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(54) Title: THERAPEUTIC AND DIAGNOSTIC METHODS USING CELL-FREE CD35

The present invention is directed to a polyclonal sandwich assay with improved sensitivity for the detection and/or measurement of soluble CD35 (complement receptor Type I, CRI) or fragments thereof, and the use of such measurements in the diagnosis and therapy of diseases and disorders. The sCD35 concentrations in normal and various diseased individuals are shown.

(57) Abstract
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+ Any designation of “SU” has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.
THERAPEUTIC AND DIAGNOSTIC METHODS

USING CELLFREE CD35

1. INTRODUCTION

The present invention is directed to a polyclonal sandwich immunoassay which provides an improved sensitivity for the measurement of soluble CD35.

2. BACKGROUND OF THE INVENTION

2.1. SOLUBLE CD35 ANTIGEN

CD35 (complement receptor Type I or CRI) is the receptor for C3b and C4b and can inhibit the C3/C5 convertases of the classical and alternative complement pathways. It can also act as a cofactor for the cleavage of C3b and C4b by factor I. CD35 is a glycoprotein comprising multiple short consensus repeats (SCRs) arranged in 4 long homologous repeats (LHRs). The C-terminal LHR, called LHR-D, is followed by two additional SCRs, a transmembrane region consisting of a hydrophobic stretch of amino acids, and a hydrophilic stretch of amino acids forming the cytoplasmic region (Klickstein et al., 1987, J. Exp. Med., 165:1095; Klickstein et al). Erythrocyte CD35 appears to be involved in the removal of circulating immune complex in autoimmune patients, and its levels may correlate with the development of AIDS (Inada et al., 1986, AIDS Res. 2:235; Inada et al., 1989, Ann. Rheu. Dis. 4:287). Solid phase assay methods for detecting human C3b receptor have been disclosed (U.S. Pat. 4,672,044, dated June 9, 1987). Antibodies to CD35 (Wong, et al., 1985, J. Immunol. Methods 82:303; Yoon & Fearon, 1985, 134:3332; Schrieber, U.S. Patent No. 4,673,044, issued June 9, 1987) have also been reported.

A soluble form of CD35 has been detected in human plasma (Yoon & Fearon, 1985, J. Immunol. 134:3332) from normal individuals and in patients with
systemic lupus erythematosus (SLE). It is reported to be functionally active and structurally similar to intact CD35 on membranes.

A recombinant soluble form of CD35 has been produced which is able to inhibit activation of both the classical and alternative pathways in vitro. It also can suppress complement activation in vivo and is effective in animal models of inflammation and reperfusion injury associated with myocardial infarction (International Patent Publication Nos. WO 89/09220, published October 5, 1989 and WO 91/05047, published April 18, 1991 both entitled "The Human C3b/C4b Receptor (CR1)"; Weissman et al., 1990, Science 249:146-151).

2.2. DISEASES AND DISORDERS INVOLVING COMPLEMENT

The complement activation pathways play a fundamental role in many human diseases and disorders. Some complement-mediated diseases and disorders are discussed infra.

Autoimmune Disease. Diminished expression of CD35 on erythrocytes of patients with SLE has been reported by investigators from several geographic regions, including Japan, the United States, and Europe. Taken as a group, patients have an average number of receptors per cell that is 50-60% that of normal populations, although there is considerable overlap because of the genetically determined marked variation in this characteristic among normal individuals.

An early report noted that CD35 number on erythrocytes varied inversely with disease activity, with lowest numbers occurring during periods of most severe manifestations of SLE, and higher numbers being observed during periods of remission in the same patient (Inada, et al, 1982, Clin. Exp. Immunol.

Recently acquired loss of erythrocyte CD35 in the setting of active SLE and hemolytic anemia was demonstrated by observing the rapid loss of the receptor from transfused erythrocytes (Walport et al., 1987, Clin. Exp. Immunol. 69:501-7).

Immune complexes are found in many pathological states including but not limited to autoimmune diseases such as rheumatoid arthritis or SLE, hematologic malignancies such as AIDS (Taylor et al., 1983, Arthritis Rheum. 26:736-44; Inada et al., 1986, AIDS Research 2:235-247) and disorders involving autoantibodies and/or complement activation (Ross et al., 1985, J. Immunol. 135: 2005-14).

Erythrocyte CD35 is reported to have a functional role in the removal of circulating immune complexes in autoimmune patients and may thereby inhibit the deposition of immune complexes within body tissue constituents (Inada et al., 1989, Ann. Rheum. Dis. 4:287). Additional findings suggest detrimental loss of CD35 activity progressing from asymptomatic seropositive homosexual volunteers to the prodromal spectrum of ARC and finally progressing to a total disappearance in overt AIDS (Inada et al., 1986, AIDS Res. 2:235).

**Hemodialysis.** In vitro studies have demonstrated that CD35 can be elicited from the intracellular vesicular compartment to the plasma membrane within seconds following stimulation of neutrophils with chemotactic peptides, such as C5a and formylmethionyl-leucylphenylalanine, certain cytokines and endotoxin. In studies of patients undergoing hemodialysis for chronic renal failure, there was a three to five-fold increase in the amount of CD35 at the cell surface of neutrophils taken during dialysis.
with membranes that activated complement but not with non-complement activating membranes.


Terminal complement complex was demonstrated in the plasma (Kojima et al., 1989, supra), and on the membranes of granulocytes during dialysis (Deppisch et al., 1990, Kidney. Int. 37:696-706).

**Cardiopulmonary Bypass.** Elevated C3a has been demonstrated in patients undergoing prolonged extracorporeal circulation (Chenoweth et al., 1981, Complement 3:152-165). Increased plasma levels of SC5b-9 (Dalmasso et al., 1981, Complement Inflamm. 6:36-48), and terminal C5b-9 complex deposits on erythrocytes and polymorphonuclear cells (Salama et al., 1988, N. Engl. J. Med. 318:408-414) have been observed in these patients.

Several lines of evidence showed that the complement system in cardiopulmonary bypass is activated primarily via the alternative pathway, e.g., the C3a level was increased, but not C4a, suggesting that alternative pathway was predominantly responsible (Cavarocchi et al., 1986, Circulation 74: III 130-133; Chenoweth et al., 1986. Complement 3:152-165).


Two types of oxygenators (bubble and membrane) have been used for cardiopulmonary bypass. The bubble oxygenator generated more C3a production
than the membrane oxygenator (Cavarocchi et al., 1986, J. Thorac. Cardiovasc. Surg. 91:252-258). In an experimental model, the dogs which had a bubble oxygenator developed higher risk of infection with *Staphylococcus aureus* than those with the membrane oxygenator, suggesting the decreased complement levels may impair the host defense during cardiopulmonary bypass (van Oeveren et al., 1987 Ann. Thorac. Surg. 44:523-528).

**Thermal Injury (burn).** It has been shown that massive activation of the alternative complement pathway, but not the classical pathway, was observed in a model of burn injury in mice. Cobra venom factor (CVF) pretreatment reduced burn mortality (Gelfand et al., 1982, J. Clin. Invest. 70: 1170-1176).

Elevations of plasma C3a des Arg and C4a des Arg were also detected in burn patients (Davis et al., 1987, Surgery 102:477-484).

Activated C3 was detected in plasma of a guinea pig model of thermal injury (Bjornson et al., 1986, J. Infect. Dis. 153:1098-1107). Complement-depletion diminished the generation of C5a (Oldham et al., 1988, Surgery 104:272-279) and burn edema (Friedl et al., 1989, Am. J. Pathol. 135:203-217) in a rat cutaneous burn model. In another rat model, complement depletion also attenuated the systemic hemodynamics depression (Schirmer et al., 1989, J. Trauma 29:932-938). Furthermore, it has been suggested that neutrophil sequestration occurs very early in a sheep thermal injury animal model, probably as a result of oxidant initiated complement activation (Demling et al., 1989, Surgery 106:52-59).

Neutrophil activation was also demonstrated in patients with thermal injury whose neutrophils have increased expression of CD35 for up to three weeks following the initial burn, and in whom infectious disease complications, such as sepsis or pneumonia,
were associated with transient, further increases in CD35 expression by neutrophils.

**Adult Respiratory Distress Syndrome (ARDS).** ARDS is a fulminant form of respiratory failure affecting many critically ill patients. It has been reported that in the first 48 hours, complement activation occurred via the alternative pathway only and was later followed by activation via the classical pathway (Zilow et al., 1990, Clin. Exp. Immunol 79: 151-157).Clr Cls–Cl inhibitor complex, C3b–P complex (Langlois et al., 1989, Heart Lung 18:71-84), and terminal complement complex (Langlois et al., 1988, Am. Rev. Respir. Dis. 138:368-375) were suggested to be useful to distinguish patients with ARDS from those without ARDS. Moreover, the amounts of C3a and C5a in patients with respiratory failure correlated with the severity of the eventual pulmonary insult (Weigelt et al., 1988, J. Trauma 28:1013-1019).

Decomplementation with cobra venom factor (CVF) in an animal model of ARDS protected against lung injury (Guice et al., 1989, Ann. Surg. 210:740-747). Similarly, complement-depletion by CVF in a porcine model produced by infusion of live *Pseudomonas aeruginosa* developed less septic acute respiratory disease (Dehring et al., 1987, J. Trauma 27:615-625). When cynomolgus monkeys (*Macaca fascicularis*) were made acutely septic with infusions of *E. coli*, severe sepsis and ARDS resulted (Stevens et al., 1986, J. Clin. Invest. 77:1812-1816). Three major early events occurred: generation of C5a, decrease in peripheral leukocyte counts, and increase in the sequestration of leukocytes in the lungs (Hangen et al., 1990, J. Surg. Res. 48:196-203). In this model, ARDS was prevented with a rabbit anti-human polyclonal antibody to C5a des arg (Stevens et al., 1986, *supra*). This antibody was also shown to be able to inhibit polymorphonuclear chemotaxis and
reduce the release of lysosomal enzymes (Hatherill et al., 1989, J. Biol. Response Mod. 8:614-624).

**Sepsis.** Activation of the complement system via the classical pathway is suggested to be involved in the development of fatal complications in sepsis (Hack et al., 1989, Am. J. Med. 86:20-26).

**Barotrauma.** It has been hypothesized that the phenomena of decompression sickness (DCS) are mediated by complement. The complement system is activated in rabbits with DCS. When these rabbits were pharmacologically decomplementing *in vivo*, they did not develop DCS (Ward et al., 1990, Undersea Biomed. Res. 17:51-66).

**sCD35 Therapy.** It has been demonstrated that a recombinant produced soluble complement receptor type I (sCD35) functions as an *in vivo* inhibitor of complement and acts to suppress post-ischemic myocardial inflammation and necrosis (Weisman et al., 1990, Science, 340: 146-151).

Although there have been some publications of other CD35 assays (Schreiber, U.S. Patent Number No. 4,672,044 issued June 9, 1987; Yoon & Fearon, 1985, J. Immunol. 134, 3332; Fearon et al., International Patent Application Number PCT/US89/01358 published October 5, 1989), none has achieved the level of sensitivity of the polyclonal based sandwich immunoassay. As indicated *infra*, the polyclonal based assay has a minimum detectable CD35 concentration of about 0.5 ng/ml.

3. **SUMMARY OF THE INVENTION**

The present invention is directed to a more sensitive polyclonal antibody sandwich immunoassay for the detection and/or measurement of soluble CD35 (sCD35; soluble complement receptor type I) or fragments thereof, and the use of such measurements in the diagnosis and therapy of diseases and disorders.
Preferably, the polyclonal antibodies are purified polyclonal antibodies. The polyclonal antibody assay can be used to detect as little as 0.5 ng/ml sCD35 in a sample.

The measurement of soluble CD35 is valuable in monitoring the effect of a therapeutic treatment on a subject, in predicting therapeutic outcome and in evaluating and monitoring the immune status of patients. Monitoring soluble CD35 levels is especially useful in disorders associated with inappropriate complement activation, including but not limited to AIDS and diseases characterized by inflammation and immune complex disorders.

In other embodiments, the levels of soluble CD35 can be measured in samples obtained from body fluids, including but not limited to plasma, serum, synovial fluid, spinal fluid, pleural effusions, tumor and tissue infiltrates.

3.1. DEFINITIONS

As used herein, the following terms will have the meanings indicated:

HTLV III/LAV/HIV = Human T cell Leukemia Virus Type I/Lymphadenopathy Associated Virus/Human Immunodeficiency Virus

OPD = O-phenylenediamine

mAb = monoclonal antibody

Spontaneous release = release by normal or pathologic physiological processes of the cell

AIDS = Acquired immunodeficiency disease syndrome

CD35 = Complement receptor type 1

sCD35 = soluble CD35
4. DESCRIPTION OF THE FIGURES

FIG. 1. Soluble CD35 assay standard curve.
FIG. 2. Detection of soluble CD35 in normal and patient sera.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the measurement of soluble complement receptor type I (sCD35), or fragments thereof, by an improved CD35-specific immunoassay, and the use of such measurements in the diagnosis and therapy of diseases and disorders.

As used herein, the term "soluble" shall mean those molecules that are "spontaneously released"; i.e., released by normal or pathologic physiological processes of the cell, and those molecules present in soluble form in a sample by virtue of their in vivo administration to the patient. Such molecules are to be distinguished from "solubilized" cell surface forms of the molecules, whose solubilization is brought about by in vitro manipulation such as cell lysis by detergent. The soluble CD35s of the invention are molecules which carry antigenic determinants of the cell-surface CD35 counterparts.

The measurement of soluble CD35 can be valuable in monitoring the effect of a therapeutic treatment on a subject and detecting and/or diagnosing a disease in a subject. These measurements can also aid in predicting therapeutic outcome and in evaluating and monitoring the immune status of patients. The soluble molecules can be measured in any body fluid of the subject including but not limited to whole blood, serum, plasma, urine, saliva, pleural effusions, synovial fluid, spinal fluid, tissue infiltrations and tumor infiltrates.
5.1. ASSAY FOR SOLUBLE CD35

The invention is directed to an improved immunoassay based upon polyclonal antibodies that are able to detect both soluble CD35 (sCD35) and soluble recombinant CD35.

The instant invention provides a method of measuring sCD35 using polyclonal antibodies to CD35. The polyclonal antibody assay can be used to detect as little as about 0.5 ng/ml sCD35. In a preferred embodiment, a sandwich enzyme immunoassay can be used. The immunoassay can be run in a one-step format, in which the sample suspected of containing sCD35 is simultaneously contacted with a capture polyclonal antibody and a detection polyclonal antibody under conditions that allow immunospecific binding of both the antibodies to any sCD35 in the sample. In another embodiment, the immunoassay can be run in a two-step format, in which, in a first step, the sample is contacted with the capture antibody under conditions that allow binding; and, in a second step, the sample is contacted with the detection antibody under conditions that allow binding to any immobilized capture antibody-sCD35 complex. The detection antibody used in the sandwich immunoassay can be detectably labeled. In another embodiment, the sandwich immunoassay can be indirect, i.e., the detection antibody can be detected by a secondary reagent. An example of a secondary reagent system is avidin (or streptavidin) and biotin.

One description of such a sandwich immunoassay follows: An antibody (capture antibody, Ab1) directed against the soluble antigen, e.g., sCD35, is adsorbed onto a solid substratum. The antigen present in the sample binds to the antibody, and unreacted sample components are removed by washing. An enzyme-conjugated antibody (detection antibody, Ab2)
directed against a second epitope of the antigen binds
to the antigen captured by Ab1 and completes the
sandwich. After removal of unbound Ab2 by washing, a
substrate solution is added to the wells. A colored
product is formed in proportion to the amount of
antigens present in the sample. The reaction is
terminated by addition of stop solution and absorbance
is measured with a spectrophotometer. A standard
curve is prepared from known concentrations of the
antigen, from which unknown sample values can be
determined. In a preferred embodiment for the
measurement of CD35 levels, functionally identical
anti-CD35 Polyserum R1 and Polyserum R2 (quality
control tested anti-CD35 polyclonal antibodies), which
differ only in that Polyserum R1 is adsorbed to a
solid phase and Polyserum R2 is enzyme-labeled, can be
used as the capture and detection antibodies,
respectively, in a sandwich immunoassay (such as the
CELLFREE\textsuperscript{\textregistered} assay, T Cell Sciences Inc., Cambridge, MA)
described in Section 6 infra.

Various procedures known in the art may be
used for raising polyclonal antibodies to epitopes of
CD35. Using methodologies described herein, extremely
sensitive antibody preparations can be obtained which
exhibit extremely low lower limits of detection of
sCD35. The polyclonal antibodies of the invention can
be characterized by the ability to detect as little as
about 0.5 ng/ml sCD35 in a direct sandwich
immunoassay. For the raising of antibody, various
host animals can be immunized by injection with a CD35
surface molecule, either in a cell, e.g., erythrocyte,
preparation or more preferably purified from cells, or
a recombinant version thereof, a synthetic protein, or
a fragment of CD35. Suitable host animals include,
but are not limited to rabbits, mice, rats, etc. In a
preferred embodiment, the immunogen is a truncated
recombinant soluble form of the CD35 produced by
recombinant Chinese hamster ovary (CHO) cell line containing recombinant expression plasmid pBSSCRlc as deposited with the ATCC, assigned accession no. CRL 10052 (see International patent publications WO 89/09220 and WO 91/05047). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, liposomes, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Antibody molecules may be purified by various techniques, e.g., immunoabsorption or immunoaffinity chromatography (protein A or G affinity columns), chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc. In a preferred embodiment, antibodies are purified by affinity chromatography using recombinant CD35 attached to the column matrix.

Antibody fragments which contain the idiootype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of F(ab')2 fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Once specific polyclonal antibodies are demonstrated to be suitable for use, other such suitable antibodies may be selected by comparison to the characteristics of the former antibodies.

Polyclonal anti-CD35 antibodies can be used for detecting spontaneously released CD35 in patients'
sera and can also be used to monitor the effectiveness of therapeutic treatments in patients where CD35 is either spontaneously released due to the pathological condition or where recombinant CD35 has been administered to the patient as a therapeutic agent. The CD35 assay can be used to detect soluble CD35 in patients with certain diseases or disorders. The CD35 levels so obtained can then be correlated with disease detection, treatment regimen, and disease or disorder prognosis. Changes in soluble CD35 values over time may be more predictive than the absolute level of soluble CD35 at any point in time. Changes in sCD35 values can be compared with predisease values, pretreatment or disease remission values, values observed in normal individuals, etc.

5.2. CLINICAL APPLICATIONS
The measurement of sCD35 can be used in the diagnosis or detection of disease, in monitoring therapy, and in the prediction of therapeutic outcome in diseases or disorders involving complement. These include but are not limited to the disorders and diseases described in Table I.
TABLE I

DISEASES OR DISORDERS INVOLVING COMPLEMENT

I. Diseases with Circulating Immune Complexes & Autoimmune Diseases
   - Systemic lupus erthematosus
   - Rheumatoid arthritis
   - Glomerulonephritis

II. Inflammatory disorders
   - ARDS
   - Sepsis

III. Infectious Disease
   - AIDS

IV. Thermal Injury
   - Burns
   - Frostbite

V. Transplantation

VI. Complement Activation in
   - Transfusion
   - Hemodialysis

   - Cardiopulmonary Bypass
   - Post Pump? Syndrome
   - Post Perfusion Syndrome
   - Barotrauma

VII. Red Blood Cell Diseases
   - Autoimmune hemolytic anemia
   - Transfusion
   - Paraxismal nocturnal hemoglobinurea
   - Familial Mediterranean fever

The invention is directed to measuring the amount of soluble CD35 in disease states involving circulating immune complexes, inflammatory disorders, or disorders of inappropriate complement activation.

The invention is also directed toward the monitoring of treatment of diseases involving circulating immune complexes or complement activation that involve sCD35. For example, erythrocytes are
involved in the removal of circulating immune complexes via adherence to erythrocyte-CD35 and may function to inhibit deposition of immune complexes in body tissue. A method of treatment for the removal and immobilization of circulating immune complexes involves the transfusion of packed erythrocytes with high CD35 activity. This process is dependent upon complement consumption. Monitoring soluble CD35 levels during this treatment can aid in determining the effectiveness of the procedure.

In further embodiments, the invention is directed toward monitoring treatment of diseases or disorders of inappropriate complement activation, such as in myocardial infarction, ARDS, thermal injury of frostbite or burns, sepsis, cardiopulmonary bypass, or hemodialysis, where the patients are being treated with soluble recombinant CD35.

5.3. KITS AND ASSAYS FOR MEASUREMENT

Kits for conveniently carrying out the assays of and used in the practice of the present invention are also within the scope of the invention. Such a kit can comprise a pair of polyclonal CD35 antibodies in suitable containers: one capture polyclonal antibody and one detection polyclonal antibody, together with standards for generating a standard curve from which sample values are determined and positive control samples known to contain a specific amount of sCD35. Preferably the kit can comprise a reagent reactive with a detectable label on the detection antibody. For example, when the label is an enzyme, the kit can provide substrate.

5.4. MONITORING THE EFFECT OF A THERAPEUTIC TREATMENT

The present invention provides a method for monitoring the effect of a therapeutic treatment on a
subject who has undergone the therapeutic treatment. This method comprises measuring at suitable time intervals the amount of a soluble CD35 or soluble fragment thereof. Any change or absence of change in the amount of the soluble molecule can be identified and correlated with the effect of the treatment on the subject. In a specific embodiment of the invention, soluble CD35 can be measured in the serum of patients by a sandwich enzyme immunoassay (for an example, see Section 6, infra) in order to predict disease prognosis, for example, in AIDS, or to monitor the effectiveness of treatments such as AZT, interferon, CD4, or pentamidine administration. Soluble CD35 may itself be used as a therapeutic treatment, and the course of therapy may be followed by detecting soluble CD35. In another example, the response of patients with non-lymphatic cancers to therapy with IL-2 can be monitored. This et al. (1990, J. Immunol. 144:2419-2424) reported an activation of the complement system in patients treated with IL-2. Levels of soluble CD35 can be used to predict a response to IL-2 therapy.

In another embodiment, the level of sCD35 can be compared with the level of one or more additional soluble leukocyte marker to monitor the effect of a therapeutic treatment. In specific embodiments, the level of one or more of soluble IL-2R or soluble CD8 (described in International Patent Publication WO 87/05912), soluble CD4 (described in International Patent Publication WO 90/04180), or soluble T cell antigen receptor (described in U.S. Patent No. 4,845,026) can be compared with the level of sCD35. In a specific embodiment when the disease is AIDS, the level of sCD4 and sCD8 can be measured in addition to sCD35. The ratio of the amount of sCD35 to the amount of one or more of the leukocyte markers
can be compared to the ratio in the patient at an earlier time or to the ratio in a normal individual.

5.5. DETECTING AND/OR DIAGNOSING A DISEASE IN A SUBJECT

In another embodiment of the present invention, measurement of soluble CD35 can be used to detect, diagnose or stage a disease or disorder in a subject. The measured amount of the soluble molecule or of the total marker is compared to a baseline level. This baseline level can be the amount which is established to be normally present in the body fluid of subjects prior to the onset of disease or the amount present during remission of disease.

Disease or disorders that may be detected and/or diagnosed in a subject according to the present invention include but are not limited to those described in Section 2.2, supra, and those listed in Table I.

In a particular embodiment, diseases and disorders caused by HIV (the causative agent of AIDS) infection may be monitored by measurements of sCD35 according to the method of the present invention. AIDS therapies include the treatment of AIDS patients with drugs such as AZT (azido-deoxythymidine), γ or β interferons, and with soluble CD4, or its fragments and derivatives, and the production of potential AIDS vaccines, such as gp120 peptides. Practitioners in AIDS therapy very much need a procedure that can be used to monitor the efficacy of these treatments or vaccines. To date, the levels of the HIV antigen p24 have not proved sensitive enough. Soluble CD35 can be identified and detected in HIV-infected patients with different manifestations of disease, providing a sensitive immunoassay to monitor AIDS therapies and vaccines.
In a preferred embodiment, the level of sCD35 can be compared with the level of one or more additional soluble leukocyte markers to detect, diagnose or stage a disease. In specific embodiments, the level of one or more of soluble IL-2R or soluble CD8 (described in International Patent Publication WO 87/05912), soluble CD4 (described in International Patent Publication WO 90/04180), or soluble T cell antigen receptor (described in U.S. Patent No. 4,845,026) can be compared with the level of sCD35. In a specific embodiment when the disease is AIDS, the level of sCD4 and sCD8 can be measured in addition to sCD35. The ratio of the amount of sCD35 to the amount of one or more of the leukocyte markers can be compared to the ratio in a normal individual.

The present Example describes a more sensitive assay for soluble CD35 based on use of polyclonal antibodies for capture and detection reagents.

6. EXAMPLE: A POLyclONAL sCD35 ASSAY; DETECTION OF sCD35 IN PATIENTS

6.1. MATERIALS AND METHODS
Production of Polyclonal Rabbit anti-CD35.
Two rabbits (#30 and #31) were subcutaneously injected with 250 µg of purified sCD35 prepared by expression of recombinant plasmid pBSCRlc in CHO cells, deposited with the ATCC and assigned accession no. CRL 10052, in 0.5 ml of CFA (Complete Freund's adjuvant) followed by five intramuscular injections of CD35. The first intramuscular injection of 250 µg of CD35 in 0.5 ml of IFA (Incomplete Freund's adjuvant) was given two weeks after the initial subcutaneous injection. Four weeks later (on week 6) an injection of 125 µg CD35 in 0.5 ml IFA was administered, followed by three more identical intramuscular injections six weeks apart.
At 26 weeks the rabbits were exsanguinated. One prebleed of 10 ml, one test bleed of 5 ml on week 4, and six production bleeds of 40 ml each (weeks 8, 10, 14, 16, 20, 22) were also collected during the immunization schedule and assayed for anti-CD35 antibody production by ELISA. Briefly, a 96 well plate was coated with each rabbit serum at 1:1000 followed by several serial dilutions in PBS. After blocking and washing the plates, purified sCD35 or PBS alone was added. This step was followed by the addition of HRP-conjugated Y21 monoclonal antibody (section 16.3.2, *infra*). The plate was developed with OPD substrate and absorbance read at 490 nm.

Polyclonal antibodies that are produced from multiple bleedings of different rabbits can be tested to ensure CD35 reactivity of sufficient titer. Antibodies which yield an assay sensitivity down to a level of about 0.5 ng/ml are preferred for use.

**Purification of Rabbit Polyclonal anti-CD35**

**Serum.** The rabbit polyclonal anti-CD35 serum was purified as follows. Ten ml of frozen rabbit serum were thawed. At 4°C, 10 ml of saturated (NH₄)₂SO₄ (76.1 g in 100 ml distilled H₂O) were added dropwise to the serum. After 2 hours at 4°C the serum was spun at 3,000 x g for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml of PBS. The resuspended pellet was then dialyzed in Spectrapor #2 membrane (12,000-14000 molecular weight cutoff) in 4L of PBS overnight, changing the PBS one time.

To remove any salts still present after dialysis, a Sephadex G-25 column with a 30 ml bed volume was assembled and equilibrated with PBS. The dialyzed sample was applied to the column and washed with PBS. Fifteen 3 ml fractions were collected and samples read at O.D. 280. Samples with an O.D. > 0.7 were pooled and subsequently run over an Affi-Gel column coupled to CD35.
A CD35 conjugated affinity column was prepared as follows. Three ml of Affi-Gel resin (Affi-Gel Active Ester Agarose, Bio-Rad, Richmond, Ca.) were washed 3 times with 10 ml each of isopropanol, followed by three 10 ml washes with cold (4°C distilled H₂O. Five ml of sCD35 (11.5 mg) were added to the washed resin and incubated overnight at 4°C at 4°C. After nearly quantitative coupling of CD35 of the activated resin, 0.1 ml of 1 M ethanolamine pH 7.0 was added to block and incubated for one hour residual active sites. The coupled resin was then packed in a Bio-Rad Econo-column and allowed to settle. The resin was washed twice with 5 ml of 0.1 M hepes, pH 8.0, once with 5 ml of 20 mM NaH₂PO₄ with 0.7 M NaCl, pH 12.0, and once with 20 ml of 0.1 M HEPES + 0.15 M NaCl pH 7.4. The column was stored until use at 4°C in 0.1 M HEPES + 0.15 M NaCl pH7.4 + 0.1% NaN₃.

After washing the CD35 coupled Affi-Gel column with elution buffer (0.7 M NaCl, 20 mM Na₂HPO₄, pH 12 with 10N NaOH) and reequilibrating with PBS, the pooled fractions from the G-25 column were added to the washed gel and incubated on a rocker overnight at 4°C. The column was repacked and washed with 10X bed volumes with PBS (bed volume was 3ml). The column was then eluted with 10X the bed volume elution buffer, and 30 0.5 ml fractions were collected. The fractions collected were read at O.D. 280. Peaks were pooled separately, and dialyzed overnight in PBS at 4°C. Antibody concentration was then determined from the dialyzed fractions by the following formula:

\[
\text{O.D.280} = \frac{\text{concentration of antibody (mg/ml)}}{1.4}
\]

The purified polyclonal rabbit anti-CD35 was then frozen in aliquots at -70°C until needed.
Horse Radish Peroxidase Conjugation of anti-CD35 Antisera. The affinity purified polyclonal anti-CD35 (e.g., from rabbit #31) was concentrated by placing the antibody in dialysis tubing with a molecular weight cutoff of 12-14,000 (Spectrapor). The loaded dialysis tube was then buried in PEG 8,000 and the volume in the tubing allowed to reduce. The tubing was then washed clean of PEG 8,000 and dialyzed against 50 mM NaCO₃ in distilled H₂O (Baker Analytical), pH 9.5, overnight at 4°C.

Ten mg of the HRP (horse radish peroxidase, Boehringer-Manheim) was reconstituted in 1 ml of 1 mM Na acetate (equal volumes of 1 mM sodium acetate and 1 mM acetic acid, pH to 4.4 with acetic acid), sheltering the HRP from light. Then 0.6 ml of reconstituted HRP was added to 0.4 ml of the 1 mM Na acetate for a final concentration of 6 mg/ml of HRP. Na periodate were (24.1) dissolved in 1 ml of the 1 mM Na acetate buffer and 0.2 ml of this solution were added to the 1 ml HRP solution. After incubating for 20 minutes at 20°C on a rocker platform, the Na periodate/HRP solution was dialyzed against Na acetate buffer overnight at 4°C in the dark.

After determining the concentration of the antibody to be conjugated according to the above equation, the dialyzed HRP was added to the antibody at a ratio of 1:2 (mg of HRP:mg of antibody) and allowed to incubate on a rocker platform for two hours at 20°C, sheltered from the light.

Just before use, 10 mg of sodium borohydride (Sigma) in distilled H₂O was prepared and 11 μl were added for every ml of antibody-HP solution. After incubating on ice for one hour, the mixture was dialyzed against PBS overnight at 4°C in the dark. After dialyzing, 10% Thimerosol (Bio-Rad) solution was added for a final concentration of 0.01%.

Heat-inactivated Fetal Bovine Serum (30 minutes at
57°C, Irving Scientific) was then added for a final concentration of 10%. The mixture was then spun at 5,000 x g for 10 minutes to remove aggregated antibody, aliquoted, and stored at 4°C away from light.

Production and Purification of soluble CD35.

Soluble CD35 Bead Assay Protocol. A solid-phase (bead) sandwich immunoassay was used. The assay is carried out according to the following protocol.

Into designated 12 x 75 mm polystyrene tubes, 50 µl of standard, control or serum specimen in duplicate was pipetted. The standards contained recombinant human CD35 in a buffered solution containing bovine serum and thimerosal at the following concentrations: 0, 5, 10, 20, 40, 60, and 80 ng/ml. To each test tube were added 150 µl of the HRP conjugated rabbit polyclonal anti-human CD35 antibody. Using non-metallic forceps, one rabbit polyclonal anti-human CD35 coated bead was then added to each tube. After shaking the tubes to ensure proper mixing of all reagents, they were incubated for 120 minutes at room temperature (22-26°C) on a rotator set at 150 rpm. At the end of the incubation, the beads were washed three times with 2 ml of deionized water. Just prior to use the chromogen solution was prepared by dissolving one chromogen tablet (containing O-phenylenediamine + 5.3 mM urea peroxide +
thimerosal) per 5 ml of substrate diluent. Into each tube and into two empty test tubes to be used as substrate blanks 200 µl of the chromogen solution were pipetted. After incubating the tubes for 30 minutes at room temperature (22-26°C) without shaking, 1.0 ml of the stop solution was added to each tube as well as the two blank chromogen tubes. The stop solution consisted of 1N sulfuric acid, reagent grade, in deionized water. All tubes were then read on a spectrophotometer at 492 nm after adjusting absorbance to 0 using the chromogen solution blank tubes.

A standard curve was constructed by plotting mean absorbance on the vertical (Y) axis versus the corresponding CD35 concentration on the horizontal (X) axis, using rectilinear graph paper. An example of the standard curve is demonstrated in Figure 1. Mean absorbance values of the specimen samples were be determined from the standard curve.

**Soluble Human CD35 Plate Assay.** The development of another soluble CD35 Assay was initiated with monoclonal capture and detection antibodies utilizing a two-step solid phase (plate), enzyme immunometric assay. The evaluation of polyclonal antibodies used in combination with other polyclonal antibodies or monoclonal antibodies was evaluated in this plate assay according to the following procedure.

One hundred µl of monoclonal antibody J3D3 at 0.4 µg/ml in PBS or for example, purified Rabbit serum #30 at 1.0 µg/ml in PBS were added to each well of an Immulon II plate (Dynatech) for 24 hours at 4°C. The J3D3 antibody was obtained from AMAC, Inc., Westbrook, Me. (Cook, J., et al., 1985, Mol. Immunol. 22:531). The coating antibody was then discarded from each of the wells and 300 µl of blocking buffer were added and incubated for 2 hours at 37°C. After washing three times in washing buffer, 100 µl of
primary calibrators I and II (p.c.I and p.c.II) were added to the appropriate wells. Primary calibrators were comprised of recombinant soluble human CD35 diluted to 120 ng/ml and 80 ng/ml, respectively, in standard diluent. The starting concentrations of p.c.I and p.c.II were diluted to 120, 100, 80, 40, and 20 ng/ml, and 80, 40, and 20 ng/ml respectively. After incubating for two hours at 37°C, the plates were washed three times with washing buffer and 100 μl of either 1:5000 dilution of polyclonal anti-human CD35 (rabbit serum #31)-HRP conjugated or a 1:4000 dilution of monoclonal anti-human CD35 (YZ1)-HRP conjugated was added to the appropriate wells for 2 hours at 37°C. Five minutes before washing the wells, the chromogen substrate was prepared by dissolving one chromogen tablet (containing O-Phenylenediamine) per 10 ml of substrate diluent. The wells were washed three times and 100 μl of substrate added to each well. After 20 minutes at room temperature, the reaction was stopped by the addition of 50 μl of 2N H₂O₄ per well. Absorbance was read on a Dynatech MR 600 plate reader at O.D. 490, reference at 630 nm.

6.2 RESULTS

6.2.1 POLYCLONAL CD35 ANTIBODY PREPARATIONS

Polyclonal antibodies were generated in rabbits that received recombinant soluble CD35 as immunogen. The antibodies were purified using recombinant CD35 solid phase affinity chromatography. The purified polyclonal antibodies were specific for CD35, recognized multiple CD35 antigenic determinants, and were functional as evidence by their ability to bind to the CD35 affinity column. Use of a recombinant CD35 column enhanced the purification step since none of the impurities that may be carried with CD35 from red blood cells are present with the recombinant protein. The use of CD35 column-purified
polyclonal antibody is a substantial improvement over using total IgG polyclera, as the percentage of CD35 specific antibodies relative to protein concentrations is much higher (e.g., thousands-fold).

6.2.2 ASSAY CHARACTERISTICS

The soluble CD35 assay was a 1 step solid-phase sandwich immunoassay. The capture antibody is coated on a solid support such as a bead and then simultaneously incubated with the sample (or standard) and the conjugated detection antibody to form an antibody-antigen-antibody sandwich. Following the 1 step incubation, unbound reagents are washed away and the amount of sandwich is quantified by the development of a color reaction. Both capture and detection antibodies are identical polyclonal antibodies.

The Characteristics of the assay are given in Table II.

| TABLE II |

CHARACTERISTICS OF A POLYCLONAL ANTIBODY BASED SOLUBLE CD35 ASSAY

* fully weighted mean intra-assay CV of 4.0%
* mean inter-assay CV of 6.4%
* minimum detectable concentration (MDC) observed range of 0.35 - 0.62 ng/ml mean value of 0.47 ng/ml
* measuring range upper limit of 80 ng/ml
* dilution linearity for samples up to 2000 ng/ml
* CV of the slope of the standard curve for 65 assays performed was 3.7%

This soluble CD35 assay of the invention was compared with assays based on monoclonal capture and
detection antibodies. Basic assay calibrators developed for this purpose ranged from 20-120 ng/ml of CD35. Monoclonal antibodies that were analyzed included YZ-1 (Changelian et al., 1985, J. Immunol. 134:1851) and J3D3 (Cook et al., 1985, Mol. Immunol. 22:531). Monoclonal antibody J3D3 is available from AMAC, Maine, catalog no. 0195B. These reagents were either tested as capture antibodies by coating them on microtiter plates during the 24 hours prior to the assay or tested as detection antibodies. In addition, two polyclonal antibody preparations (R30 and R31) were similarly analyzed and compared to the monoclonal antibody performances. Combinations of monoclonal and polyclonal antibodies in either capture or detection configurations were evaluated. Surprisingly, the configuration of polyclonal antibodies as both capture and detection antibodies provided a more sensitive assay than any other combination studied and yielded more consistent results. The results of 2 configurations are shown in Table III.
TABLE III
SOLUBLE CD35 ASSAY
POLYCLONAL vs. MONOCLONAL ANTIBODIES*

<table>
<thead>
<tr>
<th>CD35 ng/ml</th>
<th>Polyclonal o.d.</th>
<th>%C.V.</th>
<th>Monoclonal o.d.</th>
<th>%C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.006</td>
<td>-</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.657</td>
<td>2.7</td>
<td>0.292</td>
<td>3.6</td>
</tr>
<tr>
<td>40</td>
<td>0.902</td>
<td>3.1</td>
<td>0.580</td>
<td>6.5</td>
</tr>
<tr>
<td>80</td>
<td>1.163</td>
<td>3.6</td>
<td>1.179</td>
<td>8.3</td>
</tr>
<tr>
<td>100</td>
<td>1.226</td>
<td>3.0</td>
<td>1.417</td>
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<tr>
<td>120</td>
<td>1.321</td>
<td>3.5</td>
<td>1.619</td>
<td>5.3</td>
</tr>
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</table>

* Polyclonal capture and detector antibodies versus monoclonal capture and detector antibodies in a 2-step plate assay.

We believe this unexpected result arises from two sources. Firstly, the polyclonal antibodies recognize multiple determinants on CD35 and may effectively capture or detect significantly more analyte. Secondly, we specifically selected for functional polyclonal antibodies that are specific for CD35 by purifying antiserum on CD35 affinity columns. It is likely that this selection resulted in a final product with a greater proportion of functional antibody as compared to the overall protein concentration.

The polyclonal assay is a very reliable and reproducible assay for the measurement of soluble CD35. It was noted however, that the polyclonal CD35 specific antibodies required slightly more time than the monoclonal antibodies used in other 1 step based assays to reach maximum binding. To correct for this, the primary incubation period (Capture Ab on bead + standard/specimen + conjugated antibody) was extended
from 90 minutes to 2 hours. This assured that the sandwich reaction would proceed to completion over the entire measuring range. Optimization was achieved by comparing the observed optical density changes over the range of the standard curve in fifteen (15) minute added increments.

As can be seen in Table IV, the reaction began to stabilize during the 105 minute incubation period (111.3% change) and was fairly stabilized by 120 minutes (105.5% change).

Evidence that the soluble CD35 bead assay is functional was derived in a purified recombinant spike and recovery study. A normal human serum pool was initially assayed and found to contain 25.7 ng/ml of soluble CD35. Aliquots of this pool were then spiked with 500 or 250 ng/ml of CD35 and diluted with specimen diluent to three concentrations within the measuring range. The two spike specimens were diluted to different concentrations and yielded a mean recovery of 94.5% (Table V).
TABLE IV

SOLUBLE CD35 BEAD ASSAY
INCUBATION KINETICS

\[
\begin{array}{ccc}
\text{CD35} & \text{ng/ml} & \text{105-120 min} & \text{120-135 min}^* \\
\hline
5 & 10 & 111.7 & 105.6 \\
10 & 20 & 116.6 & 104.4 \\
30 & 50 & 109.4 & 106.1 \\
10 & 80 & 107.3 & 106.8 \\
\hline
\text{Mean} & & 111.3 & 105.5 \\
\end{array}
\]

* Primary incubation period (bead+standard+conjugate)

TABLE V

SOLUBLE CD35 ASSAY
SPIKE AND RECOVERY FROM
NORMAL HUMAN SERUM

<table>
<thead>
<tr>
<th>CD35 (ng/ml)</th>
<th>Dilution</th>
<th>( \frac{\text{Amount Recovered}}{\text{Theoretical}} )</th>
<th>( \frac{\text{Observed}}{% \text{Recovery}} )</th>
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<tbody>
<tr>
<td>25</td>
<td>0</td>
<td>-</td>
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<tr>
<td>500</td>
<td>1:6.25</td>
<td>80</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
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<td>40</td>
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<tr>
<td>250</td>
<td>1:3.33</td>
<td>75</td>
<td>65.0</td>
</tr>
<tr>
<td>30</td>
<td>1:5.00</td>
<td>50</td>
<td>48.6</td>
</tr>
<tr>
<td></td>
<td>1:10.0</td>
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<td>23.8</td>
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n=8 per dilution
Mean % Recovery: 94.5%

The range of the assay for measuring soluble CD35 is seen from the standard curve (FIG. 1). As the
assay is configured, the standard curve is generated from six standards of 5, 10, 20, 40, 60 and 80 ng/ml of CD35. The observed linear response is subjected to standard regression analysis. The results indicate a slope of approximately 1.8-1.9, an $r^2$ value of 0.997 and a $Y$-intercept of about zero. The standard curve evidenced excellent reproducibility with a CV of the slope of the standard curve for 65 assays performed of 3.7%. Replicate assays indicated a minimum detectable concentration of about 0.5 ng/ml as calculated by:

$$MDC = \frac{x\ OD_0 + 2\ SD_0}{x\ OD_{10} - x\ OD_0} \times 10\ ng/ml$$

$SD_0 =$ Standard deviation observed for 0 ng/ml point
$x\ OD_0 =$ mean of the optical density at 9 ng/ml point
$x\ OD_{10} =$ mean of the optical density measured for 10 ng/ml

The range of the MDC's observed was 0.35-0.62 ng/ml with a mean value of 0.47 ng/ml

6.2.3 **DETECTION OF SOLUBLE CD35 IN PATIENT SERA**

Following the development of a reliable CD35 immunoassay with greater sensitivity, various patient samples were analyzed. Normal serum values were evaluated from fifty-two normal serum specimens to determine the normal serum range and mean value of soluble CD35 in serum. The normal range obtained based upon a 95% confidence interval is 22.5-65.1 ng/ml with a mean value of 44 ng/ml. Two specimens yielded values below this range and one above it. The observed range was 16.4 ng/ml to 68.3 ng/ml (Figure 2).

Once the normal range had been accurately determined, clinical specimen values were evaluated (FIG. 2). Serum samples from patients with systemic
lupus erythematosus (SLE), renal transplants, Hodgkin's disease, osteosarcoma and unclassified leukemias were tested. Samples were tested undiluted and at a 1:3 dilution in the soluble CD35 assay. Of the thirty-one specimens evaluated, none produced values above the upper limit of the standard curve (80 ng/ml). Two of nine SLE sera evidenced a soluble CD35 level below the normal range, while one of nine yielded a value above the normal range. One of seven Hodgkin's Disease and two of five leukemia sera demonstrated levels above the normal range. These results clearly demonstrate that soluble CD35 levels can be detected in randomly selected patients with renal transplant, Hodgkin's disease, osteosarcoma, leukemia and lupus.

6.3 DISCUSSION

It has been clearly demonstrated that soluble CD35 can be detected in the sera of randomly selected patients with lupus, renal transplant, osteosarcoma, Hodgkin's disease and leukemia. Given this positive result, it is now possible to subdivide each patient population into subgroups by disease stage, type of disease, type of treatment, acute versus chronic symptoms such as the acute flareups of rheumatoid arthritis versus the more chronic longterm symptoms etc. The levels of sCD35 alone or in combination with other soluble or total markers can then be correlated with the disease state or treatment income.
WHAT IS CLAIMED IS:

1. A method for detecting or measuring soluble CD35 in a sample comprising:
   (a) contacting the sample with a first anti-CD35 polyclonal antibody under conditions which allow immunospecific binding to occur;
   (b) contacting the sample with a second anti-CD35 polyclonal antibody under conditions which allow immunospecific binding to occur; and
   (c) detecting or measuring any immunospecific binding that occurs of soluble CD35 in the sample with both the first and second polyclonal antibodies, such immunospecific binding indicating the presence or amount of the soluble CD35 in the sample, in which the first and second polyclonal antibodies can detect soluble CD35 present in the sample in an amount as low as about 0.5 nanograms per milliliter.

2. The method according to claim 1 in which the sample comprises a biological fluid from a patient in which the biological fluid is selected from the group consisting of blood, serum plasma, saliva, urine, spinal fluid, synovial fluid, amniotic fluid and cranial fluid.

3. The method according to claim 1 in which the first and second antibodies are immunoaffinity purified prior to use by adsorption to immobilized CD35.

4. The method according to claim 1 in which the first and second antibodies are added to the sample at about the same time, and then the sample is incubated in order to allow immunospecific binding to occur.

5. The method according to claim 1 in which the soluble CD35 is spontaneously released soluble CD35 in which the soluble CD35 is recombinant soluble CD35.
6. A method for monitoring the effect of a therapeutic treatment on a patient, which treatment comprises administration to the patient recombinant soluble CD35, comprising:
   (a) measuring soluble CD35 in a sample from the patient according to the method of claim 1, 3 or 4; and
   (b) comparing the amount of soluble CD35 measured in step (a) to the amount found in normal individuals or in the patient prior to the treatment.

7. A method for monitoring the effect of a therapeutic treatment on a patient having a disease associated with alterations in soluble sCD35 levels, comprising:
   (a) measuring soluble CD35 in a sample from the patient according to the method of claim 1, 3 or 4; and
   (b) comparing the amount of soluble CD35 measured in step (a) to the amount found in normal individuals or in the patient prior to the treatment.

8. A method for detecting, diagnosing or staging a disease or disorder in a subject suspected of having or having a disease associated with alterations in soluble CD35 levels, comprising:
   (a) measuring soluble CD35 in a sample from the patient according to the method of claim 1, 3 or 4; and
   (b) comparing the amount of soluble CD35 measured in step (a) to the amount found in normal individuals, a difference in the amount so detected indicating a disease state.

9. A method for detecting, diagnosing or staging a disease or disorder in a subject suspected of having
or having a disease associated with alterations in soluble CD35 levels, comprising:

(a) measuring in a sample from the patient soluble CD35 according to the method of claim 1, 3 or 4 and one or more soluble leukocyte markers; and

(b) comparing the ratio of the amount of sCD35 to the amount of the soluble leukocyte marker in the sample to the same ratio in normal individuals, a difference in the ratios indicating a disease state.

10. The method of claim 8 or 9 in which the disease or disorder is AIDS.
# INTERNATIONAL SEARCH REPORT

## I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC:

- **IPC(5):** G01N 33/542, 33/564, 33/566, 33/571
- **U.S.Cl.:** 435/5, 7.21, 7.24, 7.25, 7.94, 974; 436/501, 506, 510, 518, 811, 821

## II. FIELDS SEARCHED

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## III. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>The Journal of Immunology, Vol. 134, No. 5, issued May 1985, Yoon et al., &quot;Characterization of a Soluble Form of the C3b/C4b Receptor (cR1) in Human Plasma&quot;, pages 3332-3338, see page 3333, Col. 1; page 3336, Col. 1; and Fig. 1.</td>
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### Definitions
- **X** document defining the general state of the art which is not considered to be of particular relevance
- **Y** earlier document but published on or after the international filing date
- **L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document of same or special reason (as specified)
- **D** document relating to an oral disclosure, use, exhibition or other means
- **O** 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
- **W** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- **L** 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.
- **Z** document member of the same patent family

## IV. CERTIFICATION

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International Searching Authority: ISA/US

Signature of Authorized Officer: David Saunders