(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012026 A1

(51) International Patent Classification[7]: C12M 1/00

(21) International Application Number: PCT/US01/23943

(22) International Filing Date: 31 July 2001 (31.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/628,585 31 July 2001 (31.07.2001) US

(71) Applicant and
(72) Inventor: **WEBBER, Robert** [US/US]; 4872 East 2nd Street, Benicia, CA 94510 (US).

(74) Agents: **BIELEN, Theodore, J., Jr.** et al.; Bielen, Lampe & Thoeming, Suite 720, 1990 N. California Blvd., Walnut Creek, CA 94596 (US).

(81) Designated States *(national)*: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012026 A1

(54) Title: APPARATUS AND METHOD FOR DETERMINING THE ONSET AND PRESENCE OF SEPSIS CONDITIONS

(57) Abstract: An apparatus and method for determining the onset and presence of sepsis from the liquid portion of the blood of a mammalian subject utilizing an assay. The assay includes a chemical detector for determining the presence of iNOS in the liquid portion of the blood of the mammalian subject, without detecting eNOS or nNOS.

APPARATUS AND METHOD FOR DETERMINING THE ONSET AND PRESENCE OF SEPSIS CONDITIONS

BACKGROUND OF THE INVENTION

The present invention relates to a novel and useful apparatus and method for rapidly determining the onset and presence of a sepsis condition.

Infections in a mammalian subject such as a human can occur directly by different types of organisms and indirectly through medical trauma. For example, gram-negative and gram-positive bacteria, viruses, and fungi may cause such infections. In certain cases, "sepsis conditions" may result from such infection. Such "sepsis conditions" include systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock. The latter condition causes multiple organ failure in a subject. It is estimated that hundreds of thousands of deaths occur each year due to septic shock in the United States alone.

An early prognostication of a sepsis condition is extremely important in order to initiate therapies to treat patients having this malady. For example, current therapies for the treatment of sepsis conditions include the administering of combined antibiotics and fluid replacement. It is known that the initial systemic inflammatory response syndrome (SIRS) is defined as a condition in which two or more of the following are present in a human:

1. Temperature less than 36°C or greater than 38°C;
2. A heart rate of greater than 90;
3. A respiratory rate of greater than 20; or
4. A white blood cell count of less than 4 x 10^6/ml or greater than 12 x 10^6/ml.

"Sepsis" is a condition of SIR plus a culture-documented infection. [Sepsis patients possess an increase in cardiac output, such as that found in systemic vascular resistance diseases, but with low organ perfusion.] "Severe sepsis" is a condition of sepsis with the addition of organ dysfunction, hypotension, or hypoperfusion. "Septic shock" is defined as hypotension (despite fluid resuscitation) plus hypoperfusion abnormalities. Organ failure occurs as a result of cell death due to tissue hypoperfusion.

Causative agents of sepsis conditions above described point to the overproduction of nitric oxide (NO) by the inducible form of nitric oxide synthase (iNOS). It has been demonstrated that the lipopolysaccharide (LPS) component from the cell wall of a gram-negative bacteria and the combination of lipoteichoic acid and peptidoglycan from the cell wall of a gram-positive bacteria result in the release of inflammatory cytokines such as TNFα, IL-1β, and IL-6. The interaction of the bacterial components and the inflammatory cytokines, in turn can induce the production of iNOS. The increased formation of NO generated by the iNOS then leads to increased vascular permeability, vasodilatation, hypotension, tissue hypoperfusion, and ultimately organ failure. As discussed above, these states are the manifestations of severe sepsis and septic shock. Thus, it is generally believed that it is the action of NO on smooth muscles and the vascular endothelium that results in systemic vasodilatation and increased vascular permeability. This, in turn, is responsible for the hypotension and tissue hypoperfusion resulting in multiple organ failure.

Although the therapies used to treat sepsis conditions are useful, meaningful prognosticators are needed to allow for the earliest possible intervention. It has been noted in an article entitled "Gram-Negative Bacteremia, IV: Reevaluation of Clinical Features in Treatment in 612 Patients" by Kreger et al that the early initiation of antibiotics reduce the frequency of septic shock and death by 50 percent.

At present, a definitive rapid clinical diagnostic for sepsis conditions such as SIRS and sepsis does not exist. Among the current procedures is one described in an article entitled "Epidemiology of Sepsis Syndrome in 8 Academic Medical Centers" by Sands et al includes blood culture procedures. Unfortunately blood culture techniques are slow, taking 24 to 48 hours for a result. In addition, blood culture tests are inaccurate, only yielding positive results indicating the presence of a sepsis condition in approximately 28 percent of patients with SIRS.

An article entitled "Assessment of the Safety and Efficacy of the Monoclonal Anti-Tumor Necrosis Factor Antibody-Fragment, MAK 195F, in Patients with Sepsis and Septic Shock: A Multicenter, Randomized, Placebo-Controlled, Dose-Ranging Study" by Rinheart et al, and another article entitled "Interleukin-6 Measurements in Selection of Sepsis Patients for Anti-cytokine Therapy", by Fischkoff indicate that the presence of interleukin-6 (IL-6) greater than 1,000 pg/ml is associated with hyperinflammatory conditions and is only predictive of poor outcome in septic patients receiving monoclonal treatment, MAK 195F.

United States Patent 5,639,617 describes the early detection of a sepsis in a patient by the recognition of the presence of procalcitonin in a biological liquid of the patient. However, the existence of procalcitonin only indicates a single source of the sepsis condition i.e. gram-negative bacteria.

Increases in the concentration of nitrate or nitrite in the blood has also been linked to sepsis. However, the presence of nitrate/nitrite in the blood has not been positively correlated to the status of patients with SIRS or sepsis conditions.

The detection of iNOS, in sepsis patients from induced leukocytes of sepsis patients has been accomplished. However such determination reveals a highly variable amount of iNOS and is not necessarily correlated to the presence of a sepsis condition.

An assay for rapidly determining the onset and presence of SIRS, sepsis, severe sepsis, and/or septic shock, would be a notable advance in the medical field.

BRIEF SUMMARY OF THE INVENTION

In accordance with the present invention a novel and useful apparatus and method for performing an assay to determine the presence of sepsis conditions is herein provided.

The immunoassay of the present invention is especially useful for measuring the level of iNOS in the liquid portion of blood such as whole blood, plasma, and serum. However, other biological samples may be employed. It has been found that the intracellular enzyme iNOS is released in small quantities into the circulatory system of mammalian subjects, such as human patients, 24 to 48 hours prior to the onset of severe sepsis, during the early stages of SIRS. Thus, the detection and measurement of iNOS in the liquid portion of blood by the immunoassay of the present invention serves as a useful prognosticator for the onset of sepsis conditions such as SIRS, sepsis, severe sepsis, and septic shock. It has also been found that the quantitative measurement of iNOS in plasma, serum, and whole blood can be used to monitor the course of a sepsis condition and to assesses the course of treatment of a sepsis condition.

The present invention involves an immunoassay for use in detecting and measuring the concentration of inducible nitric oxide sythase (iNOS) in the blood of a mammalian subject. Specifically, the liquid portion of the blood is employed in this regard. One aspect of the invention involves the use of a sandwich solid phase immunoassay which employs two sites. The first site uses an anti-iNOS monoclonal antibody that may be anti-iNOS clone identified as 21C10-1D10, which is found in United States Patent Application Serial Number 08/833,506 filed

7 April 1997, incorporated by reference, in whole, hereto. Such monoclonal antibody is known as the "capture" antibody, such that it binds any iNOS contained in a sample of the liquid portion of the blood of the mammalian subject. A labeled second monoclonal antibody is also employed as the "detection" antibody in a one step or two step reaction sequence. For example, biotinylated anti-iNOS monoclonal antibody identified as clone 2A1-F8 in the above-identified patent application suffices in this regard. The assay takes place on a solid support or carrier which can be any solid material capable of binding iNOS or anti-iNOS antibodies. Such solid support is well known is immunoassays and includes, but is not limited to, polystyrene, polypropylene, polyethylene, polyacrylamides, agarose, glass, dextrans, nylon, magnetite, cellulose, modified cellulose, and amylases. The support may be spherical, as a bead, or flat such as a sheet or test strip. In addition, the support may be cylindrical, akin to the inner surface of a test tube. In essence, the solid support or carrier may have virtually any structural configuration as long as the capture antibody and the iNOS can bond together thereupon. For example, a micro titer plate well has been found to perform this function successfully.

In one form of the invention, the labeled detection antibody is bound to a "binding partner" such as streptavidin, avidin, avidin-biotin complex (ABC), an anti-biotin antibody or the like. The binding partner is, in turn, conjugated to an enzyme such as horseradish peroxidase in an enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) format.

Alternatively, the purified detection antibody or its binding partner can be directly conjugated to an enzyme or other

labels such as fluorochromes, luminescent compounds, radioactive elements, fluorescent metal-chelate complexes, and the like. For example, fluorescent emitting metals such as Europium, Terbium, Samarium, Dysprosium, and others found in the lathanide series may be employed. Such metals are chelated to groups such as ethylene-diaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), or tetra-azacyclododecanetetraacetic acid (DOTA). Detection of fluorescent light emitted by these metal chelate complexes, following excitation, is accomplished by either fluorometry, or time resolved fluorometry.

Likewise, the detection antibody, or its binding partner, can be labeled with a fluorescent compound. Again, fluorescent light emanating from such items, after excitation, may be detected and measured by fluorometric devices.

Fluorochromes useable in the EIAs or ELISAs of the present invention include, but are not limited to, fluorescine, isothiocyanate (FITC), rhodamine, tertamethyl rhodamine isothiocyanate, (TRITC), Texas Red, phycoerythrin, allophycocyanin, phycocyanin, fluorescamine, and o-phthaldehyde.

In any case, enzymes that may be used as labels for conjugation to the purified detection antibody are chosen to produce products which can be detected by spectrophotometric, fluorometric, luminescent, visual or other means. Enzyme labels which are useful in EIAs and ELISAs include, but are not limited to, horseradish peroxidase, alkaline phosphatase, glucose oxidase, glucose-6-phosphate dehydrogenase, catalase, beta-galactosidase, glucoamylase, acetylcholinesterase, malate dehydrogenase, urease, alcohol dehydrogenase, ribonuclease,

asparaginase, staphylococcal nuclease, triose phosphate isomerase, delta-5-steroid isomerase, and alpha-glycerophosphate dehydrogenase.

The detection antibody may be labeled directly to the fluorochrome luminescent compounds and the like. In the case of an indirect reaction sequence, the labeled detection antibody is bound to an appropriately binding partner, and unbound material is removed by washing. Chemiluminescent substrates, for example, may be added such that the bound enzyme converts the substrate into products which emit photons of light that can be detected and measured by a luminometer. Consequently, the amount of light that is produced during a fixed length of time is directly proportional to the amount of iNOS contained in the liquid portion of the blood serving as the sample or specimen.

Luminescent compounds which are useful in EIA or the ELISA formats include, but are not limited to, luminol, isoluminol, theromatic acridinium ester, oxalate ester, acridinium salts, imidazole, and the like.

Luminescent enzymes which release photons of light during catalysis conjugated to the detection antibody or its binding partner useful in EIA or ELISA formats, include, but are not limited to, luciferase, luciferan, and aequorin.

In addition to the sandwich EIA or sandwich ELISA formats, the method and apparatus of the present invention may measure the level of iNOS in the liquid portion of a blood specimen in a single site competitive binding assay format. In one such single site competitive binding assay format, the iNOS in the specimen competes for binding with a labeled peptide, peptide analog, or peptidomimetic that contains the epitope of

the anti-iNOS monoclonal antibody clone 21C10-1D10. Such epitope is identified in the above-identified patent application, serial number 08/833,506 as residues number 39-45 of iNOS (VTQDDLQ). The peptide, peptide analog, or peptidomimetic can be labeled with an enzyme, luminescent compound, a luminescent generating enzyme, a fluorochrome, or a fluorescent metal chelate as described above. Again, light emitted or fluoresced from or absorbed by these compounds or products may be detected by a photometer and quantified to determine the amount of iNOS present in a liquid blood sample. Radio labeling of the peptide, peptide analog, or peptidomimetic requires the measurement of the level of iNOS through a radioimmunoassay. Any radioisotope may be detected and quantified by use of a gamma spectrometer, scintillation, photographic film, or any other known technique to determine the amount of iNOS in a liquid blood sample.

Other test formats may be employed such as dipsticks, lateral flow devices, auto-analyzers, point-of-care devices, and the like. In any case, the developments of SIRS, sepsis, severe sepsis, or septic shock may be detected prior to onset or during occurrence of such sepsis conditions, as well as during a treatment phase following occurrence.

It may be understood a novel useful apparatus and method for the detection of SIRS, sepsis, severe sepsis, or septic shock has been described.

It is therefore an object of the present invention to provide an apparatus and method to detect a sepsis condition which employs the liquid portion of the blood of a mammalian subject.

Another object of the present invention is to provide an apparatus and method for detecting the onset and/or existence of a sepsis condition which is relatively quick and permits rapid therapeutic treatment of patients.

A further object of the present invention is to provide an apparatus and method for detecting the onset and/or presence of a sepsis condition in a mammalian subject which is accurate.

A further object of the present invention is to provide a method and apparatus for detecting the onset and/or presence of a sepsis condition which is capable of monitoring therapies applied to patients detected as having a sepsis condition.

Another object of the present invention is to provide an apparatus and method for detecting the onset and/or presence of a septic condition in a mammalian subject which permits the application of therapies to a patient at an early stage, thus increasing the probability of survival of a patient and reducing the length of treatment in an intensive care unit.

The invention possesses other objects and advantages especially as concerns particular characteristics and features thereof which will become apparent as the specification continues.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Fig. 1 is a graph illustrating the purification of the IgG fraction of anti-hiNOS monoclonal antibodies described in Example 1.

Fig. 2 is a graph showing a relation between chemiluminescence and iNOS detected in Example 2.

Fig. 3A is a graphical representation of a patient's iNOS level during the onset phase of SIRS/sepsis, as described in Example 3.

Fig. 3B is a graphical representation of a patient's iNOS level following treatment for sepsis, as described in Example 3.

Fig. 4 is a reproduction of a western blot analysis of plasma samples of individuals with SIRS and/or sepsis confirming the presence of iNOS, described in Example 3.

Fig. 5 is a graph representing the non-correlation of the concentration of nitrite or nitrate in plasma to the presence of iNOS in the plasma of patients with SIRS and/or sepsis as described in Example 4.

Fig. 6 is a graph representing the concentration of iNOS in plasma between a group of patients with SIRS or sepsis compared to healthy volunteers, as described in Example 5.

For a better understanding of the invention reference is made to the following detailed description of the preferred embodiments and examples which should be taken in conjunction with the herein above described drawings.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

Various aspects of the invention will evolve from the following detailed description of the preferred embodiments and examples thereof which should be viewed in conjunction with the prior described drawings.

The apparatus and method of the present invention rapidly determines the onset or presence of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, or septic shock based on analysis performed on the liquid portion of the blood of a mammalian subject such as a human patient. The apparatus includes means for detecting the existence of inducible nitrio oxide synthase (iNOS) in the liquid portion of the blood of the human patient or mammalian subject without also detecting, or cross reacting, with eNOS or nNOS. Specifically, such means may include an immunoassay that can rapidly detect and quantitate the presence or change in amounts of iNOS in plasma, serum, or whole blood. It has been found that iNOS is generated by a human patient early in an episode of SIRS and prior to the onset of severe sepsis and septic shock.

The assay of the present invention utilizes a monoclonal antibody, preferably the IgG fraction of anti-iNOS monoclonal antibody clone 21C10-ID10 which binds to residues number 39-45 of human iNOS. Such clone is identified in prior noted patent application serial number 08/833,506. The antibody is adsorbed onto a solid support or carrier, preferably the wells of a white EIA microtiter plate. This antibody is known as the "capture antibody" and is used to bind the iNOS contained in a sample or specimen of the liquid portion of the blood of

the mammalian subject. Preferably such liquid sample may be whole blood, plasma, or serum. Simultaneously a labeled detection antibody, anti-iNOS monoclonal antibody, is bond to form a sandwich in a one step reaction. Such label detection antibody is preferably a biotinylated anti-iNOS monoclonal antibody as the IgG fraction of clone 21A1-F8 identified in the above-mentioned patent application. Following the formation of the sandwich, unbound labeled detection antibody is removed by washing and an excess amount of an appropriate binding partner, such as enzyme conjugated streptavidin or enzyme conjugated anti-biotin antibody is bound. Again, unbound material is removed by washing and a chemiliuminecent substrate is added. The bound enzyme converts the substrate into certain products with the emission of photons of light. Such photons are detected and measured by a luminometer. The amount of light that is produced during a fixed length of time is directly proportional to the amount of iNOS contained in the sample or specimen of the liquid portion of the blood of the mammalian subject. In other words, as the amount of iNOS increases in the liquid portion of the blood of the mammalian subject, the amount of light generated by the assay of the present invention increases.

The method of the present invention which determines the existence and quantity of iNOS in the liquid portion of the blood of the mammalian subject may be performed rapidly, namely between one and two hours. To aid in the reduction of time necessary to perform the method using the apparatus of the present invention, absorption of the capture antibody onto a

solid support is performed in advance. The capture antibody and solid support are then stored until needed.

Where the assay takes the form of a sandwich enzyme immunoassay (EIA), a monoclonal antibody such as the IgG fraction of anti-iNOS monoclonal antibody clone 21C10-1D10 is absorbed onto the wells of a white 96 well-IEA microtiter plate for two hours or longer. The unbound material is removed in a washing step and unreacted sites are blocked with an excess of a non-specific protein such as bovine serum albumin or gelatin. The unbound material is then washed off and the plate is stored until needed. When a measurement of the amount of iNOS contained in a liquid portion of blood of a mammalian subject is to be made, the sample of the same and the IgG fraction of biotinylated anti-iNOS monoclonal antibody clone 2A1-F8 are added to each well and kept there for 60 minutes. During this time, the iNOS in the sample binds to the immobilized capture antibody and the biotin-2A1-F8 binds to the iNOS in the blood sample, in a one step reaction sequence. Alternatively the biotin 2A1-F8 conjugate can be added separately after the iNOS contained in a sample has been bound and the unbound materials are washed off, a two-step reaction sequence. In either the one or two steps reaction sequence, after incubation, the unbound materials are removed by washing and a binding partner such as enzyme labeled streptavidin, and preferably, horseradish peroxide conjugated streptavidin is added and allowed to bind for 20 minutes. Again, unbound material is removed by washing and a chemiluminescent substrate is added. Preferably, the chemiluminescent substrate takes the form of luminol with chemiluminescent enhancer and hydrogen peroxide. Reaction

between the immobilized enzyme labeled component and the chemiluminescent material will result in the production of light which is measured in a microtiter plate luminometer. Moreover, the quantity of light is directly proportional to the quantity of iNOS in the sample of the blood portion of the mammalian subject.

Although chemiluminescent materials have been described above, fluorescent-emitting materials such as metals may be used as a binding partner and fluorescent light may be detected following excitation through the process of fluorometery or time-resolved fluorometery. Moreover, other types of labels such as radioisotopes are may be employed such that a quantity radioisotope activity may be quantified by the use of a gamma spectrometer, a scintillation spectrometer, or a photographic film. Also colorimetric substrates or products that absorb light may be produced and measured by a photometer.

It should also be noted, that in addition to the sandwich EIA or the sandwich ELISA, the assay of the present invention may use a single site competitive binding assay format. Such immunoassay, the iNOS in the sample competes for binding with a labeled peptide, peptide analog, or peptidomimetic that contains anti-iNOS monoclonal antibody clone 21CD10-1D10 epitope, residues numbers 39-45 of iNOS, prior noted. Again, the peptide, peptide analog, or peptidomimetic can be labeled with an enzyme, luminescent compound, luminescent generating enzyme, fluorochrome, or fluorescent metal chelate. Moreover, radioisotopes may also be used as described hereinabove.

The following examples are deemed to be illustrative of the invention but are not intended to limit the scope of the same in any manner.

EXAMPLE 1

Mouse IgG from two anti-iNOS monoclonal antibody producing clones identified in United States Patent serial number 08/833,506 as clones 21C10-1D10 and 2A1-F8 were obtained. The antibodies were purified either from culture supernatant fluid by protein G affinity purification or from ascites fluid by ammonium sulfate precipitation followed by Sephadex G 200 gel filtration chromatography. Fig. 1 illustrates the gel filtration chromatography results. The purified 21C10-1D10 antibody was absorbed onto microtiter plates for use as the "capture" antibody in a sandwich EIA. 5.0 mg of purified IgG from clone 2A1-F8 was reacted with a ten fold molar excess of sulfosuccinimidyl 6-(biotinamido) hexanoate, obtained from the Pierce Chemical Co. of Rockville, Illinois. A reaction took place in a sodium bicarbonate buffer pH 8.5 for 16 hours and the reaction was stopped with the addition of 0.1 ml of 1.0 molar Tris buffer pH .5. The unbound biotin was removed by dialysis against 0.1 molar Tris buffer, pH 8.5, for 4 hours and against three changes of phosphate buffered saline. The biotin-2A1-F8 conjugate was used as the "detection" antibody in the sandwich EIA with a suitable binding partner.

EXAMPLE 2

In an attempt to measure iNOS directly in the plasma and/or serum obtained from patients in a clinical study by EIA immunocytochemical staining and western blots, iNOS was found in the non-liquid portion of blood samples, i.e. in induced

leukocytes. Free iNOS of the plasma portion was not detected. It is believed that the initial inability to detect iNOS free of plasma was due to the fact that iNOS is an intracellular protein and cells that express iNOS are reported to die by apoptosis. The apoptosis mechanism results in the cell remnants being phagocytosized by macrophages. It was theorized that none of the intracellular components of the leukocytes should be released under this process. It was decided to attempt the detection of iNOS in plasma and/or serum of patients with sepsis by other methods possessing greater sensitivity. Plasma from normal volunteers which should not have contained any iNOS and iNOS purified from induced DLD-1 cells were employed. The latter derived from a human colorectal epithelial adenocarcinoma, which had been reported to express human iNOS after induction with a combination of inflammatory cytokines. The stock solution of purified human iNOS was calibrated by a competitive binding radioimmunoassay using an anti-peptide polyclonal antibody, known amounts of unlabeled free peptide, and ^{125}I-labeled peptide. The stock iNOS solution was used to spike normal human plasma with known quantities of iNOS for the development of a highly sensitive EIA. The initial assay employing the EIA of this Example, using ABC as a binding partner with a colorimetric readout, failed to detect iNOS in plasma of SIRS and septic patients. However, substitution of HRP conjugated streptavidin as a binding partner and a chemiluminescent readout resulted in the detection of iNOS spiked into the normal human plasma samples. It is believed that the sensitivity increased 80 fold by this substitution. Fig. 2 represents the results of the chemiluminescent readout.

A kit was assembled to perform the immunoassay developed above:

1. One 96 well white EIA microtiter plate coated with the IgG fraction of anti-iNOS clone 21C10-1D10 monoclonal antibody ("capture antibody").

2. Washing buffer.

3. Sample application buffer.

4. Calibrated iNOS standard.

5. Biotinylated IgG fraction of anti-iNOS clone 2A1-F8 monoclonal antibody ("detection antibody").

6. Horseradish peroxide conjugated streptavidin (labeled "binding partner").

7. Chemiluminescent substrates comprised of two components:

Enhanced chemiluminescent substrate solution (luminol Or isoluminol with enhancer)

Stabilized hydrogen peroxide solution.

8. Chart/graph for determining the plasma concentration of iNOS.

9. Instruction booklet (operating manual).

The instruction booklet (operating manual), chart/graph, and other data may be provided in computer readable form, such as floppy disk, compact disk, or downloadable Internet file.

EXAMPLE 3

The sandwich EIA described in Example 2 was standardized for the length of each incubation, buffer composition, and concentration of enzyme label binding partner, for the chemiluminescent readout. iNOS plasma samples were

obtained from eight normal healthy volunteers and from ten patients with SIRS/sepsis or patients "at risk" for the development of SIRS/sepsis. Informed consent was obtained from the adults and from the parent or guardian of children prior to enrollment of all volunteers and patients in this study. The eight normal healthy volunteers ranged in age from 18 to 50 years. Three of the healthy volunteers were female and five were male. No iNOS was detected in the plasma obtained from the eight normal healthy volunteers. It was summarized that the level of free iNOS in the plasma of the eight normal healthy volunteers, if existing at all, must be below the normal limit of detection of the assay described in Example 2, i.e. 0.375 fmol/ml.

The ten patients with SIRS and sepsis, or "at risk" for developing such pathophysiology were then studied following approved protocol. Three different types of samples were obtained from such patients: Plasma (liquid portion of unclotted blood), serum (the liquid portion of clotted blood), peripheral blood mononuclear cells (PBMCs) fixed on glass slides, and in phosphate buffered saline. Free iNOS was discovered in plasma in the patients with SIRS, sepsis, or "at risk" for developing SIRS or sepsis. Figs. 3A and 3B represent this finding for particular patients in this group. The fact that iNOS is present in the plasma of patients with SIRS, sepsis, and at risk for developing SIRS or sepsis, and not present in the plasma of normal healthy individuals was confirmed by western blot analysis of plasma samples for the presence of iNOS using monoclonal antibody specific for human iNOS. Fig. 4 represents this result.

With reference to Fig. 3A, iNOS from the plasma of a particular patient at risk for the development of SIRS or sepsis was detected 24 to 48 hours prior to the onset of the clinical symptoms of SIRS/sepsis. As may be seen from Fig. 3A, this "at risk" patient possessed elevated plasma iNOS levels 24 hours prior to becoming SIRS positive on day one and 48 hours prior to becoming septic positive on day two. A total of ten ICU patents were tested. One other patient displayed the same pattern of elevated iNOS in plasma 24 to 48 hours prior to the onset of the clinical symptoms of SIRS and sepsis as that shown in Fig. 3A. Moreover, three patients with SIRS and/or sepsis, exhibited a decrease in the level of iNOS in plasma as effective therapy was applied and the patients' condition improved. Fig. 3B shows an example of one of these patients. Two other patients with confirmed sepsis of the ten ICU patients also showed a decreasing level of plasma iNOS as effective treatment was applied, improving the patients' condition.

EXAMPLE 4

The data obtained for the plasma concentration of iNOS in patients with SIRS and/or sepsis or "at risk" for developing SIRS or sepsis of Example 3 was compared to the plasma concentration of nitrite (NO_2) and nitrate (NO_3). Fig. 5 indicates that there was no statistical significant correlation between the existence of nitrite or nitrate in plasma from these patients. The regression line for this data is defined by $y=0.014x + 60.27$ and the correlation coefficient $r = 0.1038$.

EXAMPLE 5

The concentration of iNOS in the plasma, as determined by the chemiluminescent EIA of Example 2, from the 14 samples that were obtained from ten patients with SIRS and/or sepsis prior to treatment, were compared to the concentration found in the plasma of the eight healthy normal volunteers, Fig. 6 shows such results. The data was statistically analyzed by Student's t-Test. The average concentration for the 14 samples from SIRS and septic patients was 404 ± 212 fmol/ml (mean ± standard deviation) and the average for the eight control healthy normal volunteers was zero. By the Students t-Test, the probability that these two populations are the same is less than 0.001 ($p < 0.001$). Thus, the differences of iNOS measured in the plasma between normal healthy volunteers and patients with SIRS and/or sepsis prior to treatment is highly statistically significant.

While in the foregoing, embodiments and examples of the present invention have been set forth in considerable detail for the purposes of making a complete disclosure of the invention, it may be apparent to those of skill in the art that numerous changes may be made in such detail without departing from the spirit and principles of the invention.

WHAT IS CLAIMED IS

1. An apparatus for rapidly determining the onset or presence of systemic inflammatory response syndrome, sepsis, severe sepsis, or septic shock from the liquid portion of the blood of a mammalian subject,

comprising:

means for detecting the existence of inducible nitric oxide sythase enzyme (iNOS) in the liquid portion of the blood of the mammalian subject, without cross reacting with eNOS or nNOS.

2. The apparatus of claim 1 in which said means for detecting the existence of inducible nitric oxide synthase further comprises a capture monoclonal antibody which binds to iNOS in the liquid portion of the blood of the mammalian subject, and a label conjugated to said capture monoclonal antibody.

3. The apparatus of claim 2 in which said label conjugated to said capture monoclonal antibody is selected from the group consisting essentially of:

an enzyme, a luminescent compound, a luminescent generating enzyme, a fluorochrome, a fluorescent metal chelate, and a radioisotope.

4. The apparatus of claim 1 in which said means for detecting the existence of inducible nitrogen oxide synthase further comprises:

a. a capture monoclonal antibody bound to iNOS in the liquid portion of the blood of a mammalian subject; and

b. a labeled detection antibody bound to iNOS in the liquid portion of the blood of the mammalian subject.

5. The apparatus of claim 4 in which said labeled detection antibody bound to iNOS comprises a labeled detection monoclonal antibody.

6. The apparatus of claim 5 in which said labeled detection antibody comprises a labeled detection anti-iNOS monoclonal antibody.

7. The apparatus of claim 4 which additionally comprises a binding partner bound to said labeled detection antibody and a further label bound to said binding partner.

8. The apparatus of claim 7 in which said binding partner is selected from the group consisting essentially of:

an enzyme-conjugated strepavidin, an enzyme conjugated avidin, an enzyme conjugated anti-biotin antibody, and an enzyme conjugated avidin-biotin complex.

9. The apparatus of claim 7 in which said further label bound to said binding partner is selected from the group consisting essentially of:

an enzymes a luminescent compound, a luminescent generating enzyme, a fluorochrome, a fluorescent metal chelate, a colored compound, a color generating compound and a radioisotope.

10. A method of rapidly determining the onset or presence of systemic inflammatory response syndrome, sepsis, severe sepsis or septic shock from the liquid portion of the blood of a mammalian subject,

comprising the step of:

determining in a sample of the liquid portion of the blood of the mammalian subject the existence of inducible nitric oxide

synthase enzyme (iNOS), apart from the existence of eNOS and nNOS.

11. The method of claim 10 in which said step of determining in a sample of the liquid portion of the blood of the mammalian subject the existence of inducible nitric oxide synthase enzyme (iNOS) apart from the existence of eNOS or nNOS, further includes a capture monoclonal antibody which binds to iNOS in the liquid portion of the blood of the mammalian subject and a label conjugated to said capture monoclonal antibody.

12. The method of claim 11 in which said label conjugated to said capture monoclonal antibody is selected from the group consisting essentially of:

an enzyme, a luminescent compound, a luminescent generating enzyme, a fluorochrome, a fluorescent metal chelate, a color compound, a color generating compound, and a radioisotope.

13. The method of claim 10 in which said step of determining in a sample of the liquid portion of the blood of the mammalian subject the existence of inducible nitric oxide synthase enzyme (iNOS) apart from the existence of eNOS or nNOS further comprises:

a. a capture monoclonal antibody bound to iNOS in the liquid portion of the blood of a mammalian subject; and

b. a labeled detector antibody bound to iNOS in the liquid portion of the blood of the mammalian subject.

14. The method of claim 13 in which said labeled detection antibody bound to said capture monoclonal antibody comprises a labeled detection monoclonal antibody.

15. The method of claim 14 in which said labeled detection antibody comprises a labeled detection anti-iNOS monoclonal antibody.

16. The method of claim 13 which additionally comprises a binding partner bound to said labeled detection antibody and further label bound to said binding partner.

17. The method of claim 16 in which said binding partner is selected from the group consisting essentially of:

an enzyme-conjugated strepavidin an enzyme conjugated avidin, an enzyme conjugated anti-biotin antibody, and an enzyme conjugated avidin-biotin complex.

18. The apparatus of claim 1 in which said means for detecting the existence of nitric oxide synthase enzyme (iNOS) in the liquid portion of the blood of the mammalian subject,

comprises:

a. an immobilized capture monoclonal antibody, binding to the iNOS; and

b. a labeled entity competitively binding to said immobilized capture monoclonal antibody selected from the group consisting essentially of:

a peptide, a peptide analogue, and a peptidomimetic.

19. The method of claim 10 in which said step of determining in a sample of the liquid portion of the blood of the mammalian subject the existence of inducible nitric oxide synthase enzyme, apart from the existence of eNOS or nNOS further includes:

a. an immobilized capture monoclonal antibody binding to the iNOS; and

b. a labeled entity competitively binding to said immobilized capture monoclonal antibody selected from the group consisting essentially of:

a peptide, a peptide analogue, and a peptidomimetic.

20. The apparatus of claim 18 in which said further label bound to said binding partner is selected from the group consisting essentially of:

an enzymes a luminescent compound, a luminescent generating enzyme, a fluorochrome, a fluorescent metal chelate, a colored compound, a color generating compound and a radioisotope.

21. The apparatus of claim 19 in which said label conjugated to said capture monoclonal antibody is selected from the group consisting essentially of:

an enzyme, a luminescent compound, a luminescent generating enzyme, a fluorochrome, a fluorescent metal chelate, a color compound, a color generating compound, and a radioisotope.

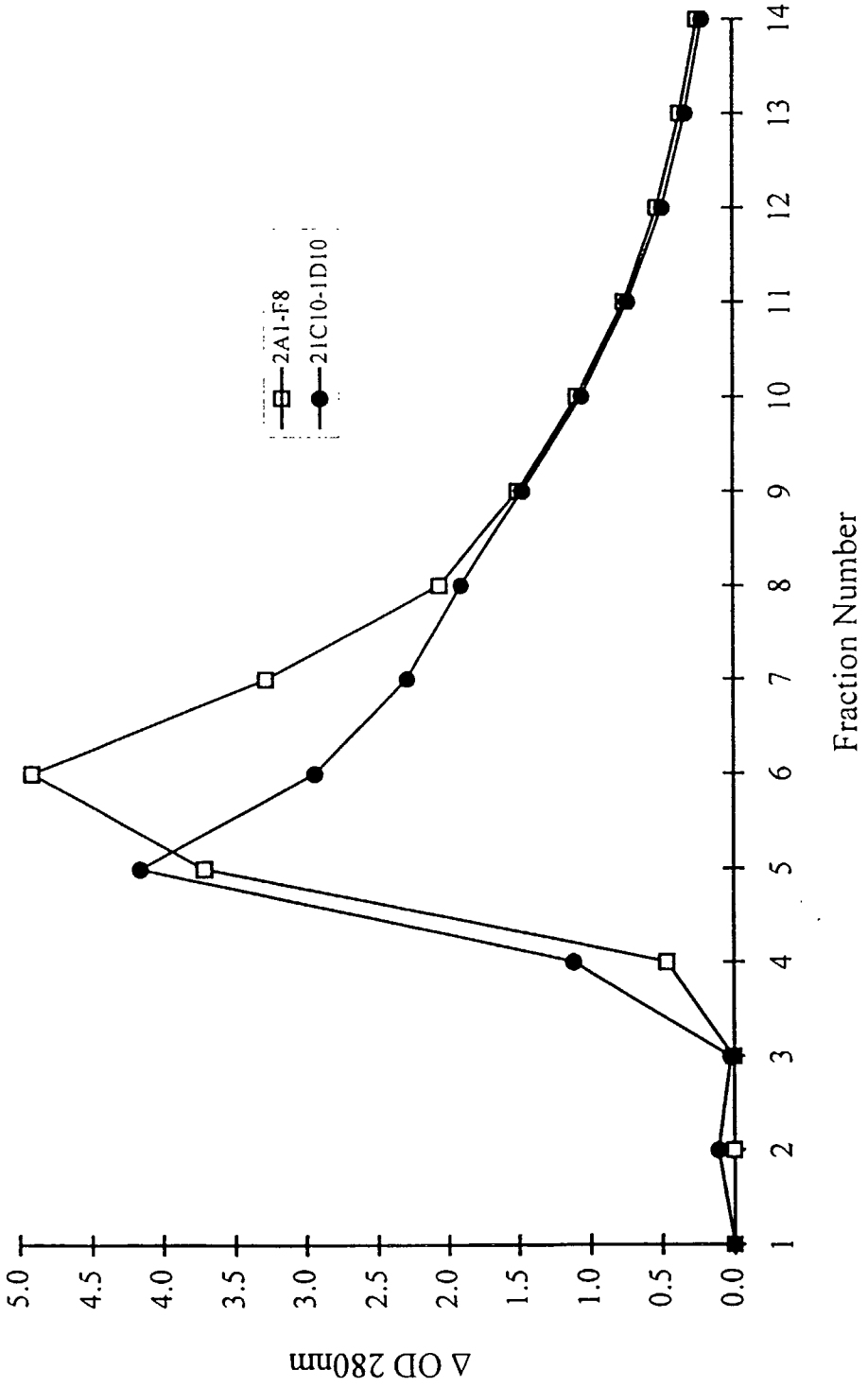
Δ OD 280nm
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Fraction Number
2A1-F8
2IC10-1D10

Fig. 1

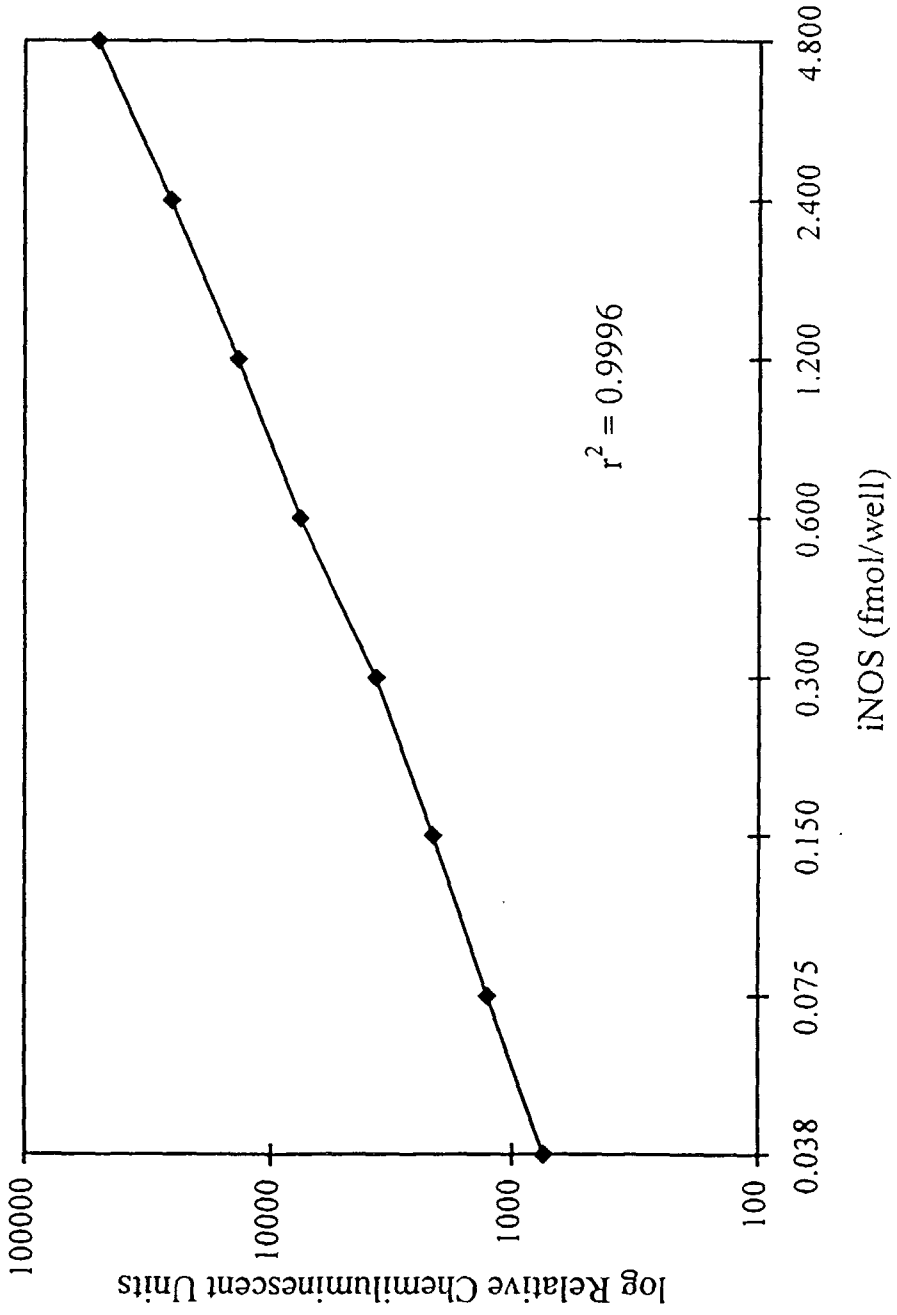
log Relative Chemiluminescent Units
100000
10000
1000
100
0.038
0.075
0.150
0.300
0.600
1.200
2.400
4.800
iNOS (fmol/well)
$r^2 = 0.9996$

Fig. 2

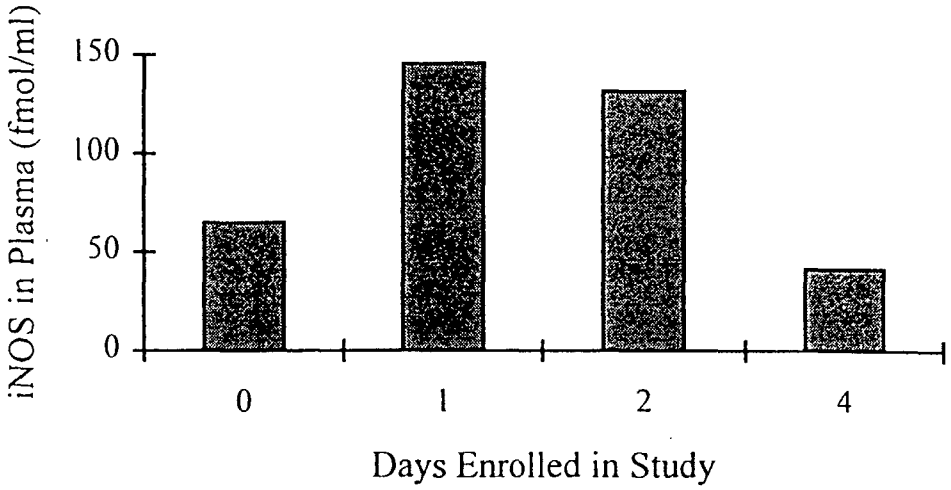
iNOS in Plasma (fmol/ml)
150
100
50
0
0
1
2
4
Days Enrolled in Study

Fig. 3A

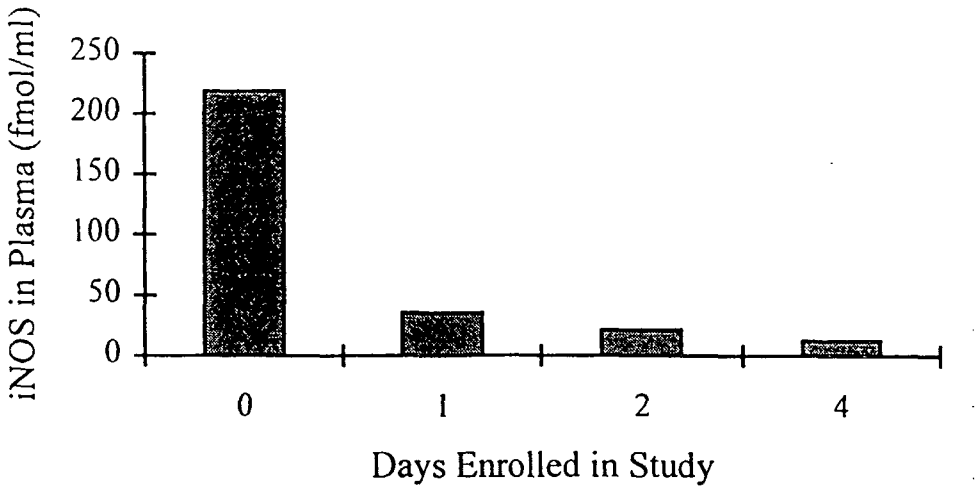
iNOS in Plasma (fmol/ml)
250
200
150
100
50
0
0
1
2
4
Days Enrolled in Study

Fig. 3B

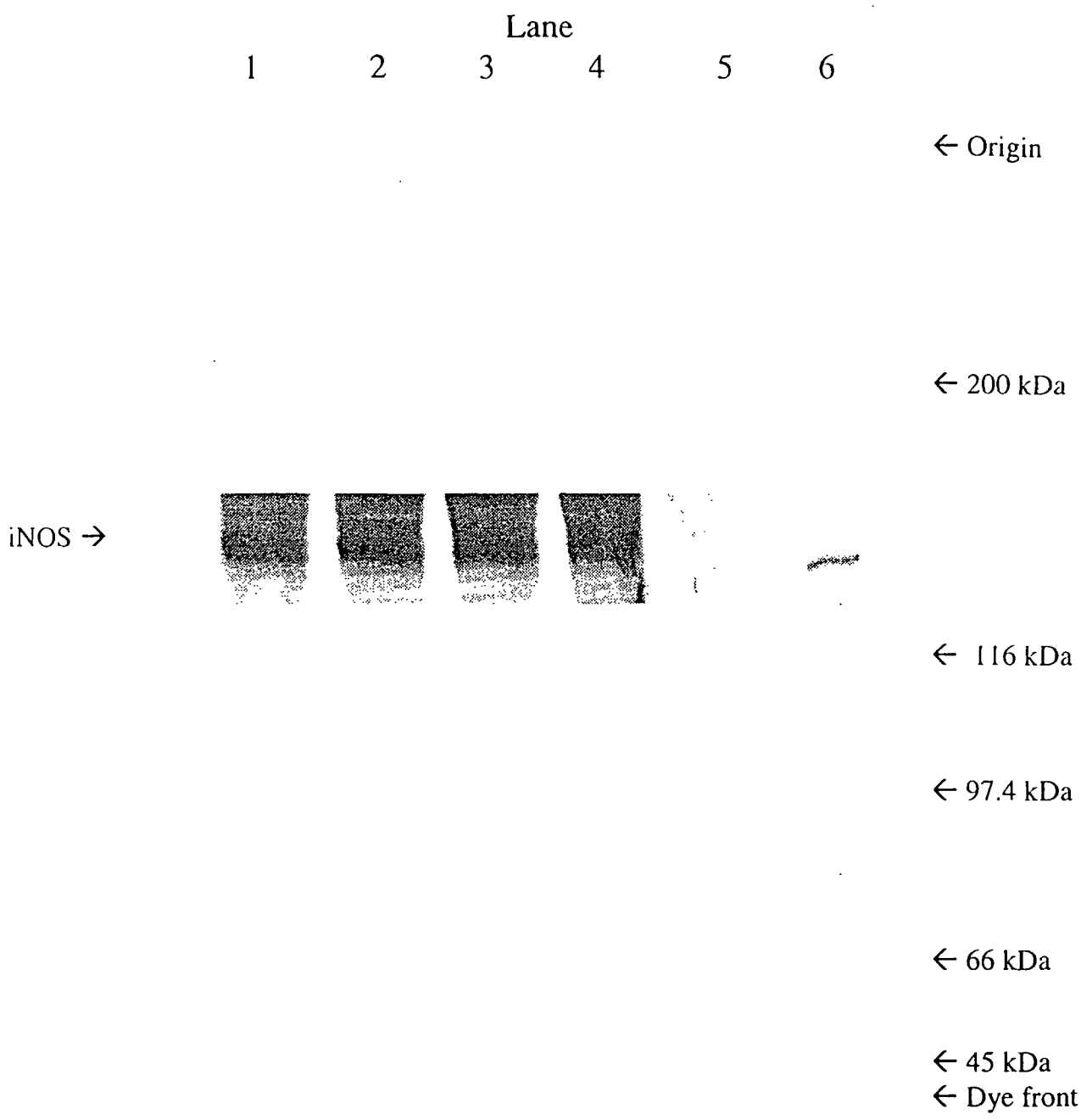
Lane
1 2 3 4 5 6
← Origin
← 200 kDa
iNOS →
← 116 kDa
← 97.4 kDa
← 66 kDa
← 45 kDa
← Dye front

Fig. 4

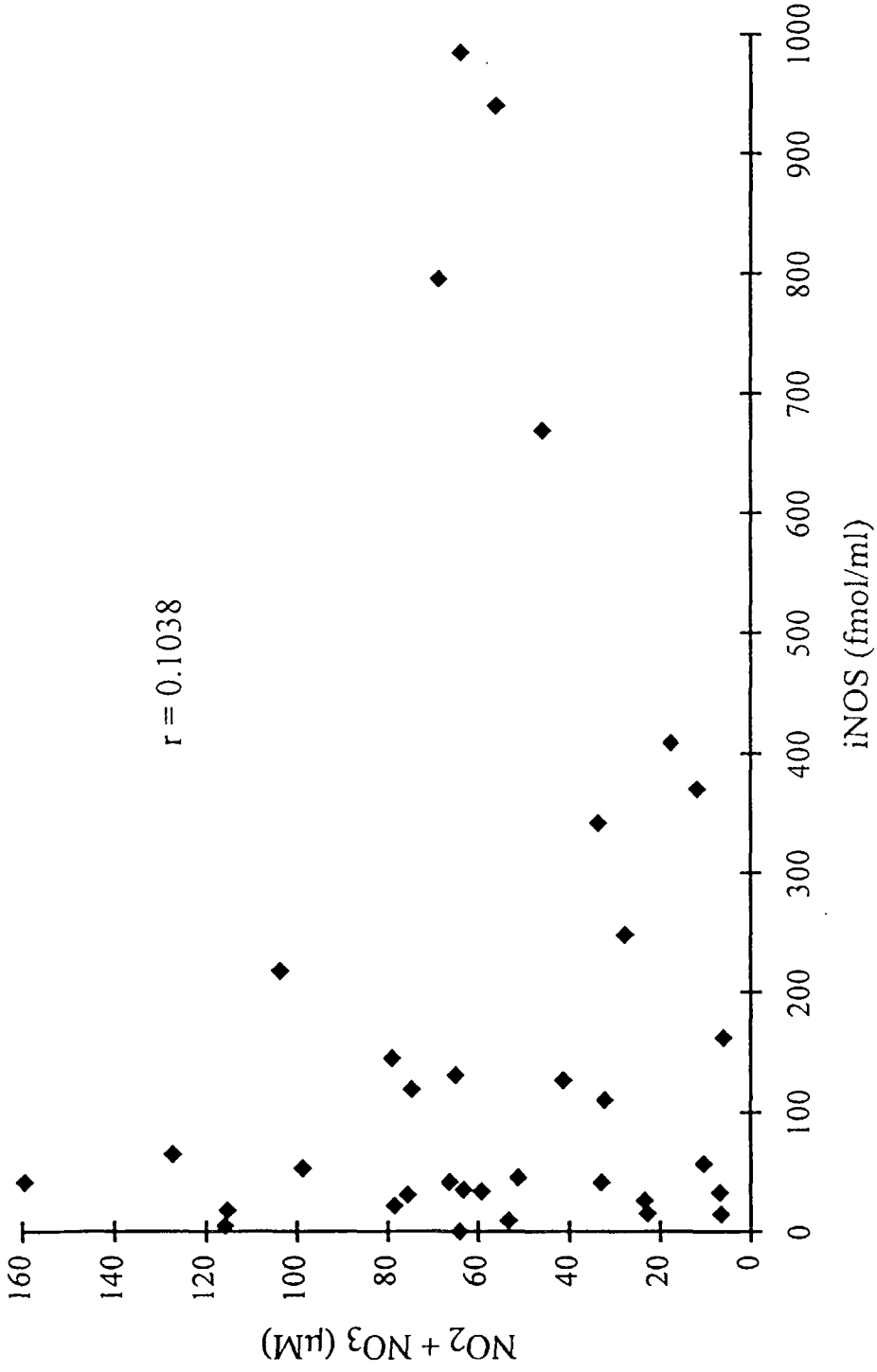
r = 0.1038
iNOS (fmol/ml)
0
100
200
300
400
500
600
700
800
900
1000
NO2 + NO3 (μM)
0
20
40
60
80
100
120
140
160

Fig. 5

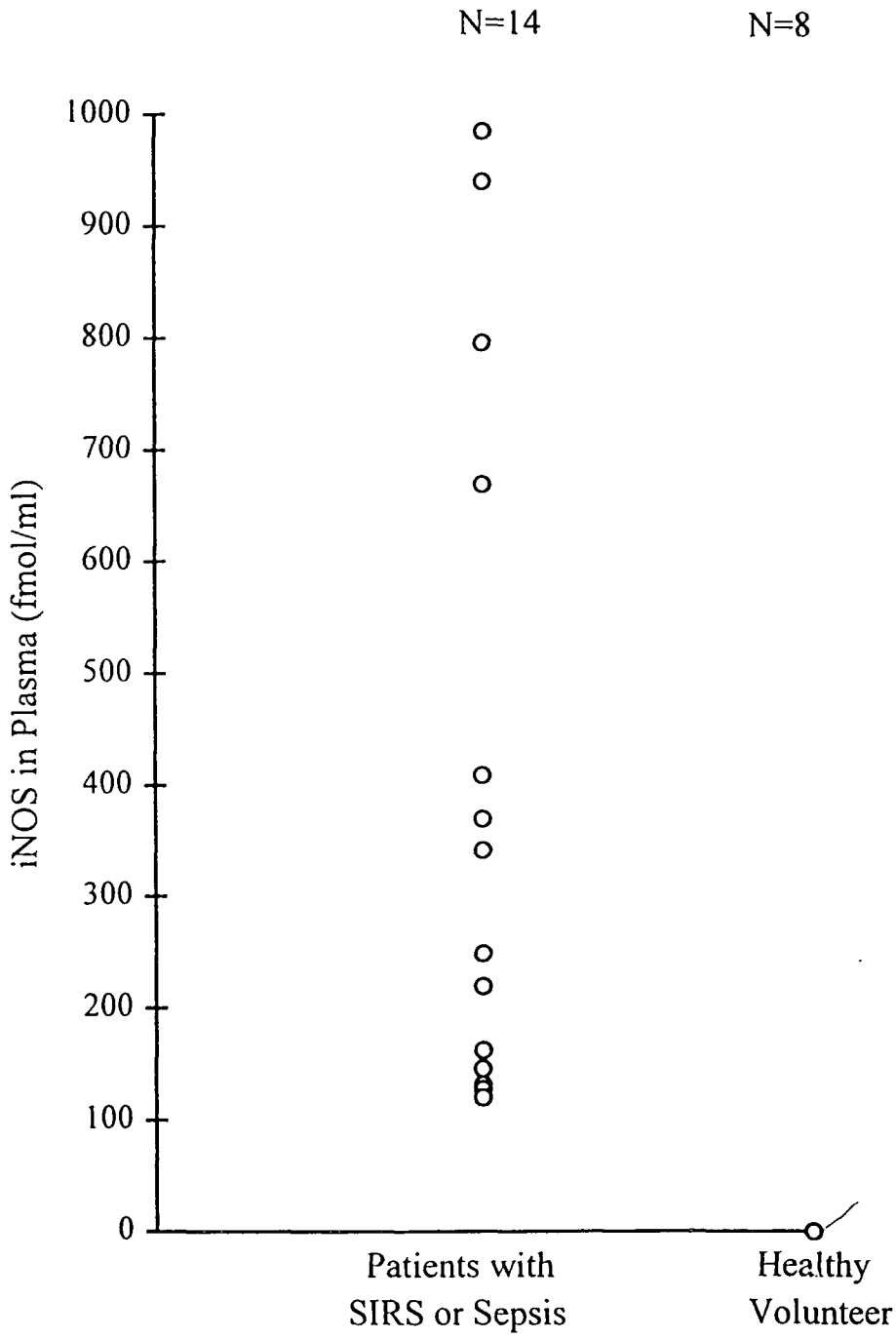
N=14
N=8
1000
900
800
700
600
500
400
300
200
100
0
iNOS in Plasma (fmol/ml)
Patients with SIRS or Sepsis
Healthy Volunteer

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/23943

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12M 1/00
US CL : 435/283.1

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. : 435/283.1; 435/4

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/39858 A1 (MERCK AND CO., INC.) 19 December 1996, see entire document.	1-21
X	WO 98/45710 A1 (WEBBER) 15 October 1998, see the entire document.	1-7, 9-16, 18-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent 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 citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2001 (30.09.2001)	31 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Vanessa Ford Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/23943

Continuation of B. FIELDS SEARCHED Item 3: MEDLINE, STN: BIOSIS, CABA, CAPLUS, EMBASE, WPIDS, JICST□search terms nitric oxide synthase, immunoassay, antibody, monoclonal, polyclonal,

专利名称(译)	用于确定败血症状况的发作和存在的装置和方法		
公开(公告)号	EP1412471A1	公开(公告)日	2004-04-28
申请号	EP2001959345	申请日	2001-07-31
[标]申请(专利权)人(译)	罗伯特·韦伯		
申请(专利权)人(译)	韦伯，ROBERT		
当前申请(专利权)人(译)	韦伯，ROBERT		
[标]发明人	WEBBER ROBERT		
发明人	WEBBER, ROBERT		
IPC分类号	C07K16/40 G01N33/53 C12M1/34 C12Q1/04 C12Q1/26 G01N21/76 G01N21/78 G01N33/543 G01N33/573 G01N33/577 C12M1/00		
CPC分类号	C07K16/40 G01N33/573 G01N2333/90245		
优先权	09/628585 2000-07-31 US		
其他公开文献	EP1412471B1 EP1412471A4		
外部链接	Espacenet		

摘要(译)

一种利用测定法从哺乳动物受试者的血液的液体部分确定败血症的发作和存在的装置和方法。该测定法包括用于确定哺乳动物受试者血液的液体部分中iNOS的存在的化学检测器，而不检测eNOS或nNOS。