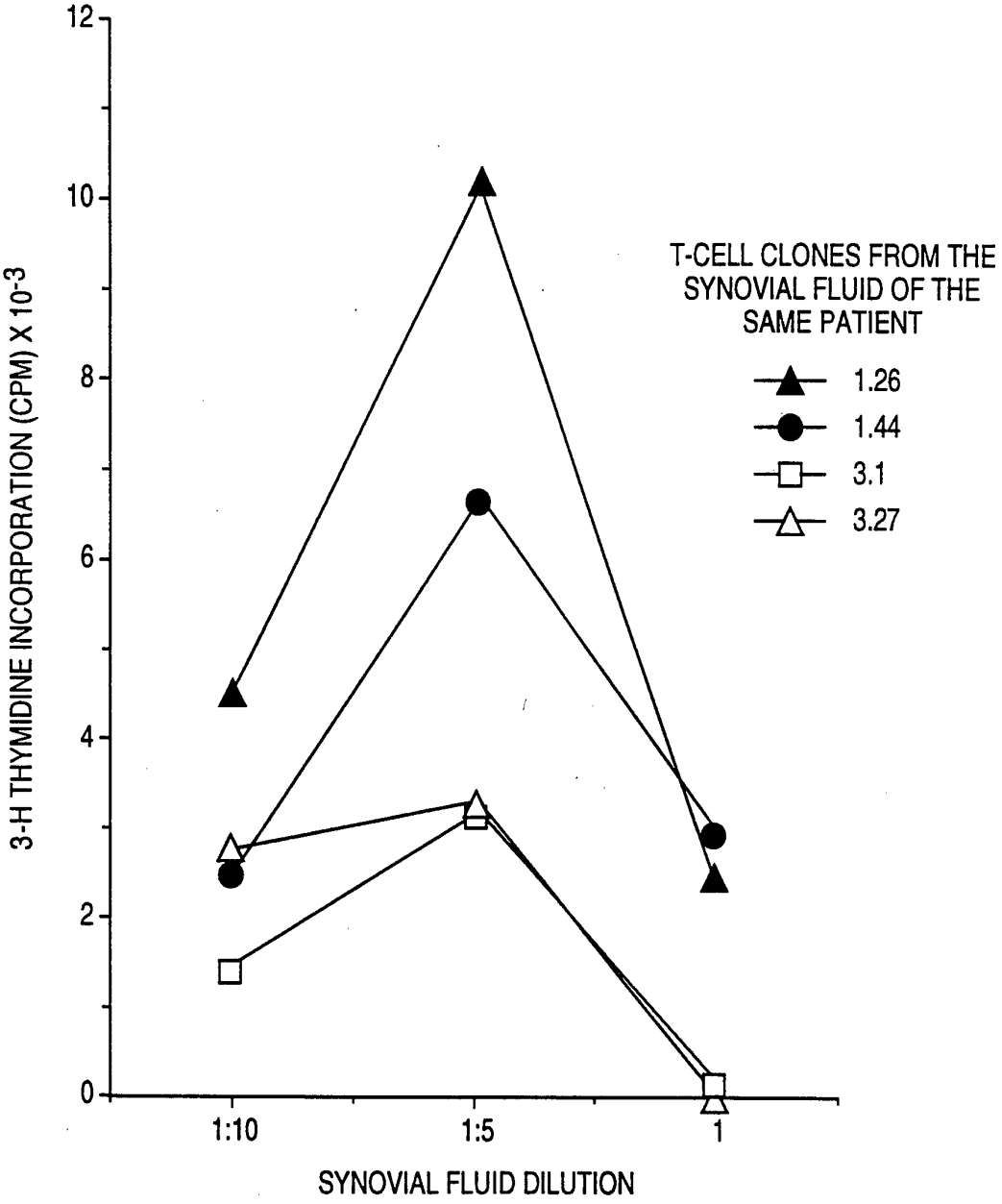
12
10
8
6
4
2
0
3-H THYMIDINE INCORPORATION (CPM) X 10-3
1:10
1:5
1
SYNOVIAL FLUID DILUTION
T-CELL CLONES FROM THE SYNOVIAL FLUID OF THE SAME PATIENT
1.26
1.44
3.1
3.27

FIG. 4

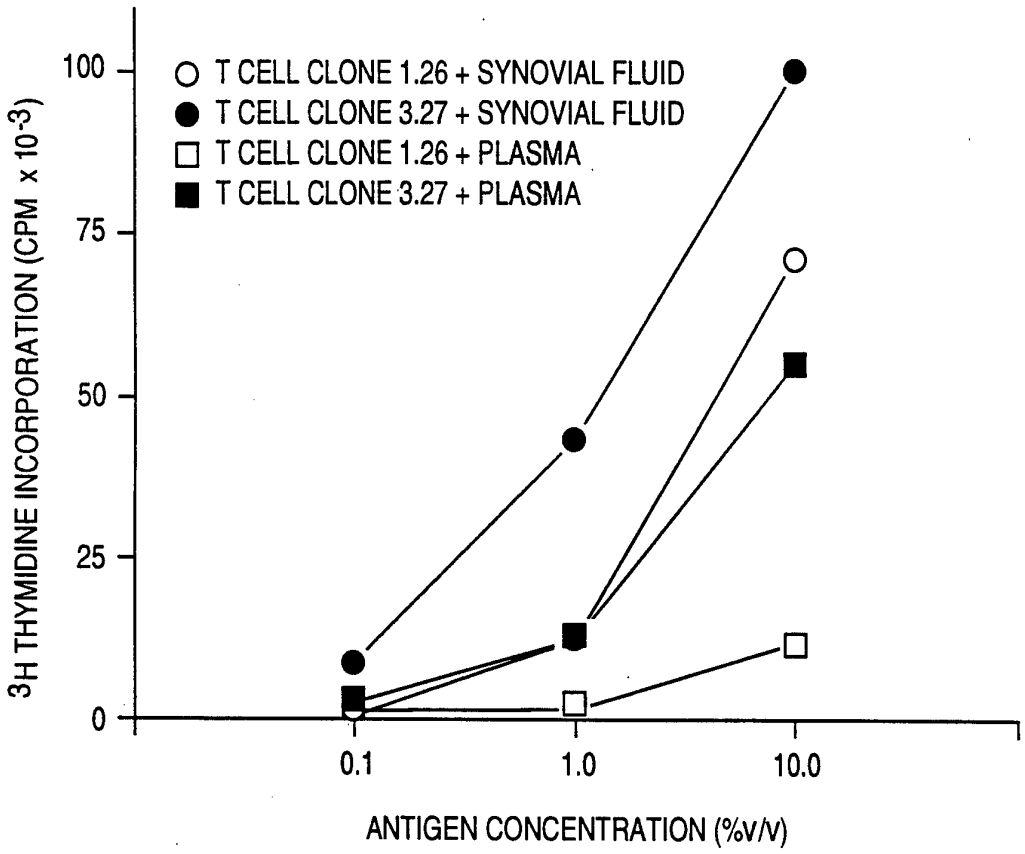
T CELL CLONE 1.26 + SYNOVIAL FLUID
T CELL CLONE 3.27 + SYNOVIAL FLUID
T CELL CLONE 1.26 + PLASMA
T CELL CLONE 3.27 + PLASMA
^{3}H THYMIDINE INCORPORATION (CPM x 10^{-3})
100
75
50
25
0
0.1
1.0
10.0
ANTIGEN CONCENTRATION (%v/v)

FIG. 5

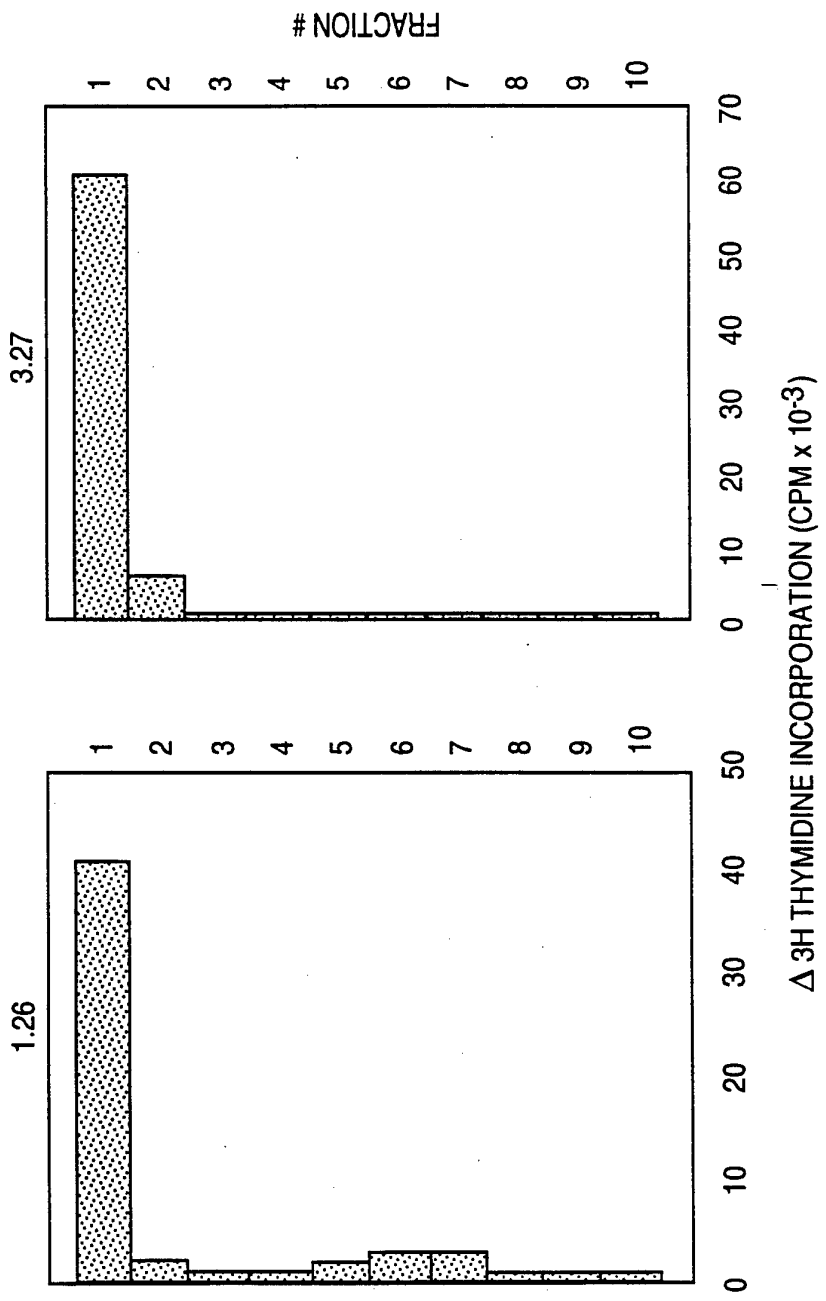
FRACTION #
1 2 3 4 5 6 7 8 9 10
3.27
0 10 20 30 40 50 60 70
1 2 3 4 5 6 7 8 9 10
1.26
0 10 20 30 40 50
Δ 3H THYMIDINE INCORPORATION (CPM x 10-3)

FIG. 6A

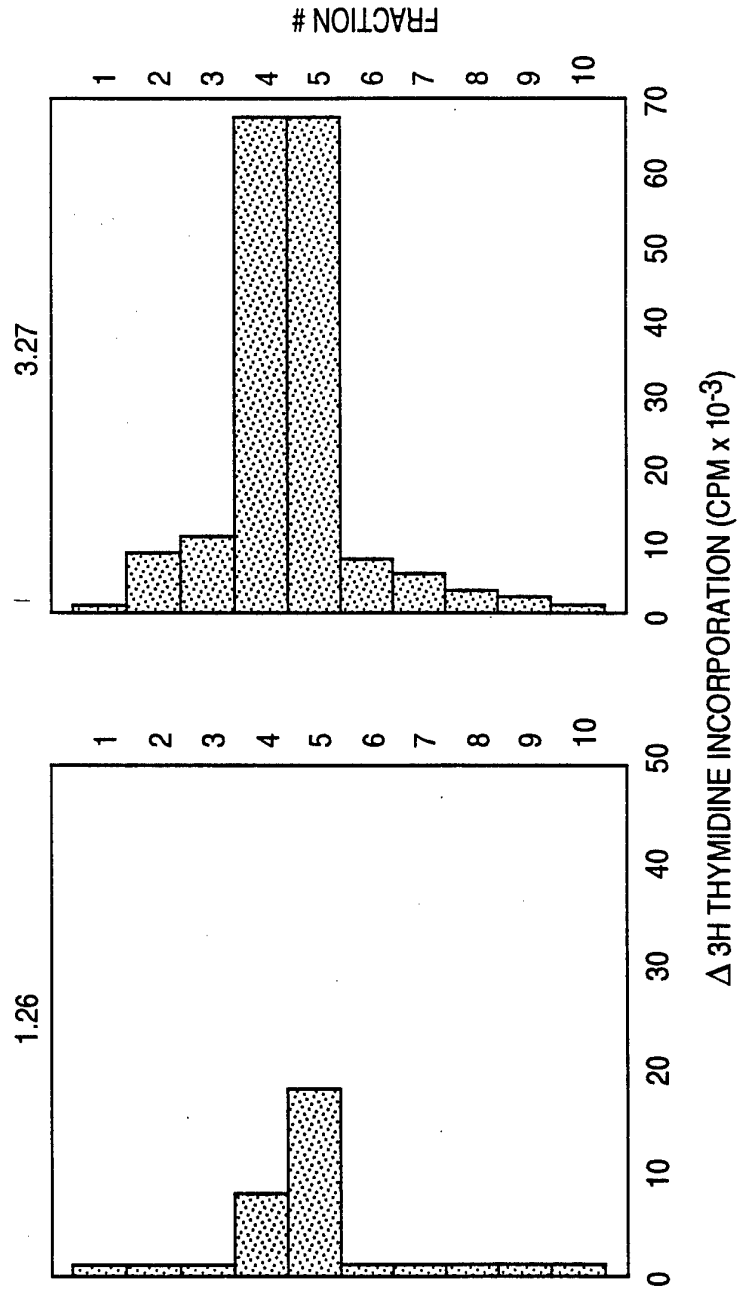
FRACTION #
1 2 3 4 5 6 7 8 9 10
3.27
1.26
0 10 20 30 40 50 60 70
0 10 20 30 40 50
Δ 3H THYMIDINE INCORPORATION (CPM x 10-3)

FIG. 6B

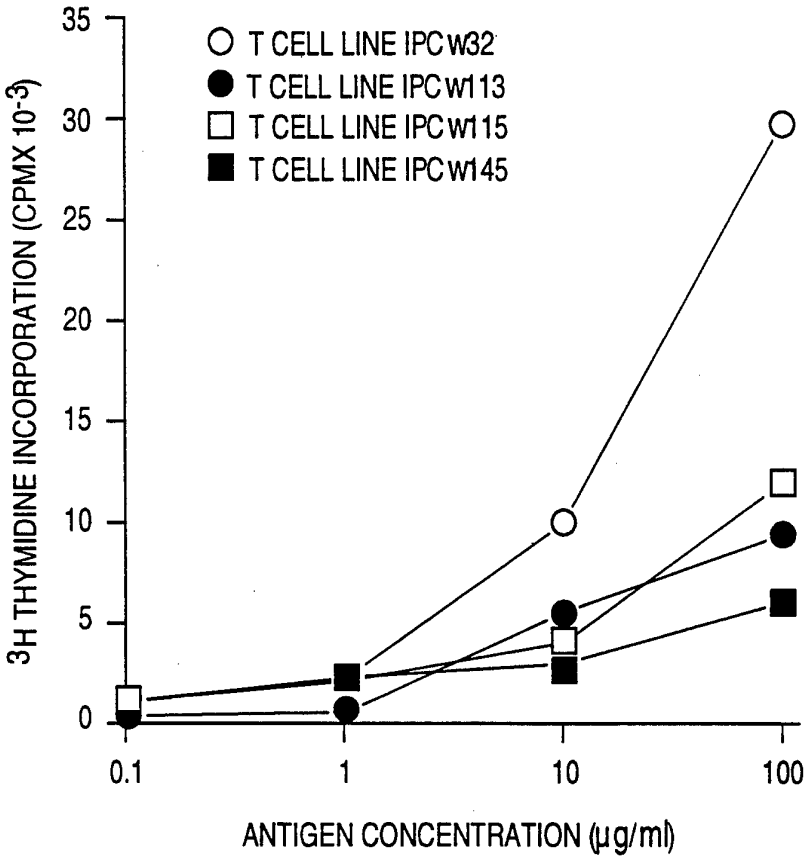
T CELL LINE IPCw32
T CELL LINE IPCw113
T CELL LINE IPCw115
T CELL LINE IPCw145
^{3}H THYMIDINE INCORPORATION (CPMX 10^{-3})
35
30
25
20
15
10
5
0
0.1
1
10
100
ANTIGEN CONCENTRATION (µg/ml)

FIG. 7

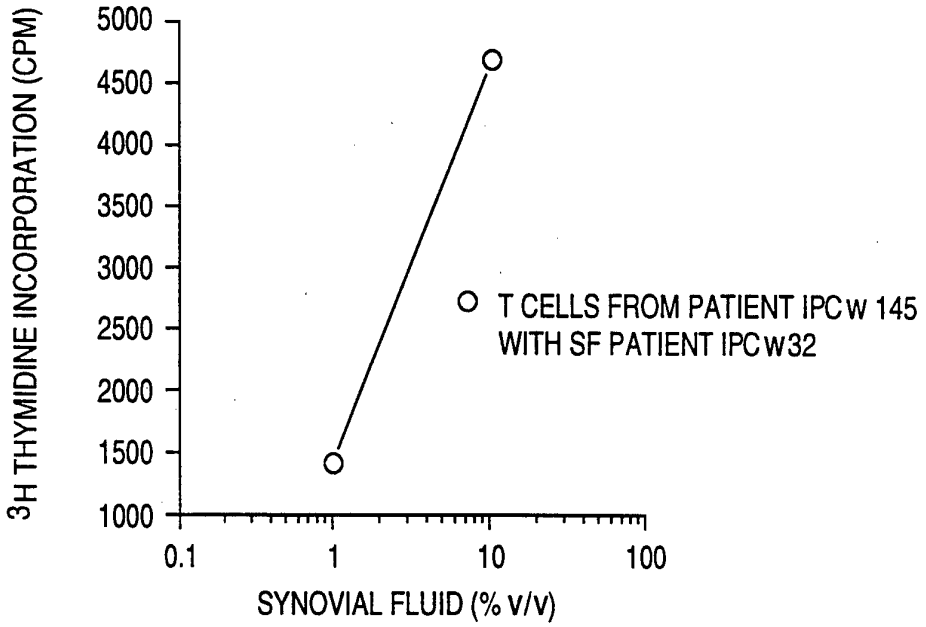
^{3}H THYMIDINE INCORPORATION (CPM)
5000
4500
4000
3500
3000
2500
2000
1500
1000
0.1
1
10
100
SYNOVIAL FLUID (% v/v)
T CELLS FROM PATIENT IPCw 145
WITH SF PATIENT IPCw32

FIG. 8A

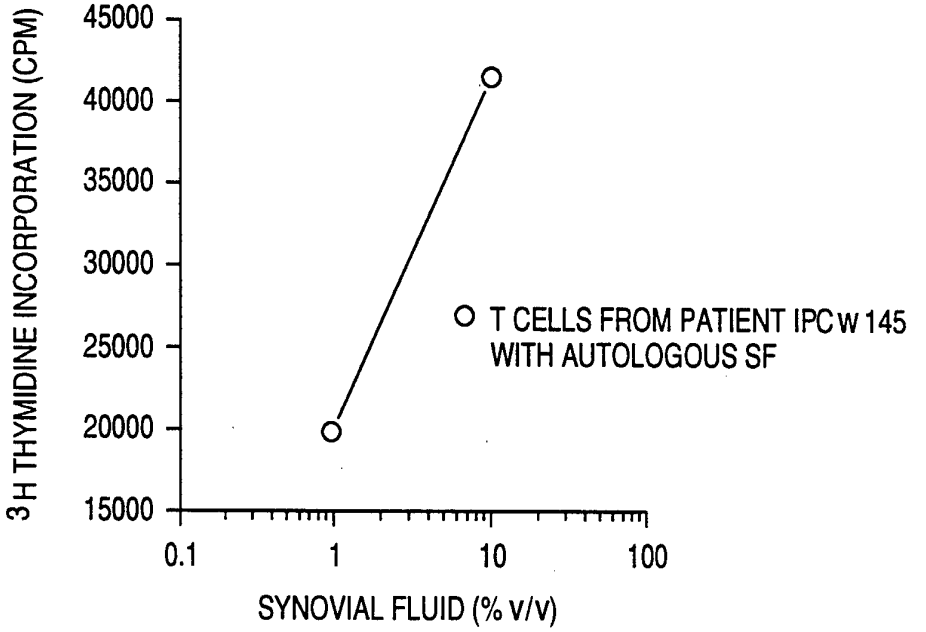
^{3}H THYMIDINE INCORPORATION (CPM)
45000
40000
35000
30000
25000
20000
15000
0.1
1
10
100
SYNOVIAL FLUID (% v/v)
T CELLS FROM PATIENT IPCw 145
WITH AUTOLOGOUS SF

FIG. 8B

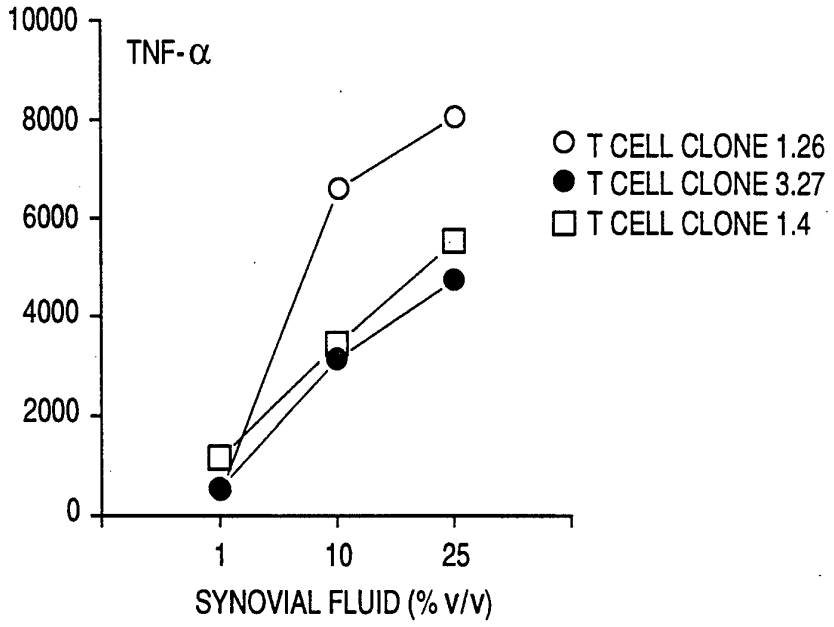
10000
8000
6000
4000
2000
0
TNF-α
1
10
25
SYNOVIAL FLUID (% v/v)
T CELL CLONE 1.26
T CELL CLONE 3.27
T CELL CLONE 1.4

FIG. 9A

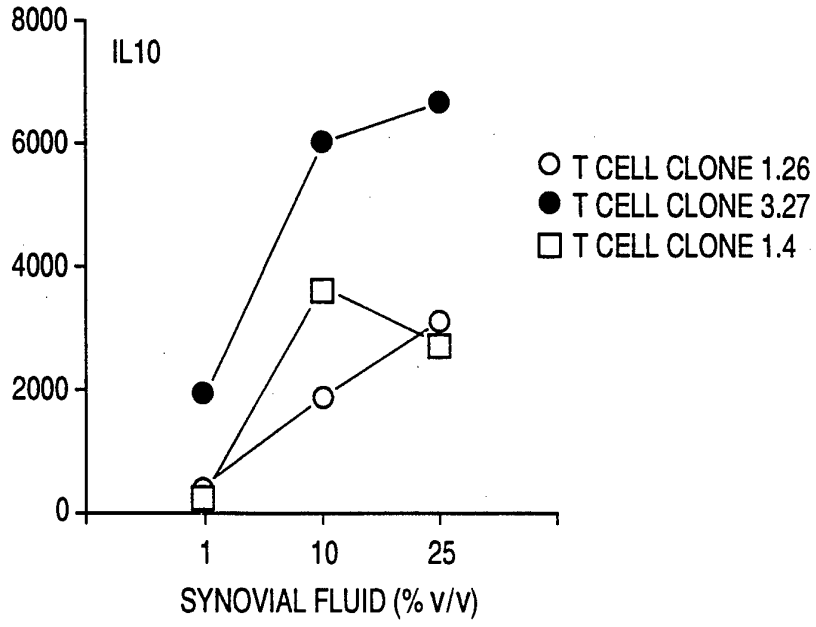
8000
6000
4000
2000
0
IL10
1
10
25
SYNOVIAL FLUID (% v/v)
T CELL CLONE 1.26
T CELL CLONE 3.27
T CELL CLONE 1.4

FIG. 9B

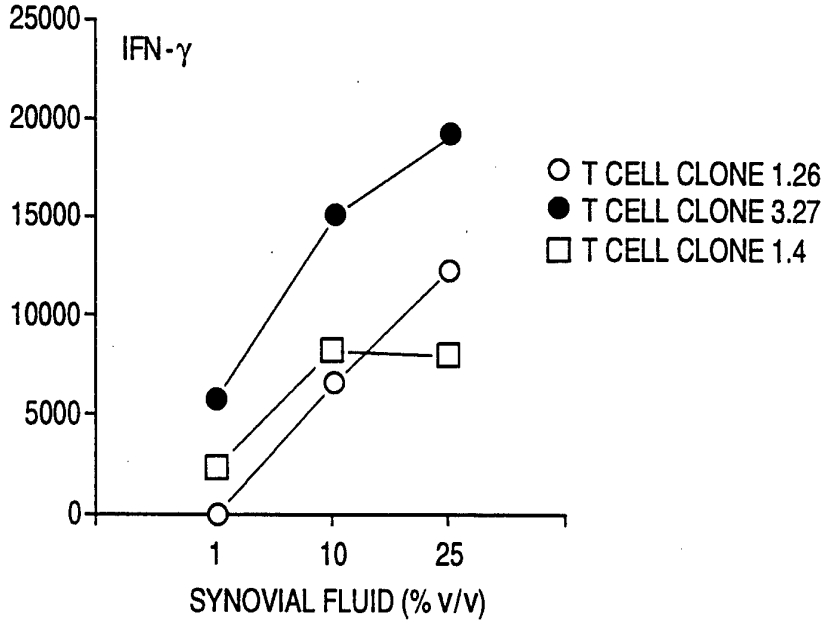
25000
20000
15000
10000
5000
0
IFN-γ
1
10
25
SYNOVIAL FLUID (% v/v)
T CELL CLONE 1.26
T CELL CLONE 3.27
T CELL CLONE 1.4

FIG. 9C

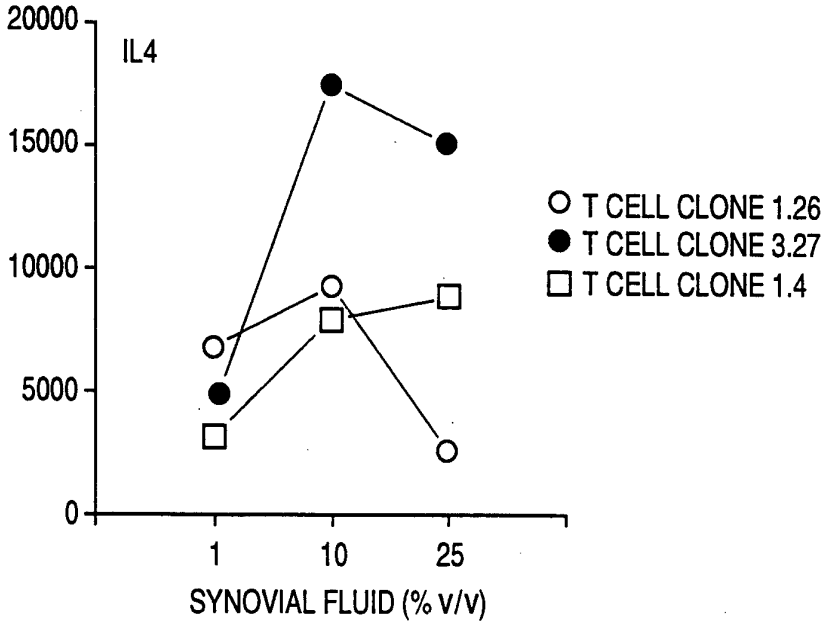
20000
15000
10000
5000
0
IL4
1
10
25
SYNOVIAL FLUID (% v/v)
T CELL CLONE 1.26
T CELL CLONE 3.27
T CELL CLONE 1.4

FIG. 9D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07218

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
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. : 435/6, 7.24, 29, 69.3, 172.3, 975; 436/503, 506, 509, 518, 543

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	European Journal of Immunology, vol. 21, issued September 1991, B. VILLARREAL-RAMOS ET AL, "Screening gene expression libraries for epitopes recognized in Mycobacterium leprae by mouse T cells", pages 2621-2624. See page 2621, col. 2 and page 2622, col. 1.	1-2, 7 3-6, 8-13, 18-19
X	US, A, 4,976,958 (SHINNICK ET AL) 11 December 1990. See col. 40, lines 17-65 and col. 44, line 18-col. 45, line 3.	7, 11 5
X Y	US, A, 5,021,342 (GREENE ET AL) 04 June 1991. See col. 4, lines 1-33 and col. 11, line 22-col. 12, line 63.	7, 11 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search
18 OCTOBER 1993

Date of mailing of the international search report
04 NOV 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer
DAVID SAUNDERS, PRIMARY EXAMINER

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07218

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X P,Y	US, A, 5,154,923 (VAN EDEN ET AL) 13 October 1992. See col. 1, line 41-col. 3, line 60.	7, 9-12 5
X	US, A, 5,114,844 (COHEN ET AL) 19 May 1992. See col. 1, line 58-col. 2, line 39; col. 4, lines 23-37 and col.5, lines 23-36.	9-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07218

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 3/12, 15/00; C12N 15/00; C12Q 1/02, 1/68; G01N 33/543, 33/564, 33/567

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.24, 29, 69.3, 172.3, 975; 436/503, 506, 509, 518, 543; 530/387.1, 412, 806, 868

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-13 and 18-19, drawn to a method to measure antigens or antibodies in vitro via T-cell proliferation classified in class 435/6+.

II. Claims 14-17, dawn to in vivo therapeutic methods for the treatment of rheumatoid arthritis classified in class 424/85.8+.

The methods of Groups I and II use no common reagents and involve no common steps. It is noted that T-cell activating antigens employed in the treatment of Group II could have been identified by methods other than that of Group I – e.g. via conventional T-cell proliferation response assays in which the antigen is not complexed with antibody on a solid phase. There is thus no special technical feature linking these Groups to form a single inventive concept.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07218

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:
(Telephone Practice)
Please See Extra Sheet.

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.