



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/546, 33/559, 33/564	A1	(11) International Publication Number: WO 93/19369 (43) International Publication Date: 30 September 1993 (30.09.93)
(21) International Application Number: PCT/US93/02901 (22) International Filing Date: 26 March 1993 (26.03.93) (30) Priority data: 07/857,764 26 March 1992 (26.03.92) US 07/857,765 26 March 1992 (26.03.92) US 08/014,549 8 February 1993 (08.02.93) US (71) Applicant: MONTEFIORE MEDICAL CENTER [US/ US]; 111 East 210th Street, Bronx, NY 10467 (US). (72) Inventor: SINGER, Jacques ; 11 Lewis Place, New Ro- chelle, NY 10804 (US). (74) Agent: COSTIGAN, James, V.; Hedman, Gibson & Costi- gan, 1185 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: TECHNIQUE FOR PREVENTION OF FALSE REACTIONS IN IMMUNOLOGICAL TESTING (57) Abstract A novel technique is disclosed for the prevention of false positive reactions in immunological testing which are caused by interference of C ₁ and C _{1q} . The method is based on heating a sample of a body fluid at a temperature of 59-64 °C in the presence of a particular neutral salt. A method for screening for and inactivation of rheumatoid factor is also disclosed.		

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TECHNIQUE FOR PREVENTION OF FALSE
REACTIONS IN IMMUNOLOGICAL TESTING

5 Throughout the specification and claims of this
application words and terms will be used which have the
following meanings:

Particle Reagent

10 A direct inhibitor of agglutination in tests
utilizing latex particles, charcoal particles, bentonite
particles when the antigen or antibody are bound to the
surface of particulate carrier by physical absorption or
covalent chemically bound to measure antibody or antigen
in human or animal fluids (blood, spinal fluid, CSF,
ascitic, urine, etc.). Manual and instrumental kits
15 being available for this technique.

Latex Fixation Test

An agglutination reaction in which latex
particles are used to passively adsorb soluble protein
and polysaccharide antigen.

20 Immunofluorescent

A technique for the detection and localization
of antigens in which specific antibody is conjugated with
fluorescent compounds, resulting in a sensitive tracer
that can be detected by fluorometric measurement.

25 Immunoassay

A ligand binding assay which employs a specific
antigen or antibody capable of binding to the analyte.
Analytes are the substance, set of substances or factors
to be assayed.

30 Radioimmunoassay

A variety of immunologic techniques in which a
radioisotope is used to detect antigens or antibodies in
some form of a immunoassay.

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Ouchterlony Double Diffusion

An immunoprecipitation technique in which antigens and antibodies are allowed to diffuse toward each other and form immunocomplexes in agar.

ELISA

5 ELISA enzyme-linked adsorbent when one immunoreagent can be immobilized on the carrier surface while retaining its activity and the reciprocal immunoreagent can be linked to an enzyme in such a manner that both the enzymatic reactivity and the immunoreactivity of this conjugate are retained.

Capillary Enzyme Immunoassay

10 Immunoassay reaction occur within glass capillary tubes, polyvinylchloride tubes and between small glass or clear plastic plates.

Particle Capture Assay (Enzyme Based Color Generating Immunoassay)

15 Latex particles are captured on filters, either by physical entrapment or by chemical adhesion to the filter fibers or capturing latex particles in suspension.

Dot Immunobinding Methodology

20 In this assay the capture reagent is bound to a microporous membrane and the specimen containing the analyte is allowed to flow over and around the capture reagent conjugate binding and color development in the DIB assay occurs as described for particle capture assay (immunofiltration assay).

25 Co-agglutination Test

Protein A rich cells of Staphylococcus aureus bind immunoglobulins from many animal cells, and these antibody coated cell are use in agglutination reactions.

Complement Fixation

30 A standard serologic assay used for the detection of an antigen-antibody reaction in which

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complement is fixed as a result of the formation of an immune complex.

Antiglobulin Test (Coombs Test)

5 A technique for detecting cell bound immunoglobulin.

Immunoglobulin

10 A glycoprotein composed of (heavy) H and (light) L chain that function as antibodies immunoglobulin class. A subdivision of immunoglobulin molecule based on unique antigenic determinant in the Fc region of the heavy chain H chains. In humans these are 5 classes of immunoglobulin designated IgG, IgA, IgM, IgD and IgE. IgG is the predominant immunoglobulin class present in serum.

15 IgM

A pentameric immunoglobulin comprising approximately 10% of normal human serum immunoglobulin with a molecular weight of 900,000 and a sedimentation coefficient of 19S(Svedberg units).

20 Fab

An antigen binding fragment produced by enzymatic digestion of an IgG molecule with papain.

F(ab')₂

25 A fragment obtained by pepsin digestion of immunoglobulin molecule. It contains antigen antibody activity. An F(ab')₂ fragment and an Fc fragment comprise an active monomeric immunoglobulin molecule.

Fc Fragment

30 A crystalizable fragment obtained by papain digestion of IgG molecule it contains antigen binding capacity.

Attenuation

When a sera sample shows reaction in a complement-fixation test but no reaction in an agar gel

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diffusion test is not detected after 24 hours but is detected after 48 hours.

BACKGROUND OF THE INVENTION

5 The diagnosis of certain diseases caused by pathogens such as bacteria or viruses is based in part of the detection of changes in the body's immune system. These changes are manifested by the detection of antibodies which are produced by the body in response to
10 the protein-glycoprotein-polysaccharide constituents of the bacteria, viruses and parasites.

 It is well known that when an antibody molecule is combined with a variety of specific antigenic determinants, an antibody-antigen reaction takes place.
15 Specificity refers to the degree to which the antibody recognition of its antigen is unique. Many body fluids contain factors, largely undefined, which gave false positive or false negative results in an antigen-antibody test system.

20 The term antibody is commonly used to describe the substance gamma globulin which is present in serum, spinal fluid and other fluids. Antibodies identified as IgG, IgA, IgM, IgD and IgE are complex proteins or glycoprotein molecules. They are elaborated by the
25 immune system in response to antigenic stimulation. Most known antigens are multivalent in that they have more than one combining site (or antigenic determinants) to which antibody may be bound.

 The precipitin reaction is basic to immunology
30 and immunochemistry because it permits the detection and quantitation of antigen and antibody in solution, in tissues, in individual cells or in region of cells.

 Techniques used for detection and quantitation of antigen or antibody include radioimmunoassay, enzyme
35 linked immunosorbent assay (ELISA), precipitin in tubes

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or by turbidimetry, light scattering, fluorescence immunoassay, precipitation on nylon or on paper membranes dot or blot assay, chromatography, neutrophile and platelet assays, acquired inhibition to clotting proteins, detection of antibody to tissue specific antigen and HLA typing, antiglobulin tests, immunodiffusion (double Ouchterlony, counterimmunoelectrophoresis, immunoelectrophoresis), complement fixation, DNA Probeimmunology and the like. Agglutination techniques are based on the use of particles (latex, charcoal, bentonite, sheep cell, etc.); on slides, in test tubes, and/or on paper or utilizing instrument particle enhanced immunoassay; light scattering assay, particle counting assay, turbidometric assay and spectroscopic assay, Petinia assay, PACIA assay, Quels instrumentation assay, and the like.

Most of these assays are for measuring antigen antibody reactions for bacterial, viral, parasitic and fungal antigen and antibodies, immunoglobulins, hormones, drugs, tumor antigen and serum proteins.

False positive results may be caused by the presence in all sera of C_{1q} which is a subcomponent of the C_1 complex. False positive results may also be caused by the presence in a large number of sera of rheumatoid factors (RF). False negative results may be caused by the presence of the undissociated C_1 complex.

The first component of the complement C_1 is largely dissociated under physiological conditions. The C_1 complement is composed of three subcomponents known as C_{1q} ; C_{1r} ; and C_{1s} which are held together in a calcium dependent complex. C_1 when diluted with glycine buffer, pH 8.2 and/or when treated with EDTA-Na will dissociate into the three components (C_{1q} , C_{1r} and C_{1s}). In the prior art, the C_{1q} component was considered to be a thermolabile substance that could be inactivated by heat at 56°C for

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30 minutes. The present inventor has discovered that C_1 and C_{1q} are only attenuated and not inactivated by heating a serum sample to 56°C for 30 minutes. It was known in the prior art that C_{1q} will react with gamma globulin in solution to agglutinate uncoated latex particles or latex particles coated with gamma globulin. C_{1q} has binding sites for IgG and IgM and initiates the activation of the classical pathway cascade. The binding sites for C_{1q} are located on the Fc portion of IgG and IgM. These binding sites are made up of known sequences of amino acids and the binding sites are sensitive to high ionic strength salts which is suggestive of electrostatic interaction. The binding between C_{1q} and immunoglobulin is known to be inhibited by inorganic ions and organic ions in concentrations of 100-300mM.

The following is the role of C_{1q} in the body:

1. Interacts with immunoglobulin through the Fc fragment.
2. C_{1q} forms an ionic bond and links many antibodies to their antigenic surface.
3. It stabilizes antigen antibody complexes.
4. It acts as a receptor for macrophages. It is through these receptors that AgAb complexes are phagocytized.

These components interfere in all immunoassay procedures by one or more of the following reactions: nonspecific agglutination of particulate carriers (latex, sheep cells, bentonite, charcoal); by interacting nonspecifically with endogenous immunoglobulin or with immunoglobulins of the antiserum added as a test reagent; by the inhibition or the amplification of many serological reactions; and by generating grossly abnormal analyte level.

C_{1q} in human sera is capable of binding to antigen antibody complexes and to immunoglobulins such a

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IgG and IgM. The normal concentration of C_{1q} is 70 mcg./ml. It will also bind to aggregated gamma globulin, will precipitate gamma globulin from solution as well as in agar gel systems. C_{1q} binds to bacteria, bacterial lipopolysaccharide, dextran, heparin, polyinosinic acid
5 carrageenan, DNA, C reactive protein, fibronectin, mitochondria, platelets, lymphocytes, monocytes, null cells, monosodium urate crystals, cytoskeletal filaments hybrid monoclonal mouse antibodies. C_{1q} will bind or
10 adsorb on glass, paper, plastic, etc.

Specific examples of particles which will bind C_{1q} include synthetic polymeric particles of various chemical composition such as polystyrene, polyvinyl toluene, styrenebutadiene, styrene-acrylic acid and the
15 like. The particles will react with C_{1q} alone or when coated with anyone of the following: immunoglobulin, aggregated immunoglobulin, antigens, specific antibodies, sheep cell sensitized with coated anti-rabbit sheep cell antiserum.

When C_{1q} is used as an exogenous reagent, to
20 detect and quantitate antigen-antibody complexes which are present in certain diseases such as lupus erythematosus, rheumatoid arthritis, lyme arthritis, acute hepatitis, etc erroneous results may occur
25 because of interference with the endogeneous C_{1q} and endogeneous immunoglobulin. Examples of the tests in which C_{1q} is added are C_{1q} binding assay, C_{1q} solid phase assay, C_{1q} inhibitory assay, C_{1q} enzyme linked immunosorbent assay (ELISA), C_{1q} AgAb assay (ELISA), C_{1q}
30 cocktail assay, (C_{1q} bovine conglutin in and low affinity rabbit IgM antihuman Fc fragments) radioimmunoassay, $^{125}\text{I}C_{1q}$ PEG precipitation test.

It will be shown herein that C_1 and C_{1q} under certain conditions can cause disaggregation or

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solubilization of AgAb of heat aggregation of gamma globulin.

The classic methodology for inactivation of complement involves heating samples to 56°C for 30 minutes. Repeated freezing and thawing of sera may cause aggregation of gamma globulin. The aggregated gamma globulin will bind C_{1q} thereby decreasing the activity of the complement. This property of the aggregated gamma globulin is called the anticomplementary property.

Heating serum at 56°C for 30 minutes has been shown to be ineffective as a procedure for the inactivation of C_{1q}. The present inventor has discovered that only when the temperature is raised to 59-64°C for about 3 to 30 minutes in the presence of organic or inorganic ions, the C₁ and C_{1q} is inactivated. The higher the temperature the shorter will be the required heating time.

It has also been discovered that heating a serum sample at 59-64°C for about 3-30 minutes prevents the interference of the complement in immunoassays even though heating at 56°C for 30 minutes reversibly attenuates the complement.

The effect of heat at temperatures of 56°C; 59°C; 61°C and 64°C was demonstrated by heating HGG in buffer #2 at a concentration of 18 mg/ml for various times. The degree of aggregation was determined in a photometer by reading the optical density of different samples at 5 minute intervals depending on the amount of aggregation obtained. The results are reported in Table I which shows that as the temperature is increased from 56°C to 64°C over a period of time the amount of aggregation increases by a factor of about forty. It is unobvious to use a temperature of more than 56°C to treat any test sample containing IgG because it has been demonstrated that higher temperature cause self-

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aggregation of IgG and would interfere in a photometric assay. This is evidence that it is unobvious to use a temperature range of more than 56°C for treating a sample before subjecting it to an immunoassay. It is only when a neutral salt is added to a sample of body fluid that it is possible to utilize the temperature range of 59°-64°C to unactivate the complement without causing increased self-aggregation of IgG.

Table I

	<u>56°C</u>	<u>59°C</u>	<u>61°C</u>	<u>64°C</u>
<u>Minutes</u>	<u>OD</u>	<u>OD</u>	<u>OD</u>	<u>OD</u>
5	0.0362	0.0038	0.0458	0.0962
10			0.0969	0.8359
15	0.0362	0.0605	0.124	1.15
20		0.0706	0.2219	
30	0.0555	0.1248	0.5229	
60		0.3979	0.8539	

When antibodies are attached to the surface of a solid state immuno test, they will aggregate on the surface and will interfere with antigen-antibody reactions. A further example of complement interference is when normal sera are added to a solid state immunoassay as a blocking agent. The normal sera contains C_1 and C_{1q} which will bind non-specifically to AgAb complexes or it may dissociate and solubilize AgAb complexes in the test sample.

C_{1q} will compete for binding sites with collagen and will bind to the platelets causing adhesion, aggregation and serotonin release.

The practical problem which results from the interference of C_{1q} is the false negative results when the presence of antibodies is masked or an increase or decrease in the quantitation of the antigen or antibody reaction causing an alteration of assay sensitivity or accuracy.

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A large number of methods have been used in the prior art to eliminate C_{1q} in immunoassay procedure. These methods include:

- 5 1. Use of antisera to C_{1q} which is intended to neutralize exogenous and endogenous C_{1q} .
2. Use of dilution of serum to minimize the amount of endogenous C_{1q} .
3. Inactivation of C_{1q} by heating the sample to 56°C.
- 10 4. Heating sample containing C_{1q} to 56°C in the presence of 0.1M EDTA Na.
5. Use of control solid adsorbent with non-immune antibodies.
6. Use of aggregated gamma globulin to trap and bind the endogenous C_{1q} .
- 15 7. In the latex test, the gamma globulin to be adsorbed on the surface of latex is replaced with a fragment of the same antibody $F(ab')_2$ fragment for coating latex particle. C_{1q} binds to Fc fragment of immunoglobulin but not to the $F(ab')_2$ fragment.
- 20 8. To eliminate nonspecific agglutinator for latex, CSF is boiled at 100°C for five minutes or urine is heated at 100°C for 3-5 minutes. Following heat treatment urine or CSF are centrifuged and the supernatants are tested.
- 25 9. In a test for antigen antibody complexes, a euglobulin fraction is used which is obtained from ascitic mouse fluid.
- None of these methods have completely prevented the interference that is caused by C_1 or C_{1q} .
- 30 It was known in the prior art that certain chemical compounds could inhibit the reaction of C_{1q} and immunoglobulin. It was surprising and unexpected that the chemical compounds such as the inorganic salts which were previously known as inhibitors of the C_{1q} - IgG or C_1
- 35

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- IgM reactions were found by using the latex photometry technique to be activators of C_1 by unmasking the C_{1q}

Humoral antibodies are in gammaglobulin fraction of serum. Five classes of immunoglobulin are recognized IgG, IgA, IgM, IgD and IgE. Papain splits IgG molecule into three fragments - two called Fab (fragment binding to antigen), one called Fc (crystallized fragments). Fab fragment is associated with antibody specificity.

IgM consists of approximately 10% of normal immunoglobulin of about 900,000 and a sedimentation coefficient in the ultracentrifuge of 19S (Svedberg) units, while those of IgG has a sedimentation coefficient of 7S and 160,000 molecular weight. In normal human adults IgG constitutes approximately 75% of the total serum immunoglobulin. The concentration range in normal serum for IgG is 8-16 mg/ml and IgM is 0.5-2 mg/ml.

Ninety-eight percent of the IgM antibody is pre-eminent in the early immune responses to most bacterial and viral antigens which react with antigen through their Fab fragments. In the antibody response to natural blood group antibodies, the major immunoglobulin expressed on the surface of B cells is formed as a receptor in small T lymphocytes, as monoclonal cryoglobulin, or as mixed polyclonal cryoglobulins IgM-IgG, IgM-IgG-IgA, on the circulating immune complexes IgG-IgM, IgA-IgG-IgM.

Rheumatoid factors present in Rheumatoid arthritis sera represent only 1-2% of the IgM molecule. These antibodies (IgM) react with IgG immunoglobulin or aggregated gamma globulins are called RF. They primarily react with the Fc portion of the IgG but some of them also react with the Fab fragment of these immunoglobulins. Some other classes of immunoglobulin

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IgA, IgE and IgD have shown to be RF because of their reactivity with IgG. They also react with the Fc portion.

By means of the latex agglutination test IgM rheumatoid factor antibodies have been found to be present in 70-80% of patients with rheumatoid arthritis; in a 0.5-2% of normal population healthy control; and in 20-30% of sera of people over 65 years of age.

Rheumatoid factor is also present in percentage varying from 1 to 30% in sera of patients with liver disease, subacute bacterial endocarditis, viral disease, leprosy, tuberculosis, syphilis, cirrhosis of the liver, hepatitis, sarcoidosis, mixed cryoglobulinemia, malignant tumor, trypanosomiasis, in heroin addiction, and in other connective tissue diseases such as lupus erythematosus, Sjogren's syndrome, scleroderma and polymyositis. Rheumatoid factor also makes a transient appearance in patient after vaccination against various diseases.

It has been suggested that RF represents normal components of the immune network. The synthesis of RF regularly accompanies all primary immune responses and is usually transient.

Rheumatoid factor is recognized to interfere in all immunoassay procedures using particulate carriers, such as: latex, sheep cells, bentonite, and charcoal particles. In these procedure particles are coated physically or chemically with human and/or animal gamma globulin (immunoglobulins). These antibody coated particles are used in the detection of rheumatoid factors in sera and fluids. The gamma globulin in these tests are obtained commercially by chemical fractionation of human or animal sera. There are other serological tests in which latex particles are coated with antibody (gamma globulins) produced by immunization of animals with bacteria, viruses, fungi and parasites. These specific antibody coated latex particles will also agglutinate

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5 rheumatoid factors present in humans or animal sera. The majority of commercial kits with latex particles are used for detection of bacterial, viral, fungal and mycotic antigens and also for other antigens such as extracts of organs, for antibiotics, drugs, etc. RF will produce false positive reactions with coated antibodies when these kits are used.

10 In precipitation reaction, using tube precipitation techniques such as nephelometry, turbidity, radial radio immuno diffusion, a higher level of the analyte is obtained as a result of RF complexing with IgG. RF which reacts with antigen containing IgG can alter the concentration of IgG.

15 In many assay procedures polyethylene glycol is used to increase the sensitivity of the tests and can lead to self-aggregation of immunoglobulins. Complexes may be formed between RF and these immunoglobulins producing false positive results.

20 The antibody used in general immunoassay procedures for the detection or quantitation of analytes are either polyclonal or monoclonal. The polyclonal are prepared by in vivo immunization while the monoclonal antibody can be prepared in tissue cultures or by ascites formation in syngeneic mice. Monoclonal antibodies appear to have excellent affinity and specificity for antigens. They are commonly used in many immunoassay procedures such as in immunology, chemistry, hematology and in microbiology. Polyclonal antibody which are produced in animals in response to bacteria, fungi, parasites and viruses may react with RF. Rheumatoid factor is known to react with gamma globulins of rabbits, sheep, goats, horses, cows, as well as mice and produce false positive reactions.

35 Monoclonal antibodies produced in mice have been shown to react with rheumatoid factors. Patients

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receiving mouse antibody therapy mount an immune response against the Fab and Fc region of murine IgG. Preexisting antimouse immunoglobulin antibodies have been detected in sera of healthy individuals and patients with various disease.

The incidence of RF antimouse IgG reactivity has been estimated in one study at 9% of the normal population of blood donors.

In immunofluorescent techniques the antibody which are used are conjugated with fluorescent dyes and have been found to react with rheumatoid factors. This reaction has been seen frequently in serological tests for syphilis, rubella, toxoplasmosis and other infectious diseases. Both the direct and indirect immunofluorescent assays may give false positive results because of the presence of RF.

In ELISA technique, the antibody which is added for the detection of the ligand, may crosslink with RF and produce a false positive result because of an elevated response signal. Similar false positive results are obtained in most other immunoassays included radioimmunoassays.

The purpose of all immunoassay procedures is to detect the reaction between antigen and antibody through the formation of antigen and antibody complexes. RF can erroneously enhance immune complex formation; it is known that the complement can solubilize antigen antibody complexes by preventing the formation of large, insoluble lattices, or it may enhance the immune complex formation. RF has multiple binding activity for the Fc portion of immunoglobulin and the complement may bind also to the same Fc fragment. Through steric hindrance RF may interfere with the binding of complement in these assays.

The competitive binding of RF in these immunoassays, will enhance precipitation and trapping of

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labeled ligand. This will produce a shift in equilibrium of assays reduce primary antibody reactions, alter assay sensitivity, falsely elevate binding and cause an overestimation of the quantitative results.

5 Cellular assays that employ human or animal antibodies and complement are susceptible to RF interference. Human sera used in microcytotoxicity assays (e.g. HLA antigen typing) must be screened for RF to minimize the possibility that false positive cell
10 killing will occur as a result of RF enhanced activation of complement.

If an autoantibody in a patients serum binds to the cell surface molecule and RF is present, it can cross-link the auto antibody with a labeled detection
15 antibody.

The non competitive, two site, solid phase immunoassay appears to be most susceptible to RF interference because it uses two antibodies, especially when human immunoglobulin are being measured. RF
20 interference is especially problematic in assays used to assess human sera for the presence of autoantibodies specific to selective tissue (e.g. skin, thyroid) when these patients also have circulating RF.

It is impossible to predict which serum will contain a RF that will bind to antibody in a particular
25 assay. In general RF can form stable complexes with IgG if either reactant is in a high enough concentration, or if these reactants produce sufficient multiple sites of reactivity which shift the equilibrium from unbound reactant toward a stable product (e.g. due to IgG
30 aggregation and/or immune complex formation and IgM RF multivalency. The subcomponent $C1_q$ of the immunomolecular complex of the complement C1 binds to IgG, IgM and to antigen antibody complexes. Rheumatoid factor binds also
35 to the same immune globulin and complexes. Binding sites

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are located on the same Fc portion of the immunoglobulin. Cl_q is present in all human and animal sera. Both of these compete with one another, and both of them produce false positive reactions in all immunoassay procedures.

5 Rheumatoid factor interference in immunological assays has been extensively reviewed by R.G. Hamilton, In Shakil, Fed. Autoantibody to immunoglobulin. Monograph Allergy Basel, Kasger 1589, 26, pp. 27-44 and in Antigen Detection to Diagnosis Bacterial Infection Vol. I
10 Methodology and Vol. II Application Ed. R.B. Kohler CRC Press 1986, Boca Raton, Ann Arbor and Boston.

The methodology which has been suggested for the elimination of RF interference in immunoassay in the prior art can be summarized as follows:

15 1. Avoidance, by prescreening sera for RF. Analysis of each serum for RF is cumbersome and expensive. If a test is positive, the problem still remains how to eliminate this interference.

20 2. Using particle carriers such as latex, bentonite or charcoal particles, the test can be performed. Two latex particles are used one with specific antibody for the antigen to be detected and another latex particle coated with nonspecific antibody from the relevant animal species.

25 3. Latex particles sensitized with human gamma globulin are used for adsorbing serum specimens for RF. This procedure may also inhibit the AgAb reaction, IgG added to the latex particles may elute from the particles and inhibit the AgAb reaction (crossreacting or
30 sterically hindrance). IgG may be eluted from the surface of latex particles which have gamma globulins covalently bound to the surface.

4. Removing RF with aggregated gamma globulin, will inhibit the AgAb reaction, aggregation cannot be

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controlled and those aggregates will bind nonspecifically also C_1 and C_{1q} .

5. Preaddition of antihuman IgM and Ig to the sera, may produce AgAb complexes which may precipitate the antibody in the test system.

6. Antibodies are produced for use in the immunoassay procedures which are obtained from a species that is immunologically distinct from human species (for example, chicken gamma globulin does not react with RF).

7. Use of a Fab or $F(ab^1)_2$ fragments of antibody in which the binding of RF to IgG Fc fragment is eliminated. In this method, Fab fragments are difficult to manufacture. Many normal sera as well as RF sera contain antibodies to these Fab fragments.

Immunocomplexes have been isolated in normal sera that contain both anti Fc and anti Fab antibody activity. This will neutralize the $F(ab^u)_2$ fragments used in the immunoassay.

8. Heating sera at 56°C or 62°C may produce aggregation in this sera which may then interact with RF and also with C_1 and C_{1q} and not eliminate the interference. Heating sera at more than 60°C up to 100°C may coagulate the serum and when this is centrifuged supernatant may be tested for antigen.

9. Inactivation of RF by heat in combination with dithiothreitol or betamercaptoethanol has not been fully successful in eliminating RF. These methods are generally uncontrollable and can interfere with the detection of antibody.

10. A preincubation with proteolytic enzyme (Pronase-CB), pepsin or trypsin.

11. Preabsorption of globulin with polymerized, pooled human plasma.

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12. Adding EDTA (1 part of serum and 3 parts of EDTA), boiling for 5 minutes, centrifuge, and test the supernatant.

5 13. Blocking tests may be used to detect nonspecific positive reactions. In this procedure, the positive test sample is retested after incubation with specific and nonspecific sera or immunoglobulin fractions.

10 14. A large number of blocking substances have been used in the tests for detection of antigen by an immunoassay procedure. The purpose of the blocker step is to fill any protein adsorbing sites on the solid phase which are not already occupied so as to prevent nonspecific adsorption of the solid phase and preventing
15 false positive results. In some of these procedures, bovine albumin, or normal human serum, or normal rabbit serum, or fetal calf serum are added to bring the C_1 and C_{1q} to the solid phase component. C_1 and C_{1q} crosslink immunoglobulins, producing AgAb complexes aggregating
20 AgAb or solubilizing AgAb complex, and thus produce false positive reactions.

25 15. Removal of RF. RF containing immunoglobulin fraction of serum can be separated from the remainder of the serum to permit their analysis in assays free from RF interference. In exchange or size exclusion chromatography IgM is separated from IgG to allow the separate analysis in an IgG or IgM assay. Mini exchange (e.g. di and quaternary aminoethyl Sephadex) columns have been used. This approach may not be successful at all
30 times. A single step absorption with Protein A Sepharose and IgG saturated protein A-Sepharose may be used. In this procedure absorption would remove 50-90% of both IgM RF and total IgG.

35 It is apparent from all data that there are great difficulties in absorbing RF from human or animal

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sera. RF-IgM antibody cannot be selectively separated from IgM antibody.

RF are present in all five class of immunoglobulin IgM, IgA, IgG, IgD and IgE, but 90-98% in the IgM class.

It is well known that IgM antibodies are split into smaller units by breaking their disulfide bonds with sulphydryl reagents such as mercaptoethanol, dithioerythrol or cysteine. The disulfide bonds joining the two heavy chains of the IgG or of the IgM are reduced to - SH groups and these two halves of the IgG are no longer covalently bond. This procedure in immunology is also associated with heating sera at 56°C or 60°C. It is well recognized that heating will produce aggregation of immunoglobulin. RF binds strongly to aggregated gamma globulin and in the same time the C_{1q} component of the human serum will also bind to aggregate gamma globulin. Having binding sites near to another RF and C_{1q} on the Fc fragment of immunoglobulin in addition to be competitive they may also crosslinked one with another or with AgAb complexes formed or endogenously present in sera. It is therefore mandatory that for the elimination of RF, C_{1q} should be simultaneously eliminated or inactivated. agglutinator.

Therefore, it is a primary object of this invention to provide an improved method of eliminating false positive and false negative results that are caused by one or more components of the complement system C₁ and C_{1q}.

It is also a primary object of this invention to provide a novel technique for the elimination of both C_{1q} and RF in body fluid to be tested in immunoassays.

It is also an object to provide a method of neutralizing or inactivating the interference of the complement in immunoassay testing.

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It is also an object of the invention to provide novel test kits which include novel reagents for use in all immunoassay procedures.

5 It is also an object of the invention to provide a method for preventing the formation of aggregates of immunoglobulin in sera.

10 It is also an object of the invention to provide a method for the prevention of the aggregation of gamma globulin by heat at a temperature of 59-64°C in the presence of a neutral salt for a period of 3-30 minutes depending on the temperature.

15 It is also an object of the invention to provide a technique for the recognition of C_{1q} in sera by the use of heat at a temperature of 59-64°C in the presence of a neutral salt for a period of 3-30 minutes depending on the temperature.

20 It is also an object of the invention to provide a technique for the detection of rheumatoid factor in sera by the use of heat at a temperature of 59-64°C for an effective period of time in the presence of a neutral salt and latex coated with gamma globulin.

25 It is also an object of the invention to provide a technique for the quantitative measurement of rheumatoid factor.

30 It is also an object of the invention to provide a technique for the quantitative measurement of C_{1q} purified protein.

It is also an object of the invention to provide a method of inhibiting aggregated gamma globulin or antigen-antibody complexes that have not been denatural by heat.

It is also an object of the invention to provide a novel apparatus for the automated performance of immunoassays.

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These and other objectives of the invention will become apparent from a review of the appended claims.

5

SUMMARY OF INVENTION

The invention provides a method for the prevention of the interfering effects of C_{1q} in a body fluid.

10

The invention in its broadest sense comprises a method for the prevention of the interfering effect of C_1 and C_{1q} from a body fluid in immunoassays. The method comprises the steps of:

15

(a) adding an effective amount of a chemical compound to a diluted sample of a body fluid, said chemical compound being capable of inactivating the C_1 or C_{1q} in said body fluid when a mixture of said body fluid and said chemical compound are heated to a temperature of 59-64°C for a sufficient period of time to inactivate said C_1 or C_{1q} without affecting the reactivity of the antigen or antibody in said body fluid; and

20

(b) heating the diluted sample containing said chemical agent and said body fluid to a temperature of about 59°C to about 64°C for a period of time which is sufficient to prevent the interference of C_1 and C_{1q} in immunoassays.

25

When it is desired to inactivate C_{1q} as well as RF in a particular sample of a body fluid, the following steps comprise a preferred method:

30

(a) adding an effective amount of a chemical compound to a diluted sample of a body fluid, said chemical compound being capable of inactivating the C_1 or C_{1q} in said body fluid when a mixture of said body fluid and said chemical compound are heated to a temperature of

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59-62°C for a sufficient period of time to inactivate said C_1 or C_{1q} without affecting the reactivity of any antigen in said body fluid and effective amount of a chemical reagent of the formula R-SH wherein R is an organic moiety which will not affect the reactivity of any antigen in said body fluid to the mixture when said reagent of the formula R-SH is heated to a temperature of 59-62° for a sufficient period of time to inactivate RF; and

(b) heating the product of step (a) to a temperature of 59-62°C for a sufficient period of time to inactivate the C_{1q} and RF.

In a useful procedure for the practice of the invention, a 0.25ml of the sample which is to be tested in an immunoassays procedure, is diluted 1:10 in glycine buffer, pH 7.8-8.2, ionic strength 0.025-0.0125. Then, 20 μ l of a solution of a neutral salt (2M NaCl or 2M KCl or 0.1M EDTA-Na) is added to the diluted body fluid is incubated at 63°C for 3 minutes. If the sample is to assayed for RF, the preferred temperature is 60°C for 30 minutes.

The invention also includes the screening method which uses latex particles to determine if a sample of a body fluid, i.e. sera, synovial fluid, pleural fluid or ascitic fluid contains C_{1q} or RF using a sequence of steps which are based on the reactivity of C_{1q} and RF with latex particles and the use of heat in the presence of a suitable chemical compound, as herein defined, to inactivate C_{1q} . A screening method has also been devised for the demonstration of the presence of C_{1q} by the Ouchterlony technique has been devised.

The invention further includes the method for the detection and semiquantitative determination of RF in human sera and fluid by the use of heat and a suitable

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chemical compound which is used to prevent interference caused by C_{1q} to make the latex test specific for RF.

A technique has also been devised for the determination of the amount of purified C_{1q} using latex particles.

The invention also provides a novel apparatus for automatically carrying out latex agglutination tests without interference from C_{1q} or RF.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph which illustrates a plot from an X-Y recorder of a photometer which shows both a baseline (negative curve) of a negative turbidometric test and a positive curve (slope >0) of a positive turbidometric test. The dashed line marked "tangent to maximum" is the line drawn with a tangent meter to measure the maximum slope as shown by the superimposed right triangle marked "measurement of maximum slope".

FIG. 2 is a flow chart which shows the sequence of steps for the screening method of the invention for determining if a body fluid is RF positive or contains C_{1q} .

FIG. 3 is a schematic of an apparatus according to the invention.

FIG. 4 is a graphical representative of the test results of Example 12B.

DETAILED DESCRIPTION OF THE INVENTION

The term "body fluid" includes cerebrospinal fluid, synovial, ascitic fluid, pleural fluid, blood and the like, urine and serum. When body fluids are tested, the interfering effect of C_1 and C_{1q} in an immunoassay may be prevented by the methods set forth herein. The preferred detection method for use in demonstrating the

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operability of the invention is the photometric latex agglutination technique.

5 The photometric latex agglutination technique is a simple, rapid, qualitative and quantitative immunological assay. It can be performed in minutes with small quantities of reagents. The sensitivity can be adjusted over a wide range and it can quantitate as little as 10 nanograms of antigen and antibody. A photometer which may be used for latex agglutination is 10 a Model 500 (Chronolog Corp.) used for blood Aggregometry. The instrument is provided with temperature controls and the heater block is controlled to 37°C. It has a stirring motor with a stirring speed at 1200 rpm.

15 The reaction between the latex antigen and antibody mixture is performed in a single tube with continuous stirring and then measured by monochromatic light transmitted through the suspension which is undergoing continual flocculation. Flocculation is 20 measured by monitoring the changes in transmission as a function of time using a single channel recorder connected with the aggregometer. The difference in light transmission is expressed as a voltage and recorded as in function of time. The ensuing flocculation is evidenced 25 by a change in transmission (T) with time (t). The variation of % transmission with time is a measure of the rate of changes of particle size during flocculation. The increase in transmission is a result of the 0.8 μ size latex particle colliding during AgAg reaction and forming 30 larger and fewer aggregates of latex particle up to 5-6 μ in diameters. When it reaches the maximum size, the sigmoidal flocculation curve become asymptotic. The point in flocculation when the particle size changes most rapidly is represented by the slope of the percent 35 transmission curve. This slope is measured with a

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tangent meter within a few minutes after the latex suspension is added. In the use of negative fluid where no antigen antibody reaction takes place, the transmission versus the time will result in a straight line. The maximum slope value is detected in less than 3 minutes. To obtain quantitative information, the sera are diluted and each aliquot is run in the photometer. A family of curves for serum samples of different dilutions is then obtained and the slopes are calculated. A standard curve is generated by plotting the maximum slope response of the various dilutions of the fluid containing a certain amount of antigen or antibody. The curves for the unknown samples are compared to the standard curve and the quantitative estimation is based on the comparison.

A typical base line or negative curve which is actually a substantially straight line is shown by the horizontal dotted line and a positive "curve" is shown in FIG. 1. The dotted line is a line which is drawn on a tangent to the sigmoid shaped curve at a point on the curve where the slope is at a maximum as recorded by the X-Y plotter. The maximum slope is the region where the rate of change of the particle size of the flocculating particles is the greatest.

It is possible to predict from the standard curve the results for any dilution of a body fluid without testing the unknown body fluid at several dilutions.

The preferred chemical compounds for use in the practice of the invention include the neutral salts of the Hofmeister series, organic acid salts or diamino compounds. Any of these compounds may be used in the practice of the invention provided that when they are added to a 1:10 dilution of a sample of a body fluid in a glycine buffer pH 7.0-8.5, pH 8.2, and a ionic strength

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of 0.0125-0.025 μ at a concentration of 40mM to 200mM, and the sample is heated to a temperature of 59-64°C for a sufficient period time, e.g. from 3 to 30 minutes, the interference of C₁ or C_{1q} is prevented without affecting the reactivity of the antigen or antibody in said body fluid.

The following is the list of salts which have been shown to be promoters of the latex agglutination test. In addition to unmasking the C_{1q} these salts will neutralize the charged group of the C_{1q} and also serve to dissociate the C_{1q} complexes from immunoglobulin and AgAb complexes:

Table II

Inorganic ions

sodium bromide
sodium chloride
sodium iodide
sodium citrate
sodium cyanate
sodium perchlorate
sodium acetate

Organic ions

ethylenediamine sodium
ethylenediamine tetraacetic
acid tetrasodium
sodium propionate
sodium butyrate
1,4 diamino butane
Suramine
heparin

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Other neutral salts of the Hofmeister series which inactivate the C_1 or C_{1q} when heated to a temperature of 59-64°C for a sufficient period of time may also be utilized.

5 The neutral salts are strong electrolytes serving as a source of ionic strength. The charge of a neutral salt influences the macromolecular confirmation by weakening attractive or repulsive inter and intra-charge=charge interaction. Therefore, reaction with
10 charge residues, the reaction with dipolar groups such as peptide bond, amino, carboxyl, hydroxyl, primary and tertiary amide, it will alter the free energy difference between folded (associated) and unfolded (dissociated) form of the macromolecule neutral salt exercising
15 striking effects on association and dissociation equilibria. It should be noted that EDTA Na_4 is used herein for its action as a neutral salt and not for its chelating or complexing properties.

20 Large numbers of studies have dealt with the effect of neutral salts on a protein. Tandford, C. Physical Chemistry of Macromolecules, Wiley, N.Y.C., Chapter 8. Herskovitz, T.T. and Kelly, T. M. J. Physical Chemistry 77:381, 1973.

25 While the exact mechanism for the reaction is not known, it is believed that it is these neutral salts which are used in this invention function as an activator to unmask and release the C_{1q} component of the C_1 complex and of the serum. The same neutral salt will be used to dissociate C_{1q} from complexes with IgG, IgM and AgAb
30 complexes and finally will also be used by heat to inactivate the C_{1q} components.

35 Protein interaction is a complex process in which shape, electrostatic potential and hydrophobicity and hydrophilicity of the interacting molecules plays a role. Stability of IgG as well as of all proteins

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depends greatly on factors such as hydrogen bonding, hydrophobic bonding, van der Waals interaction, electrostatic forces of attraction and repulsion among charged surface and bound water. Therefore, the effect of salts on C_{1q} and immunoglobulins or on any protein will depend on the salt effect on these stabilizing factors.

Because of the many stabilizing factors which exist, the salts will affect the protein molecule through different mechanisms, P. H. Hippel and T. Schleich pp. 417-574 in Structure and Stability of Biological Molecule. Ed. S. N. Timasheff and G. D. Fishman, Marcel Decker, N.Y. 1969.

The inorganic and organic salts exert a structural perturbation on protein. They modify the environment of a macromolecule and thus affect the way they operate in aqueous solution through temperature, pH, compete hydrogen bonding, hydrophobic bond effectors. Neutral salts are defined as strong electrolytes which are significantly soluble in water without imparting major charge to in solution pH. The mechanism by which neutral salts or aliphatic acids act on proteins, in association and dissociation of large protein has been postulated. The first mechanism is through reaction with charged residue. Since the charges of the proteins are mostly located at the surface of the macromolecule they should not have a major effect on the residue located inside the macromolecule.

The second mechanism is the reaction with dipolar groups in the macromolecule such as the peptide bond, amino, carboxyl and hydroxyl groups, primary, secondary and tertiary amino groups, etc.

The third mechanism is the non-polar interaction. These are compounds which are partially by non-polar or hydrophobic in character.

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The fourth mechanism is organic salts are added to the protein, both intra and intermolecular bonds are broken, the side chains of the molecule are exposed and the water (or the solvent) alters its structure to make it thermodynamically hospitable for the newly exposed non-polar group.

It is recognized that heating sera at 53°C and higher will aggregate gamma globulin.

Since C_{1q} binds strongly or complexes with aggregated gamma globulin, the present invention provides the novel methodology which is based on the use of heat and an inorganic or organic anion to produce a change in the configuration of immunoglobulin and bind C_{1q} without producing a degree of aggregation which will irreversibly bind the C_{1q} to the aggregated immunoglobulin.

The mechanism by which heat in the presence of a neutral salt prevents the interference of C_{1q} in an immunoassay can be postulated as follows:

1. C_{1q} is released from the C_1 macromolecular complex; released from the binding site of C_{1q} with IgG, IgM, IgGIgM and IgGIgG complexes or from the C_{1n} inhibitor and other binding sites by:

- a. dilution of serum with low ionic strength buffers such as glycine, borate and phosphate buffers;

- b. addition of inorganic or organic neutral salts which affect the binding sites of C_{1q} with immunoglobulins or antigen-antibody complex or C_1 inhibitor;

- c. use of heat to produce an unfolding or a conformational change in the molecule of immunoglobulin for a greater capacity of fixing C_{1q} ; and

- d. prevention of heat aggregation of immunoglobulin by the solubilizing or inhibiting effect of C_1 and C_{1q} and a neutral salt.

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C_{1q} which is released in soluble form by heat in the presence of salt, is inactivated by heat and it is prevented from interfering in immunological reaction.

5 It appears that in the heating process hydrogen bonds are broken down between the oxygen atom of carboxylic acid groups and hydrogen atoms of amino groups. Heating at 60°C for longer periods of time causes protein molecules to unfold. The neutral salts which have electrostatically neutralized the charged
10 group of C_{1q} will inactivate the C_{1q} molecule. Alteration of the binding sites of C_{1q} will prevent C_{1q} from agglutinating or binding with immunoglobulin. False positive (nonspecific) results which are due to C_{1q} in immunoassay are thereby prevented.

15 The latex fixation test is positive for 75-80% of rheumatoid patients, and is also positive in 0-25% of patient with other diseases. Approximately 25% of all sera positive patients who have RA have a titer between 1:160-1:640. Singer and Plotz have suggested that a
20 titer of 1:80 or more should be considered positive. As stated in the proficiency testing for RF, by the Communicable Disease Center, many laboratories report as significant a titer higher than 1:80. It was suspected that the false positive tests were due to the presence of
25 C_1 or C_{1q} in sera. This is the reason for false positive results in which low titers cannot be defined for RF sera and probably the reason that only 80% of RA patients have a positive test. Furthermore, in many clinical situations C_{1q} may be increased in sera and cause a
30 positive test result for RF. The clinical significance of a positive test and different titers are confusing. Physician interpretation, in many instances, is made without knowledge of the interferences of C_{1a} or C_1 in serological testing.

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It is for this reason that this invention eliminates the C_{1q} interference and any titer which obtained in the latex gamma globulin slide, tube or nephelometric test would be only due to the presence of RF.

The prevention of the C_{1q} interference makes it possible to assign a meaningful titer to the RF test which can be correlated with clinical symptomology.

For the detection or quantitation of rheumatoid factor in the latex photometric technique, it is preferred to prepare a sample by adding a chemical compound as hereinabove described to a diluted body fluid and apply heat at a temperature of 60°C for a period of about 30 minutes although higher temperatures, i.e., 63°C for a shorter period of time, i.e., 3 minutes could be utilized. Thereafter, the prepared sample is tested for the presence of RF by contacting the sample with coated or uncoated latex particles.

Any agglutination of the latex particle may be rapidly detected visually or in a spectrophotometer. The optical density of the sample increases in direct relation to the amount of RF in the sample. After standardizing the spectrophotometer by using a reference standard sample of RF, the sample is placed in the spectrophotometer and if a flocculation curve is observed within three minutes, the sample is positive for RF. This method has the advantage of providing physicians with rapid, sensitive and reproducible test results which are obtained from a sample that has been treated to eliminate any C_1 or C_{1q} that might cause a false positive result. In addition, the fact that any interference due to the C_1 or C_{1q} has been prevented makes possible the use of an automated spectrophotometer with a microprocessor controlled heater which prevented any error caused by subjective reading of a slide or tube test and provides

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a permanent printed record. This provides quality assurance and a record for proficiency testing for regulatory authorities. It should be understood that the C₁ component and RF bind to the same sites on the IgG molecule and therefore it is of primary importance that the C₁ component be inactivated. This will eliminate the possibility of false negative results which would be caused by C₁ binding at the sites where RF must bind to form agglutinated IgG. This also reduces the sensitivity of the test.

If quantitative test data is required, the sample which has been qualitatively tested is serially diluted. Each serially diluted sample is tested in the spectrophotometer to obtain a curve until the point when a serially diluted sample does not give a curve. The titer is the dilution of the most dilute sample which yields a curve.

If quantitative data is required, a series of reference standard curves in a photometer are prepared using known amounts of purified RF or standardized rheumatoid factor serum which is free of C_{1q} and coated latex particles. The reference standard curves are compared to the curve for the test sample to determine the quantity of RF present in the sample.

The following latex immunoassays are examples of the tests which may utilize the present invention:

Bacterial Infection:

Brucella
Campylobacter jejuni
Cholera toxin
Clostridium difficile
Diphtheria
Escherichia Coli
Haemophilus influenzae, Type b
Klebsiella (six types)

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- Legionella
Leprosy
Leptospirosis
Mycobacterium tuberculosis
5 Neisseria gonorrhoeae
Neisseria meningitidis (dif. groups)
Pneumococcal infection
Pseudomonas
Proteus (three types)
10 Salmonella (serological typing)
Shigella (serological typing)
Staphylococcus aureus
Staph. a. clumping factor, Protein A
Staph. enterotoxin A to E
15 Streptococcal Grouping A,B,C,D,E,F,G,Q
Streptococcus, Group A
Group B. Streptococcal (GBS) Antigen
B-hemolytic Strep., Groups A,B,C,G
Streptococcus pneumoniae
20 Syphilis/Taws
Tetanus Toxoid
Tularemia
Whooping Cough
Yersinia pestis (plague)
25
- Mycological Infection:
Actinomyces viscosus
Aspergillus fischeri
Candida albicans
30 Coccidioides immitis
Cryptococcus neoformans
Farmer's Lung
Histoplasma capsulatum
Sporothrix schenkii
35

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Mycoplasma Infection:*Mycoplasma pneumoniae* (various species)Parasitic Infection:

- 5 *Amoebiasis* (*Entamoeba histolytica*)
 Canine Heartworm
 Chagas' disease
 Chlamydia
 Filariasis (elephantiasis)
10 *Hydatid disease* (*Echinococcus granulosus*)
 Kala-azar
 Malaria
 Toxoplasma gondii
 Trichinella spiralis
15 *Trichomonas vaginalis*
 Trypanosoma congolense

Rickettsial Infection:

- Rickettsia conorii*
20 *Rickettsia prowazekii*
 Rickettsia rickettsii
 Rickettsia typhi

Viral Infection:

- AIDS
25 Avian encephalomyelitis
 Cytomegalovirus (CMV)
 Enterovirus
 Hepatitis
 Herpes
30 Infectious Mononucleosis
 Measles
 Pseudorabies in Swine (herpes)
 Rotavirus
 Rinderpest
35 Canine parvovirus

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Rubella

Toga Viruses

Autoimmune Diseases:

- 5 Anti-Nuclear Antibody
Rheumatoid Factor (RF)
Systemic Lupus Erythematosus
(Deoxynucleoprotein, DNP)
Thyroiditis

10

Hormone Assay:

- Estriol (E_3)
Estrogen
Human Chorionic Gonadotropin (HCG)
15 B-subunit HCG
Human Growth Hormone
Human Placental Lactogen (HPL)
Thyroglobulin
Thyroid Antibody

20

Drug Assays:

- Amikacin
Barbiturates
Cortisol
25 Digoxin
Gentamicin
Morphine
Netilmicin
Phenobarbital
30 Phenytoin
Primidone
Theophylline
Tobramycin

35

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Others:

- Allergy Testing
- Alpha₁-Anti Trypsin
- Alpha-Fetoprotein (AFP)
- 5 Anti-Microsome
- Anti-NADase
- Anti-Streptokinase
- Anti-Streptolysin O (ASO)
- Anti-Thrombin III
- 10 Anti-Thyroglobulin
- Anti-Microglobulin
- B₂-Microglobulin
- Carcino-Embryonic Antigen (CEA)
- Circulating Immune Complexes
- 15 Complement C₃ & C₄
- C-reactive Protein (CRP)
- Ferritin
- Fibrin/Fibrinogen Degrad. Prods. (FDP)
- Fibrinogen
- 20 Haptoglobin
- Hemoglobin
- Histamine
- Human blood (forensic use)
- Human semen (forensic use)
- 25 Immunoglobulin IgA
- Immunoglobulin IgD
- Immunoglobulin IgE
- Immunoglobulin IgG
- Immunoglobulin IgM
- 30 Lipase
- Multiple sclerosis
- Multi-Tumor Antibody (MTA)
- Myeloid Leukemia
- Myoglobin
- 35 Plasmin

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Plasminogen
Sarcoidosis
von Willebrand Factor Antigen

5 When a body fluid is heated at 59-62° for 30 minutes in the presence of these chemical compounds, aggregation of gamma globulin is prevented. By simultaneously adding a small effective amount of an R-SH compound, both the C_{1q} and RF are denatured and thus both C_{1q} and RF are inactivated.

10 The following technique is recommended for inactivation of RF as well of C_{1q} in sera or other fluids (cerebrospinal , pleural, synovial).

15 Fluid is diluted 1/10 in a low ionic strength glycine buffer 0.02 M (ionic strength) pH 8.2; to 0.25 ml serum dilution is added 10 to 20 µl microliter 0.1M solution of EDTA-tetra Na, and 2 microliters of a freshly prepared 5M mercaptoethanol solution which fluid is immediately heated at 60°C for 30 minutes.

20 These procedures are limited to testing for antigens in body fluids. Testing for antibodies is not suggested. Another limitation of this procedure is that the antigen to be detected and quantitated should not contain SS bonds. It should not be tested in those few instances where viral capsule antigens are sensitive to the denaturing effect of mercaptoethanol.

25 By this procedure, the effect of C_{1q} and RF is eliminated by dissociating the AgAb complexes and may liberate antigens if they are caught in the AgAb complexes. Antibodies may be denatured and liberate exposed antigen. Popova O. Y. and Kositikya, L.S. Dissociation of immune complexes and inactivation of bound antibody by reducing agents. Immunochemistry 14:633,635, 1977.

30 This procedure can be utilized in all tests for the detection of bacteria, viruses or parasites, which do

35

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not contain SS bonds. It can be used for tests or kits for:

- Haemophilus influenzae
- Neisseria meningitidis
- 5 Streptococcus pneumoniae
- Group B streptococci
- Neisseria gonorrhoeae
- Salmonella
- Shigella
- 10 Staphylococcus aureus
- Compylobacter jejuni
- Legionello strains
- Escherichia coli
- Pseudomonas species
- 15 Candida albicans
- Actinomyces viscosus
- Coccidioides immitis
- Cryptococcus neoformans
- Mycobacterium phospholipid
- 20 Mycobacterium tuberculosis
- Clumping factor
- Streptococcus Group A
- Trichinella spiralis
- Toxaplasma gondi
- 25 Trichomonas vaginalis
- Hepatitis A and B virus antigens
- Herpes
- Rotavirus
- Epstein Barr Virus
- 30 Respiratory syncytial virus
- Drug assay: amikacin, barbiturate, gentamycin, morphine,
Netilmicine, Phenytoin, Theophyline, Tobmomycin.
- Complement C3
- Complement C4
- 35 Polyamine (heparin)

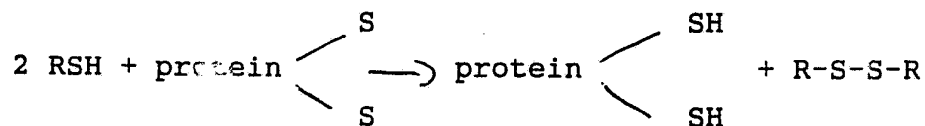
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Vitamin K and other kits for testing antigens which do not contain SS bonds.

The preferred chemical compounds for use in the practice of the invention include the neutral salts of the Hofmeister series, organic acid salts or diamino compounds. Any of these compounds may be used in the practice of the invention provided that when they are added to a 1:10 dilution of a sample of a body fluid in a 1/8 diluted glycine buffer pH 8.2, at a concentration of 40mM to 200mM, and the sample is heated to a temperature of 59-62°C for a sufficient period time, e.g. 30 minutes, the C₁ or C_{1q} are inactivated without affecting the reactivity of the antigens or antibody in said body fluid.

While the exact mechanism for the reaction is not known neutral salts which are used in this invention function as an activator to unmask and release the C_{1q} component of the C₁ complex (the main nonspecific component agglutinator in latex system).

When the RF is to be inactivated, it is necessary to use the particular compounds of the formula R-SH include mercaptoethanol, cysteine, dithioerythritol thioglycolic acid and thioalcohols, lower alkyl (C₁₋₅) mercaptans and the like. These compounds will react with proteins according to the following reaction mechanism:



Other suitable compounds may be used which also inactivate RF using the procedures described herein. The amounts of RSH compounds are not critical and from 25mM to 50mM and preferably about 30mM (in the final dilution) may be used. The sample is heated to a temperature of about 59-62°C for a sufficient period of time to

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inactivate the C_{1q} and RF. Usually a period of 30 minutes is effective.

DESCRIPTION OF THE PREFERRED EMBODIMENT

5 The following examples are presented in order to illustrate the present invention. They are not to be contrued to limit the scope of the appended claims.

10 The following two techniques are used in Examples 1-12, discussed in detail below, to generate experimental data to further demonstrate the application of this invention.

A. Latex photometric rate reaction as a representative of particulate immunoassay techniques.

15 B. Ouchterlony analysis to detect the reaction of antigen and antibody by the precipitation techniques. Immunoprecipitation is the simplest and most direct means of demonstrating antigen antibody reaction in the laboratory and can be applied to all other immunoassay procedures.

20 Since the present invention was developed using latex photometry, this procedure will be described first:

A. LATEX PHOTOMETRIC RATE REACTION TECHNIQUES

1. Glycine Buffer #1:

25 Reagents: Glycine saline buffer. To 975 ml of 0.1M glycine add 2.5 ml of 1N NaOH made up to 1000 ml with distilled water and the pH adjusted to pH 8.2 Ten gram of sodium chloride are then added to each 1000 ml buffer.

30 Glycine Buffer #2:

Dilute 100 ml glycine saline buffer to 700 ml of distilled water to make a 1:8 dilution of the buffer.

35 2. Latex particles: Seragen Diagnostic, Bang Laboratories, Indianapolis, IN., Polystyrene latex 10 percent solid particle size 0.777 u diameter.

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3. Human gamma globulin PENTEX, 0.5 gr percent solution in glycine saline buffer #1.

4. Latex particles suspension is prepared by adding 25 μ l of latex 10% particles size 0.777μ diameter to 10 ml of glycine buffer #1.

5. Latex human gamma globulin suspension. To 10 ml glycine saline buffer #1 add 25 μ l of latex 10% and 50 microliter of human gamma globulin.

6. EDTA tetrasodium 0.1M

In the general procedure of the invention, sera or fluids to be tested are diluted in 1:10 glycine buffer # 2, to 0.25 ml of the diluted serum is added 10 μ l of 0.1M EDTA tetrasodium dihydrate and incubated in a temperature controlled bath, at 59° - 64° C. for 3-30 minutes. Thereafter, latex particle, coated with IgG or uncoated, are added and tested for the presence of flocculation or aggregation of the latex particles.

Example 1

This example describes a screening method to determine if a sample of a body fluid is RF positive or if it contains C_{1q} .

Sera is diluted to a dilution of 1:10 with glycine buffer #2. The diluted sample is placed in the Chronolog photometer with continuous stirring. To this sample is added 0.25 ml of a suspension of uncoated latex particles. If the recorder of the photometer shows a straight line it indicates that there is no detectable C_{1q} and RF. If a sigmoidal curve is obtained, this indicates the the sample contains C_{1q} or rheumatoid factor.

If in the sample which has been treated with latex particles, a straight line is still observed after three mintues, a neutral salt (10 μ l 2M NaCl) is added to the mixture of sera and latex. If a flocculation curve is then obtained in the photometer,

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this indicates the presence of masked C_{1q} or RF or a mixture of C_{1q} and RF. If no flocculation curve is obtained, an aliquot of the sera, buffer, and 2M NaCl is prepared as described above. That mixture is heated at 56°C for 30 minutes and uncoated latex is added. A flocculation curve should be observed, which indicates the presence of C_{1q} and RF.

Whenever a flocculation curve is obtained, a new aliquot of the sera, buffer, and 2M NaCl is prepared and heated at 63°C for 3 minutes; and uncoated latex is added. If a flocculation curve is observed, it confirms the presence of RF; if no flocculation curve is observed, it confirms that the sera is negative for RF and by inference, the specimen did contain C_{1q} .

This example represents a screening procedure for detection of RF if present in the body fluid, which also may be used to confirm that in a sample to be used in the immunoassay procedure, any interference which may be caused by C_{1q} has been prevented.

Example 2

This example demonstrates that the C_{1q} subcomponent of the C_1 will agglutinate sera and is inactivated by 63°C for 3 minutes in the presence of salt.

A commercial preparation of a C_{1q} depleted human serum is tested with a latex IgG preparation in the photometer. No agglutination of latex particles take place when 0.25 ml of serum diluted 1:10 with glycine buffer #2 pH 8.2 is added to the latex IgG suspension. If 0.25 ml serum which is depleted of C_{1q} , is combined with 15 μ l of purified commercial C_{1q} preparation, the serum gives strong positive latex agglutination. Diluted sera was then heated at 63°C, for 3 minutes in the

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presence of 15 μ l of 2M NaCl and when tested with latex coated with IgG, no agglutination was detected.

COMPARATIVE EXAMPLE 1

This example shows that IgG agglutinate with various purified complement components and with Rheumatoid factor with added C₁ or added negative serum when the sample is not treated with heat at 59-64°C for a sufficient period of time.

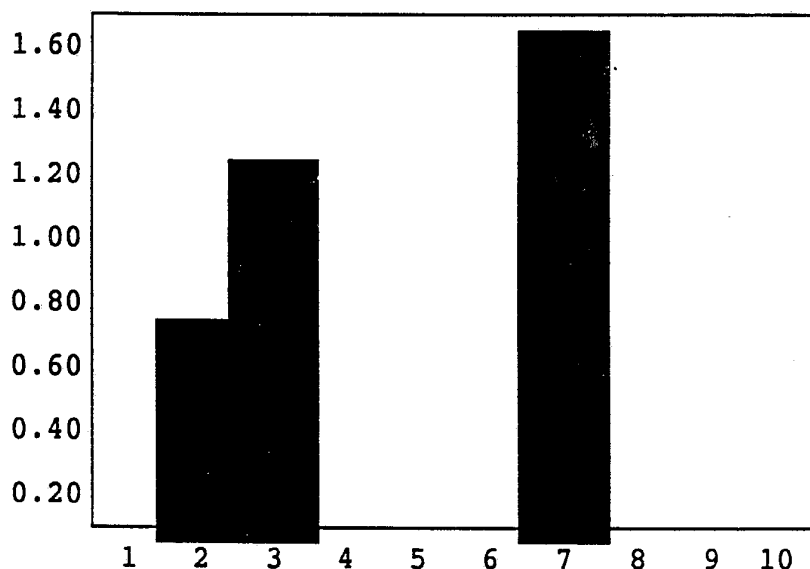
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1. C₁, 20 μ l in 0.25ml of glycine buffer #2
2. C₁+EDTA-Na, added 10 μ l of 0.1M EDTA-Na
3. C_{1q}, 10 μ l
4. C_{1r}, 10 μ l
5. C_{1s}, 10 μ l
6. C₁ inhibitor, 10 μ l
7. Purified RF, 10 μ l
8. Purified RF + 10 μ l C₁
9. Purified RF and 25 μ l Negative serum.

40

C₁ Diamedix 0.04 mg/ml; C_{1q} Sigma 0.5 mg/ml; C_{1r} human 250 μ g; Calbiochem; C_{1s} Calbiochem 500 μ g/ml; RF purified

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1mg/ml; C_{1nh} 0.5/mg Calbiochem.

This table shows that C_1 (1), C_{1r} (4), C_{1s} (5), and C_1 inhibitor do not agglutinate latex IgG particles. When C_1 is treated with EDTA-Na it releases the C_{1q} subcomponent which will agglutinate latex IgG particles. Purified RF preparation (7) agglutinates latex IgG, however, when C_1 is added to, purified RF (8) it inhibits the RF agglutination of negative sera containing the C_1 .

Example 3

This example shows that latex particles coated with HgG or uncoated can be used to detect the presence of C_{1q} .

Latex particles uncoated or latex particles coated with human gamma globulin were added to purified C_{1q} from Diamedix Corp., Sigma Lab, Behring, Atlantic Lab each in concentrations of 0.5 mg of C_{1q} /ml. All of these preparations agglutinated latex particles that were coated or uncoated with gamma globulin.

Dilutions of C_{1q} were prepared from C_{1q} obtained from Cytotec. These dilutions were quantitated in a photometer using the technique described above with uncoated latex particle. The results were as follows:

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<u>CONCENTRATIONS</u>		
	<u>mg/ml</u>	<u>Mx Slopes</u>
	0.5	1.2
	0.025	0.60
5	0.0125	0.30
	0.00625	0.18
	0.003125	0.09
	0.0015625	0.045
	0.0007812	0.027
10	0.0003906	0.014

Example 4

This Example demonstrates the effect of various concentrations of the neutral salts on normal sera which is heated at 60°C for 30 minutes.

Various amounts of EDTA tetra sodium solution added to 0.25 ml of a negative serum, tested by the photometric technique with latex IgG.

	0.1M EDTA tetra Na μl added	Slopes Unheated	Slopes Heated at 60°C
25	0	0.08	0
	10	0.958	0
	20	1.12	0
30	30	0.815	0
	40	0.786	0
35	50	0.647	0
	60	0.647	0
	70	0.660	0
40	80	0.660	0
	100	0.660	0

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Normal sera which is heated at 60°C 30' in the presence of a neutral salt (EDTA-tetra Na) gives a negative reaction even though it previously gave a positive reaction when tested in the photometric technique with latex IgG.

A subsequent test using normal sera and the same concentration of EDTA tetra Na, using uncoated latex and heat at 63°±1°C for 3' gave a zero slope for each concentration.

Example 5

This example reports the testing of 100 normal donor sera by latex photometry.

	1	2	3	4	5
		Heated	Heated	Heated	Heated
		56°C	56°C	60°C	63°±1°C
		30'	30'	30'	3'
		Salt	No Salt	Salt	Salt
	Unheated*	Added	Added	Added	Added
	<u>Percent</u>	<u>Percent</u>	<u>Percent</u>	<u>Percent</u>	<u>Percent</u>
Nonspecific Agglutination	20	100	100		
No Agglutination	80			100	100
* control					

One hundred samples of sera obtained from apparently healthy blood donors were first tested for the presence of rheumatoid factor by the slide technique (Rheumatex, Wampole Laboratories, Cranbury New Jersey and the standard latex fixation test tube method of Singer and Plotz. Am. J. Med 21:888-892 (1956). The tests were negative for all sera.

Each serum was diluted 1:10 in glycine buffer #2 and treated as follows:

1. unheated, no salt added;
2. heated at 56°C for 30 minutes and added 15μl of 0.1M sodium EDTA;
3. heated at 60°C for 30 minutes with no added salt;

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4. heated 60°C for 30 minutes with 15 μ l 0.1M EDTA Na added; and

heated at 63 $\pm$ 1°C with 15 μ l 0.1M EDTA Na added.

5 The table shows that 80% of the unheated sera are negative by this procedure for C_{1q}, however, when heated at 56°C, 30' with or without salt, the sera become positive for C_{1q}. When these sera are heated at 60°C 30' and 63 $\pm$ 1°C 3' in the presence of EDTA Na these sera become negative for C_{1q}. The C_{1q} from the normal sera
10 which previously agglutinated latex particles at 56°C become inactivated at 60°C 30' or 63 $\pm$ 1°C for 3' minutes only when heated in the presence of the salt.

The sera should not be treated at a temperature of 63 $\pm$ 1°C without added salt because if a sample has a
15 high level of gamma globulin i.e., more than 18 mg/ml, the high level of gamma globulin may cause aggregation.

Example 6

20 This example shows the effect of salt and heat in RF containing sera.

Fifty rheumatoid sera were diluted in glycine buffer #1 to a dilution of 1:10 and tested unheated, heated at 56°C for 30 minutes and with 15 μ l EDTA-Na added and heated at 60°C for 30 minutes with EDTA-Na added.
25 All these sera were positive by the slide test as well as by the latex IgG tube technique.

Fifty percent of these sera (diluted 1:10 in buffer) were negative by latex IgG photometry. This inhibition was due to inhibition of RF by the C₁ component of the complement. By the use of heat and salt the C₁ complement component is dissociated and RF is released to react with latex IgG. The C₁ component is dissociated into
30 C_{1q}, C_{1r} and C_{1s} and the agglutination of C_{1q} with latex particles may be prevented while using heat at 60°C for

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30 minutes with added 15 μ l 0.1M EDTA-Na. The results were as follows:

Percent specimen	Unheated No Salt	Heated 56° EDTA-Na	Heated 60° EDTA-Na
	50%	100%	100%
Positive/ Negative for RF	Negative	Positive	Positive

Since 63 $\pm$ 1°C for 3 minutes may decrease the avidity of RF for latex particles coated with IgG, temperatures of 60°C for 30 minutes should be used for preventing the agglutination of C_{1q} in latex IgG particles.

Example 7

Serum was obtained from a patient suffering from a Cryptococcus neoformans infection. The blood culture was positive for Cryptococcus neoformans. The serum had a titer of 1:60 when tested by the slide latex agglutination test (Meridian Diagnostics). The patients serum was serially diluted with glycine buffer #2 and a titer of 1:120 was detected using the Meridian Diagnostics latex antibody test.

An aliquot of this serum was tested with latex particles alone. The serum did not agglutinate and a straight line was obtained in the photometer recorder. After 3 minutes in the photometer 20 μ l 0.1M NaEDTA was added and a flocculation curve was obtained which was interpreted as being due to the presence of the C_{1q} component of the serum. When an aliquot of the serum was heated at 63°C for 3 minutes the Cryptococcus test in one specimen was positive but in latex alone was negative. This demonstrated that the serum heated at 63°C for 3 minutes became specific for Cryptococcus antigen by preventing the interference of C_{1q}.

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Example 8

A 30 μ l sample of serum depleted of C_{1q} is modified by adding 30 μ l of purified C_{1q} and Cryptococcus polysaccharide (0.1 mg/ml) to form a test mixture. One aliquot of the mixture is tested on uncoated latex and latex coated with Cryptococcus antibody. A second aliquot is heated at 63°C for 3 minutes prior to testing on the uncoated latex and the Cryptococcus latex. The results are as follows:

10

	Latex (Uncoated)	Latex Cryptococcus
25 μ l C _{1q} depleted serum with 30 μ l C _{1q} (0.5mg/ml) unheated and 30 μ l of Cryptococcus polysaccharide (0.1 mg/ml)	0.60	0.60
25 μ l C _{1q} depleted serum with 30 μ l C _{1q} (0.5mg/ml heated and 30 μ l of Cryptococcus polysaccharide (0.1 mg/ml)	0	0.70

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This example demonstrates that heat at 63°C for 3 minutes will prevent C_{1q} from agglutinating latex particles and that heat at 63°C for 3 minutes will not affect the agglutination of Latex Cryptococcus antibody with Cryptococcus polysaccharide.

Example 9

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The invention also includes a novel apparatus for automatically carrying out immunoassays in which it is desired to inactivate C₁ or C_{1q} in a sample of body fluid.

35

The apparatus may be used for:

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1. Screening methodology for confirming that C_{1q} has been inactivated.
2. Screening methodology for detection of C_{1q} in any body fluid.
- 5 3. Screening methodology for confirming the presence of RF in a specimen of body fluid.
4. Methodology to demonstrate the absence of RF in a body fluid after treatment with mercaptoethanol.
5. Screening methodology for detection of RF.
- 10 6. Methodology for obtaining RF titers in body fluids.
7. Methodology to quantitate the concentration of RF (IgM) in body fluids.
8. Methodology to detect bacterial, fungal, parasitic, rickettsial and virus diseases using latex agglutination
- 15 in an apparatus which eliminates C_1 and C_{1q} from the sample of body fluid.

FIG. 3 shows a schematic diagram of an apparatus of the invention. The basic structure of the invention comprises an irradiation unit comprising a

20 light source 1; a filter or prism 2; a sample cell 3 for holding a sample of body fluid; an electronically controlled variable electric heater block 4 in proximity to cell 4 which is adapted to hold a test sample tube. The electronic control on the heater

25 block 4 is capable of providing temperatures which will heat the sample in sample cell 3 to a temperature in the range of about 59-64°C for periods of time ranging from 3 minutes to 30 minutes or longer. The sample

30 cell 3 has a number of associated reagent feed lines 6, 7 and 8 which are provided with automatic metering valves which are electronically controlled to deliver the required amounts of dispersed latex particles, chemical solution, and/or antigen or antibody solution, and/or mercaptoethanol solution. As shown in FIG. 3

35 line 6 is provided for delivering the dispersion of

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latex particles, line 7 is provided for the delivery of a neutral salt solution such as 2M NaCl and line 8 is provided for delivery of mercaptoethanol solution for immunoassays in which it desired to eliminate RF from the sample of body fluid. Reference cell 3A with light source 1A is provided for holding a control sample for compensation. Heater 4A and lines 6A, 7A and 8A are provided in the event it is desired to run a parallel control. Photocells 12 and 14 are placed in such a way that they will sense the light transmitted through the cells 3 and 3A. An amplifier 16 is used to amplify the signals from the photocells 12 and 14. The signals are sent to interface 18 and are processed in a computer for display on a CRT and/or printer which are not shown in FIG. 3. Cells 3 and 3A are preferably equipped with conventional magnetic stirrers which are utilized to keep the components of the test cell in suspension.

The novel apparatus of the invention may be operated as follows: an analyte such as sera (diluted with glycine buffer #2-1:10), which is to be tested for Cryptococcus neoformans is placed in the cell 3 and 10ul of a 2M solution of KCl in water is metered into cell 3 through line 6. Heater block 4 is energized and the temperature of the cell is raised to 63°C for three minutes to inactivate the C_1 and C_{1q} .

Thereafter, 15ul of a dispersion of latex particles coated with Cryptococcus neoformans antibody is metered into cell 3 through line 6 while the magnetic stirrer is run at 1000rpm. The change in optical density is then compared with a control which is run in cell 3A to determine if flocculation has occurred. The detection of flocculation is read as a positive result for the presence of Cryptococcus neoformans antigen.

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The apparatus of the invention may possess the basic structure of prior art photometric devices such as the Chronolog Model 570VS for performing optical aggregation tests. This apparatus may be modified to include a variable heater block means and associated timer means as well as reagent feed means which have been described herein. Suitable photometers which may be modified to embody the invention are described in United States Letters Patent No. 4,118,192, which is incorporated herein by reference or photometers which are commercially available such as the LA-2000 which is manufactured by Eiken Chemical Co.

OUCHTERLONY ANALYSIS

Confirmatory experiment for the effect of salts and heat for inactivation of C_{1q} in human sera.

In the double diffusion agarose Ouchterlony technique the antibody or the antigen are applied to the center of the well and antigen or antibody are applied to 5 wells situated at the periphery of the plate. To each well is applied 10 μ l of the material to be tested and 10 μ l of antisera. The presence or absence of a precipitin line between antigen and antibody is read after the plates are kept at room temperature or in a refrigerator for 24 to 48 hours.

The following reagents were used for these series of experiments: Purified C_{1q} conc. 0.04 mg/ml obtained from Diamedix Corp; Purified C_{1q} conc. 0.5 mg/ml obtained from Diamedix Corp. Cytotec and Sigma; antisera to human C_{1q} in goats was obtained from Sigma and Atlantic labs. Negative sera were obtained from healthy blood donors and rheumatoid sera from patients who were diagnosed as having rheumatoid arthritis. The salt was 1M NaCl.

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Experiment A

Ten normal sera were tested. Normal sera were applied to the center well and 3 different antibodies were applied to the outer wells: (a) antibody to C_{1q} ; (b) antibody to C_{1r} , and (c) antibody to C_{1s} ; all antibodies precipitated at the mid-distance between the center well and outer well and the three line fused together.

Experiment B

Ten rheumatoid sera were studied by the same procedure as Experiment A and the same results were obtained.

Experiments A and B demonstrate clearly that normal serum and rheumatoid factor containing sera contains those proteins called C_{1q} , C_{1r} and C_{1s} . The fact that they fuse together indicates that they are part of the same molecule which is C_1 .

Experiment C

Purified C_{1q} , C_{1r} and C_{1s} preparations were tested according to the technique of Example 10 with their respective antisera and showed the same three precipitin lines which were observed in Example 11. This shows that C_{1q} , C_{1r} and C_{1s} react the same as C_1 .

Experiment D

To 10 normal sera were added 10 μ l of 0.1M EDTA tetrasodium according to the technique of Example 10. These samples were heated at 56°C for 30 minutes and tested with antisera to C_{1q} ; all antisera gave a distinct precipitin line after 24 hours incubation demonstrating that the addition of a neutral salt and heat at 56° for 30 minutes enhances the C_{1q} antigen precipitin line.

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Example 10

This examples is a method for the demonstration of the presence of C_{1q} in sera and the use. The Methodology utilized for preparing the latex particles has been described in PROCEDURE A.

Material:

Ouchterlony diffusion on plate. In each of 6 tubes was:

1. 50 μ l of a normal serum;
2. 50 μ l of normal sera with added 30 μ l of Na_4 -EDTA;
3. 50 μ l of rheumatoid sera with aded 30 μ l of Na_4 -EDTA;
4. 50 μ l of normal sera with added 30 μ l of Na_4 -EDTA and heated at 60°C for 30 minutes;
5. 50 μ l of normal serum with added 30 μ l of Na_4 -EDTA and heated at 63°C for 3 minutes. These sera in increments of 10 μ l are added to the 6 outer well. In the outer well 10 μ l of an antibody to C_{1q} produced in rabbits or goats are added. Plates are incubated at room temperature for 24 and 48 hours. Precipitin lines are obtained from unheated normal sera, sera heated at 56°C for 30 minutes and for normal and rheumatoid sera to which EDTA- Na_4 were added. Heating sera at 60°C for 30 minutes and 63°C for 3 minutes with the addition of salt prevented the development of precipitin lines.

Example 11

This experiment illustrates the effect of temperature on the C_{1q} molecule which in purified form is thermolabile at 56°C for 30 minutes but thermostable at that temperature when complexed with C_{1n} or in sera when heated for 10-30 minutes.

AntiseraRun:

	<u>Anti C_{1q}</u>	<u>Anti C_{1n}</u>
1* 25 μ l Normal serum unheated	ppt	ppt
2* 25 μ l Normal serum heated at 56°C for 30 minutes with 15 μ l	ppt	ppt

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		of 2M NaCl		
5	3	25 μ l Normal serum heated at 63°C for 3 minutes with 15 μ l of 2M NaCl	no ppt	very weak ppt
	4*	10 μ l C _{1q} /5mg/ml	ppt	no ppt
10	5*	10 μ l C _{1q} heated at 56°C for 30 minutes	no ppt	no ppt
	6*	10 μ l C _{In}	no ppt	ppt
15	7*	10 μ l C _{1q} + 10 μ l C _{In}	ppt	ppt
	8*	10 μ l C _{1q} + 10 μ l C _{In} heated at 56°C for 10 minutes	ppt	ppt
20	9*	10 μ l C _{1q} + 10 μ l C _{In} heated at 56°C for 20 minutes	ppt	ppt
	10*	10 μ l C _{1q} + 10 μ l C _{In} heated at 56°C for 30 minutes	ppt	ppt
25	11*	10 μ l C _{1q} + 10 μ l C _{In} heated at 63°C for 3 minutes	no ppt	ppt

*Control

30

The C_{1r} inhibitor had 10 International Units (Calbiochem).

35

The invention is illustrated by Run No. 3 which shows that in the presence of a neutral salt, the C_{1q} in serum is thermolabile at a temperature of 63°C for 3 minutes while it is not thermolabile at a temperature of 56°C for 30 minutes in the presence of a neutral salt.

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Example 12A

To a 0.75 ml of a solution of human gamma globulin, 0.50 percent solution of 0.25 ml of glycine buffer No. 2 is added to six test tubes.

5 To each of the tubes are added:

1. Control + (HgG solution) 25 μ l of buffer no. 2;
2. 25 μ l of 2M NaCl;
3. 25 μ l of C_{1q} purified (0.5 mg/ml);
4. 25 μ l C₁ purified (0.004 mg/ml);
- 10 5. 25 μ l of C_{1r} purified; and
6. 25 μ l of C_{1s} purified.

All tubes are incubated in a water bath at 64°C 5-30 minutes. In this experiment turbidometric readings are made in the photometer and no latex particles are used.

15 The control tube and the tube containing human gamma globulin and C_{1r} and C_{1s} develop strong turbidity in about 5 minutes time; all other tubes which contain 2M NaCl, C_{1a} and C₁ added to IgG did not develop any turbidity and appeared as clear as the original IgG unheated solution even
20 when heated up to 30 minutes. These experiments demonstrate that immunoglobulins heated at 64°C for about 5 minutes develop aggregation. It also demonstrates that formation of aggregates by heat is prevented by the addition of neutral salt as well by C_{1q} or C₁ to IgG previous to heating at 64°C.

25 Example 12B

The procedure of run No. 2 of Example 12A was repeated using various salts and the turbidometric readings are reported in FIG. 4. The results show that these salts substantially prevented the formation of aggregates by heat.

30 Example 13

To two test tubes, each containing 1 ml of glycine buffer (pH 8.2) are added 20 μ l of two different preparations of purified IgM RF (obtained from Dr. Blass Frangione, NYU Medical School and Dr. Ralph Heimer, Jefferson Medical College). The samples
35

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were serially diluted up to 1:8000 with glycine buffer and tested by the latex fixation tube method of Singer and Plotz for detection of RF. Titers of 1:2000 and 1:4000 were obtained respectively. When this experiment was repeated with the modification that 2 μ l of a 5M 2-mercaptoethanol solution were added to each test tube and heat was applied (60°C for 30 minutes), both preparations yielded negative results. This shows that the combination of mercaptoethanol and heat at 60°C for 30 minutes destroys the RF.

Example 14

Two sera from patients with Cryptococcal meningitis were tested by the latex agglutination test of (CALAS) Meridian Diagnostic Inc. Latex particles in these kits are coated with an anticryptococcal globulin. One sera was negative for RF and the other sera had a titer of 1:60 for RF in the latex gamma globulin test of Singer and Plotz. The technique utilized in the kit should eliminate from the fluid tested the presence of nonspecific agglutinator, the RF.

To 200 μ l of serum specimen is added 200 μ l of a Pronase solution (Meridian catalog #140050); serum pronase reagent is incubated at 56°C for 15 minutes, some solution is boiled for 5 minutes to terminate the enzymatic digestion; these sera gave agglutination titers of 1:256 and 1:512 by a tube method using latex particles coated with anticryptococcal globulin.

The technique described above has been compared with the technique of the present invention to include the elimination of C_{1q} and the RF from the samples. It includes also two controls. One control is a 25 μ l of a 1 mg C_{1q} (purified C_{1q}) which is added to a 0.25 μ l of a Glycine buffer solution; and a second

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control is a dilution of 1:50 of a RF serum obtained from patient suffering from active rheumatoid arthritis.

5 To a 0.25 ml serum 1:10 dilution and to the two controls are added 10 μ l of a 0.1M solution of EDTA tetrasodium and 2 microliters of a 5M mercaptoethanol solution. The sera and controls are heated at 60°C for 30 minutes.

10 Sera is serially diluted with 0.1M glycine buffer pH 8.2 and tested with Cryptococcal antibody (latex kit of the Meridian Diagnostic Inc.). Both sera, as well the as the control were tested with the same reagents. Both sera gave agglutination titers of 1:256 and 1:512 respectively. The titers were similar
15 with that obtained by the original procedure of Meridian Diagnostic; both controls were negative demonstrating that C_{1q} and RF are eliminated from this sera using the technique of the present invention.

20

Example 15

Two sera were obtained from patients suffering from pneumococcal pneumoniae isolated from patients with Streptococcus pneumoniae in their sputum which are identified as Pneumococcus serotype 8. The
25 patients sera gave a positive latex particle test when tested with the Welcogen antigen detection kit of Welcome Diagnostic. In this kit, latex particles are coated with S. pneumoniae rabbit antibody. The serum sample prior to testing was heated for 5 minutes in
30 boiling water after the addition of 3 volumes of 0.1M sodium ethylene diaminotetraacetate (pH 7.4) per 1 volume of serum. The samples were cooled at room temperature and clarified by centrifugation prior to testing. The supernatant from the clot was used and
35 was found to be positive for both sera.

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These two sera obtained from patients suffering from pneumococcal pneumoniae were also positive for the presence of RF as detected by the latex agglutination test of Singer and Plotz, Am J. Med. 21:88, 1956.

Patient sera are diluted 1:10 with a glycine buffer diluted 1:8 with distilled water. To a 0.25 ml of diluted serum, and to two 0.25 ml control were added 10 μ l of 0.1M solution of EDTA, and 2 lambda of a 5M mercaptoethanol solution, the sera are heated at 60°C for 30 minutes. The controls were a 1/50 dilution of serum containing RF obtained from sera of patients suffering from RA and glycine buffer (pH 8.2) containing 1mg of a C_{1q} purified preparation (Sigma Co.).

Following treatment, the sera and controls are tested for the presence of S. pneumoniae using the Welcogen, Welcome Diagnostic kit. The two sera were positive for S. pneumoniae and negative for the two controls.

This example shows that when the C_{1q} and RF are eliminated from sera containing S. pneumoniae, the sera can be positively tested for the S. pneumoniae antigen.

The invention also includes kits for the carrying out of an immunoassay.

The kits of the invention may comprise the components of any conventionally available kit and (a) a chemical agent which when added to a sample of body fluid is capable of inactivating the C₁ or C_{1q} in said body fluid when a mixture of said body fluid and said chemical compound are heated to a temperature of 59-62°C for a sufficient period of time to inactivate said C₁ and C_{1q} without affecting the reactivity of an

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antigen or antibody in said body fluid and (b) a solution containing C_{1q} .

5 The kits of the invention may comprise the components of any conventionally available kit and (a) a chemical agent which when added to a sample of body fluid is capable of inactivating the C_1 or C_{1q} in said body fluid when a mixture of said body fluid and said chemical compound are heated to a temperature of 59-64°C for a sufficient period of time to inactivate said C_1 and C_{1q} without affecting the reactivity of an antigen or antibody in said body fluid and (b) a solution containing C_{1q} .

Examples of these kits are as follows:

15 1. Reagents used for the elimination of the interference of the C_{1q} complement subcomponent of C_1 in fluid or sera:

- (a) antigen;
- (b) antibody;
- (c) control for antibody;
- 20 (d) control for antigen;
- (e) a neutral salt (preferably 2M NaCl); and
- (f) buffer for antigen antibody reaction.

25 2. For in vitro diagnostic use, a kit will contain reagents used in the respective immunoassay procedures:

- (a) latex antigen or antibody emulsion;
- (b) latex antigen control;
- (c) latex rheumatoid factor control;
- (d) latex antibody control; and
- 30 (e) a neutral salt (preferably 2M NaCl).

3. For RF identification and C_{1q} screening technique:

- (a) latex particles solution 0.70-0.90 microns dia;
- 35 (b) ~~AgG~~ 2%; OK

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- (c) Glycine buffer solution pH 8.2, μ 0.1;
- (d) RF purified protein;
- (e) RF serum standard;
- (f) Ouchterlony plate (optional);
- (g) purified C_{1q} ;
- (h) neutral salt 2M NaCl or 2M KCl; and
- (i) mercaptoethanol solution 5M in H_2O .

4. For the elimination of the interference of the C_{1q} and for the elimination of RF interference.

- (a) An 0.1M glycine buffer saline pH 8.2;
- (b) A solution of 0.1M EDTA Na_4 ;
- (c) A concentrated solution of 5M mercaptoethanol;
- (d) A dilution of 1:50 of RF serum human serum (which has been tested for HIV) as a control for RF, or a purified RF preparation;
- (e) A dilution of C_{1q} complement component (25 μ l of a 1mg C_{1q} purified protein preparation to be added to a 0.25 ml of glycine saline buffer pH 8.2 as a control for C_{1q}); and
- (f) The buffer to contain 0.1% sodium azide as a preservative.

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CLAIMS

1. A method for the elimination of the effect of C_1 and C_{1q} from a body fluid in order to eliminate the interference of C_1 and C_{1q} in immunoassays, said method comprising the steps of:
- 5 (a) adding an effective amount of a chemical compound to a diluted sample of a body fluid, said chemical compound being capable of preventing the C_1 or C_{1q} in said body fluid from interfering in an immunoassay when a mixture of said body fluid and said chemical compound are heated to a temperature of 59-64°C for a sufficient period of time without affecting the reactivity of the antigen or antibody in said body fluid; and
- 10 (b) heating the diluted sample containing said chemical agent and said body fluid to a temperature of about 59°C to about 64°C for a period of time which is sufficient to eliminate the interference of C_1 and C_{1q} in an immunoassay.
2. A method as defined in Claim 1, wherein the body fluid is serum.
3. A method as defined in Claim 1, wherein the chemical compound is a neutral salt, an organic acid salt or a diamino compound.
- 25 4. A method as defined in Claim 2, wherein the neutral salt is selected from the group consisting of sodium chloride and ethylenediamine tetraacetic acid, tetrasodium salt.
5. A method as defined in Claim 4, wherein the heating is carried out at 60°C for 30 minutes.
- 30 6. A method as defined in Claim 4, wherein the heating is carried out at 63°C for 3 minutes.
7. A method for the prevention of the formation of aggregates of immunoglobulin in a body fluid, said method comprising the steps of:
- 35

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(a) adding an effective amount of a chemical compound as defined in Claim 1 to a sample of a body fluid; and

(b) heating the sample containing said chemical agent and said body fluid to a temperature of about 59°C to 62°C for a period of time which is sufficient to suppress the formation of aggregates of immunoglobulins.

8. A method as defined in Claim 6, wherein the body fluid is sera.

9. A method as defined in Claim 2, wherein the chemical compound is a neutral salt.

10. A method as defined in Claim 6, wherein the neutral salt is selected from the group consisting of sodium chloride and ethylenediamine tetraacetic acid, tetrasodium salt.

11. In an immunoassay kit the improvement which comprises including:

(a) a chemical agent as defined in Claim 1; and

(b) a solution containing C_{1q} complement.

12. The kit as defined in Claim 11 wherein the chemical agent is a neutral salt and the C_{1q} solution is a purified C_{1q} solution.

13. The kit as defined in Claim 11 wherein the neutral salt is sodium chloride or potassium chloride.

14. A method for the detection of RF in a sample of a body fluid, said method comprising:

(a) contacting a sample of a diluted body fluid with latex particles and detecting agglutination;

(b) contacting a fresh sample of said diluted body fluid with an effective amount of a chemical compound which is capable of preventing the C_1 or C_{1q} in said body fluid from interfering in an

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immunoassay when a mixture of said body fluid and said chemical compound are heated to a temperature of 59°-64°C for a sufficient period of time without affecting the reactivity of the antigen or antibody in said body fluid; and

(c) heating the diluted sample containing said chemical agent and said body fluid to a temperature of 59°-64°C for 3-30 minutes;

(d) adding latex particles to the heat treated sample; and

(e) determining the presence of RF factor by detection of any flocculation in the heat treated sample which contains latex particles.

15. A method as defined in Claim 14 wherein the heating is carried out at 63°C for 3 minutes.

16. A method for the detection of RF in a body fluid as defined in Claim 14 wherein in step (a) prior to detecting agglutination, an effective amount of a chemical compound is added to the diluted body fluid with latex particles said chemical compound being capable of preventing the C_1 or C_{1q} from interfering in an immunoassay when the sample is heated to a temperature of 59°-64°C for a sufficient period of time.

17. A method for the detection of RF in a body fluid as defined in Claim 16 wherein in step (a) no agglutination is detected and an effective amount of a chemical compound is added to a fresh sample of diluted body fluid, said chemical compound being capable of preventing the C_1 or C_{1q} from interfering in an immunoassay when heated to a temperature of 59°-64°C for a sufficient period of time; and (b) heating the mixture of step (a) to a temperature of 56°C for 30 minutes to unmask the C_1 or C_{1q} .

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18. A photometric apparatus for the detection of agglutination, said apparatus comprising:

- (a) a light source;
- (b) a sample cell for holding a sample of body fluid;
- (c) a controlled variable heater block in proximity to said cell;
- (d) reagent feed means and valve means to meter into said sample cell measured amounts of reagents;
- (e) a photocell means to detect light transmitted through said sample cell;
- (f) amplifier means to amplify the signal from said photocell means;
- (g) a microprocessor to process said signals and control said valve means; and
- (h) display means for recording the results of the detection of agglutination.

19. A method for the quantitation of purified C_{1q} , said method comprising:

- (a) serially diluting a sample of C_{1q} ;
- (b) contacting said serially diluted samples from step (a) with latex particles;
- (c) determining the amount of flocculation of each diluted sample in a photometer;
- (d) determining the maximum slope of each sample; and
- (e) comprising the maximum slope of the samples with a reference standard.

20. A method for confirming that the interference of C_{1q} in a body fluid, in a subsequent immunoanalysis has been eliminated said method comprising:

- (a) placing a sample of C_{1q} antisera in a first well of an agar gel diffusion plate; and

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(b) placing a sample of a body fluid that has been treated to prevent the interference of C_{1q} , in an immunoassay, in a second well of an agar gel diffusion plate; and

5 (c) observing the agar gel diffusion plate for a sufficient period of time to confirm that no precipitation line has formed.

21. A method for the reduction of the interference of C_{1q} and rheumatoid factor in
10 immunoassays where the antigen to be detected does not contain S-S bonds and where the viral capsule antigens are not sensitive to the denaturing effects of mercaptoethanol which comprises pretreating a sample of a body fluid before subjecting said body fluid to an
15 immunoassay, to the following steps:

(a) adding an effective amount of a chemical compound to a diluted sample of a body fluid, said chemical compound being capable of inactivating C_{1q} in said body fluid when a mixture of said body fluid and
20 said chemical compound are heated to a temperature of 59-62°C for a sufficient period of time to inactivate the C_{1q} without affecting the reactivity of any antigens in said body fluid and an effective amount of a chemical reagent of the form R-SH where R is an organic
25 moiety which will not affect the reactivity of any antigen in said body fluid when said reagent of the formula R-SH is heated to a temperature of 59-62°C for a sufficient period of time to inactivate the RF; and

(b) heating the product of step (a) to a
30 temperature of 59-62°C for a sufficient period of time to inactivate the C_{1q} .

22. A method as defined in Claim 21, wherein the chemical compound is a neutral salt, an amino compound or an organic acid salts and the R-SH compound
35 is mercaptoethanol, cysteine or dithioerythritol.

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23. A method as defined in Claim 22, wherein the temperature is 60°C and the sample is heated for 30 minutes.

5 24. A method as defined in Claim 23, wherein a neutral salt is employed.

25. A method as defined in Claim 24, wherein the neutral salt is ethylene diamine tetraacetic acid tetrasodium salt.

10 26. A method as defined in Claim 25, wherein the R-SH compound is mercaptoethanol.

27. In an immunoassay kit for the determination of an antigen, the improvement which comprises a vial containing a chemical compound as defined in Claim 21; a vial containing an R-SH compound as defined in Claim 21; a vial containing RF; and a vial containing C_{1q}.

28. A kit as defined in Claim 27, wherein the chemical compound is a neutral salt.

20 29. A kit as defined in Claim 27, wherein the R-SH compound is 2-mercaptoethanol.

30. A method for the reduction of interference of C_{1q} and rheumatoid factor in immunoassays where the antigen to be detected does not contain S-S bonds and where the viral capsule antigens are sensitive to the denaturing effects of mercaptoethanol which comprises pretreating a sample of a body fluid before subjecting said body fluid to an immunoassay to the following steps:

30 (a) adding a glycine buffer and an effective amount of a chemical compound to a dilute sample of a body fluid, said chemical compound being capable of inactivating C_{1q} in said body fluid when a mixture of said body fluid and said chemical compound are heated to a temperature of 59-62°C for a sufficient period of time to inactivate the C_{1q} without affecting the

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reactivity of any antigens in said body fluid and an effective amount of a chemical reagent of the form R-SH where R is an organic moiety which will not affect the reactivity of any antigen in said body fluid when said reagent of the formula R-SH is heated to a temperature of 59-62°C for a sufficient period of time to inactivate the RF; and

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(b) heating the product of step (a) to a temperature of 59-62°C for a sufficient period of time to inactivate the C_{1q}.

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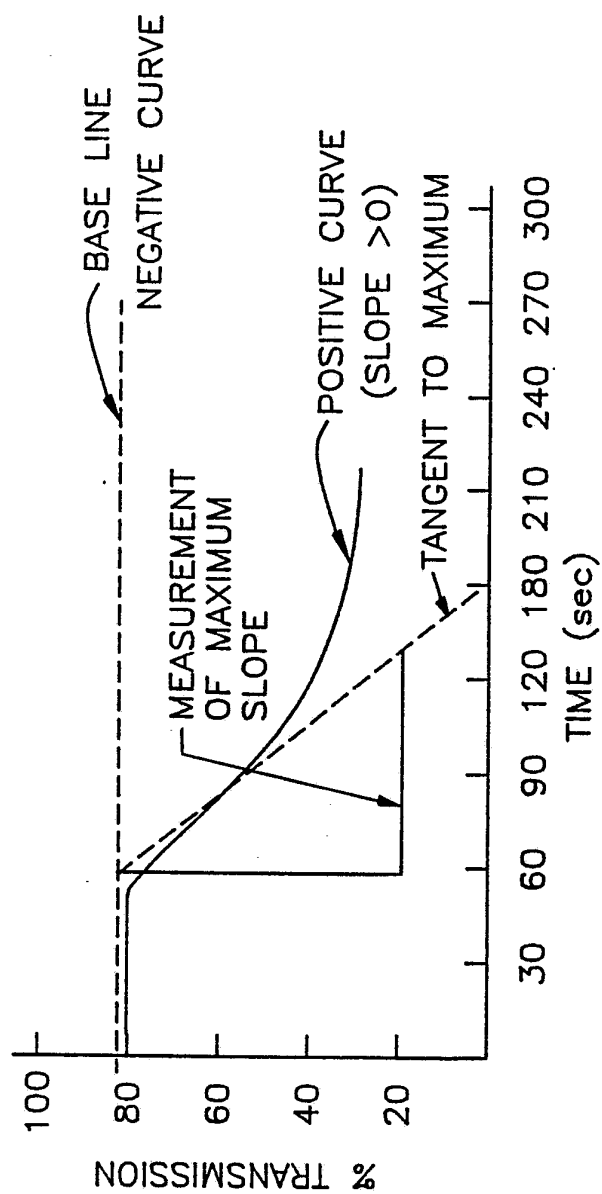


FIG. 1

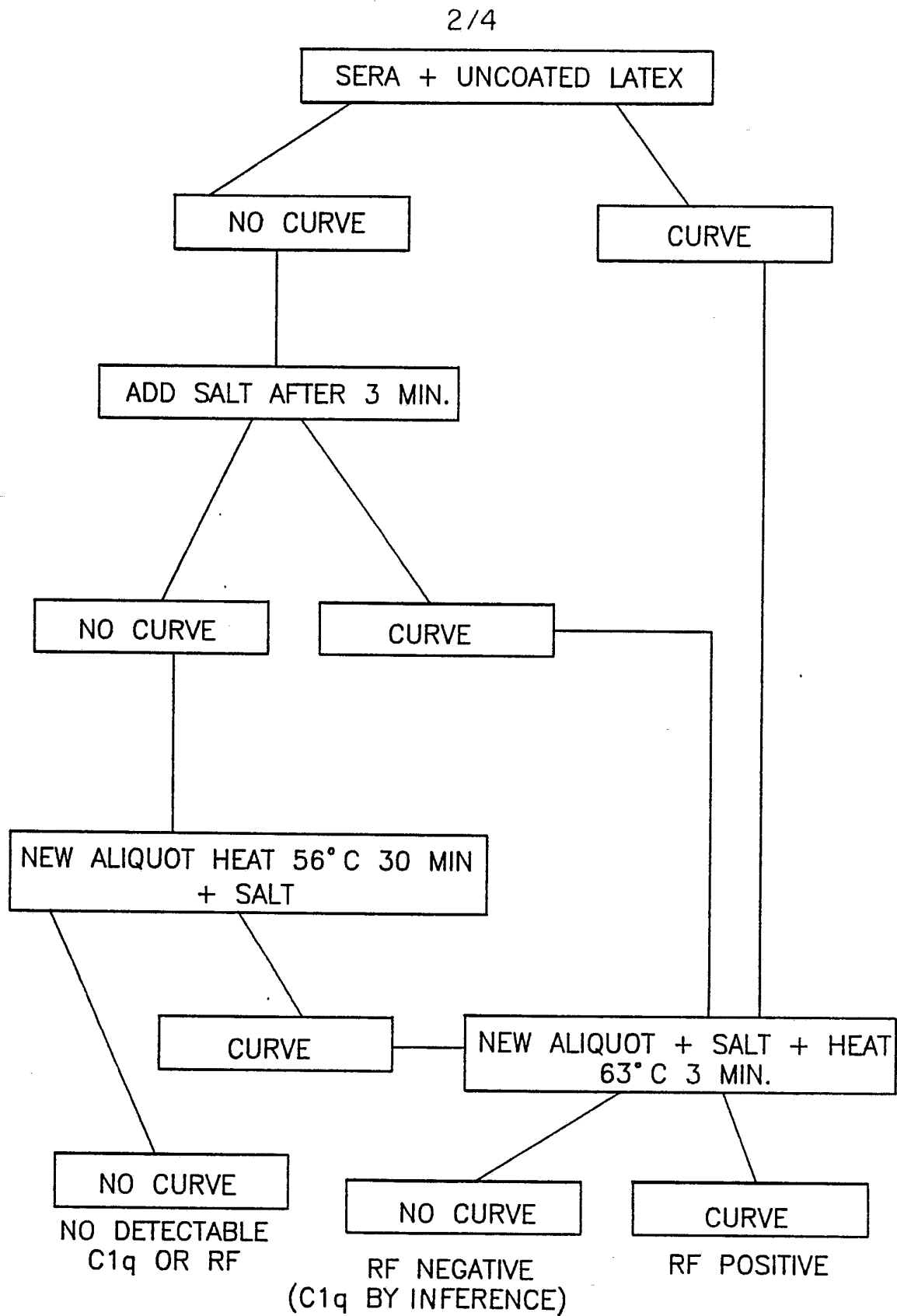


FIG. 2

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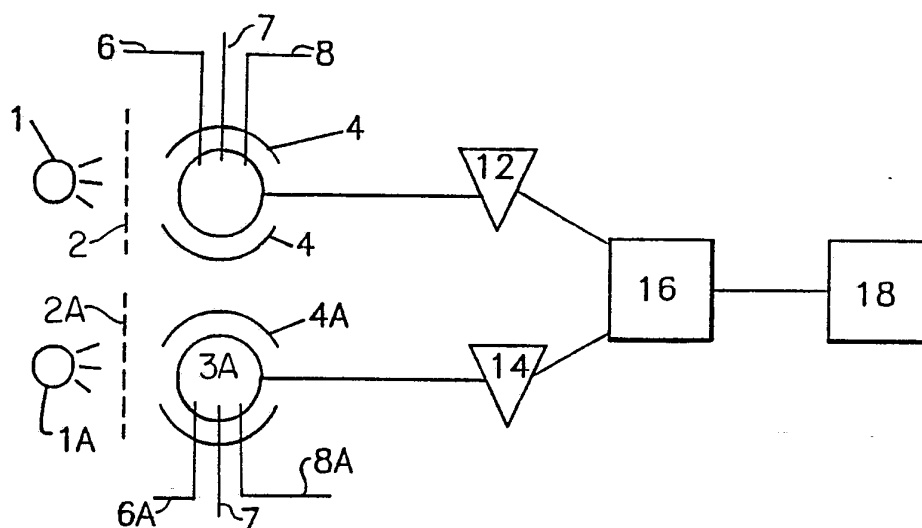


FIG. 3

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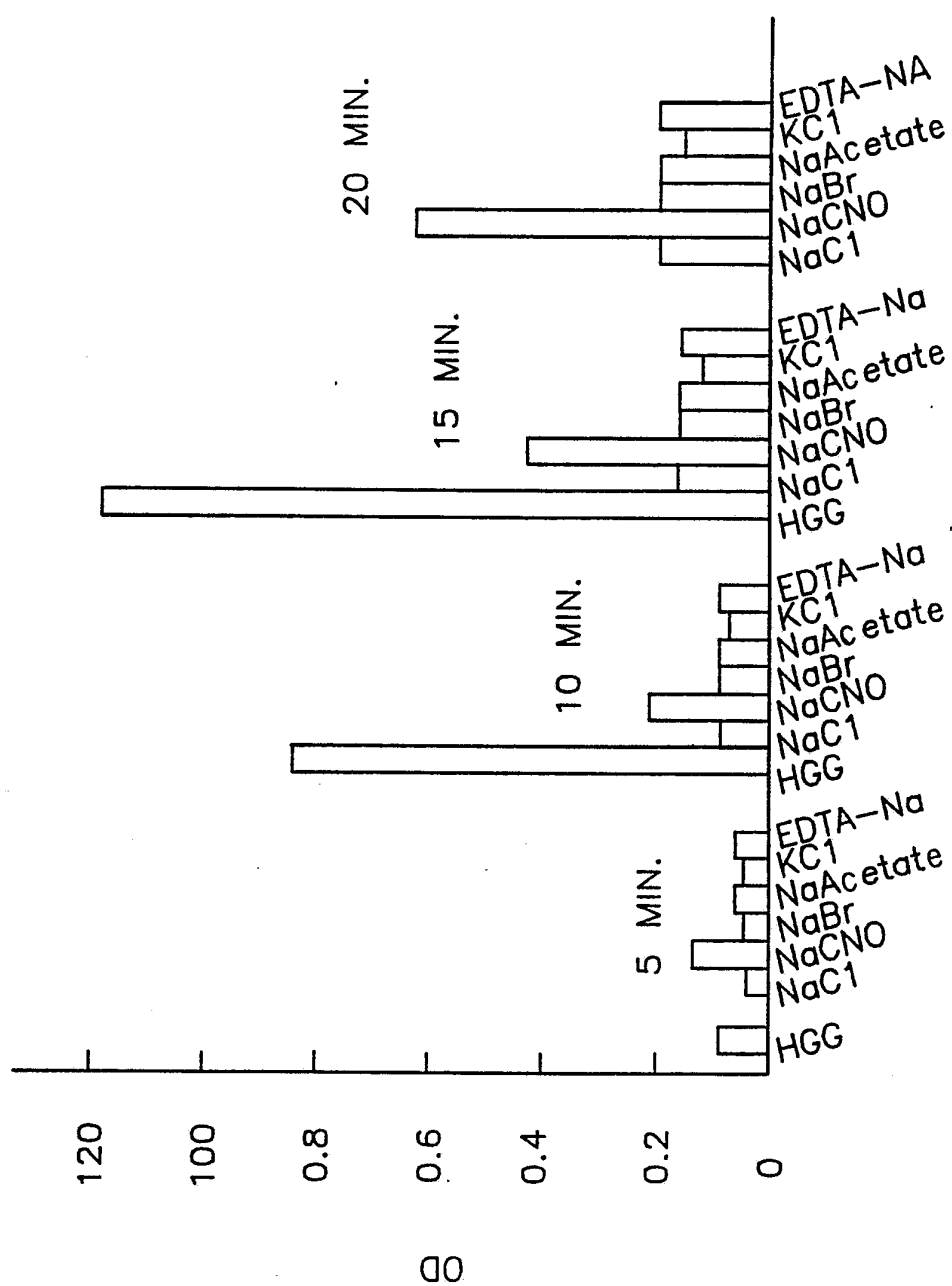


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02901

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : G01N 33/546, 33/559, 33/564

US CL : 435/7.1, 7.92; 436/509, 515, 529

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. : 435/7.1, 7.92, 962; 436/509, 515, 529, 805, 821, 825, 909

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

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

APS, MEDLINE search terms: complement, clq, cl, rf, rheumatoid factor, ouchterlony, 59-64 degrees, edta, mercaptoethanol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,062,935 (MASSON et al) 13 December 1977, col. 8, lines 57-60.	1-17, 19-26, 30
Y	US, A, 4,379,085 (WILLIAMS et al) 05 April 1983, col. 2, lines 41-54; col. 3, lines 18-28; col. 5, lines 1-3; figure 1.	1-17, 21-30
Y	N.R. ROSE et al, "MANUAL OF CLINICAL LABORATORY IMMUNOLOGY" published 1986 by American Society for Microbiology (Washington, D.C.), pages 204-210, see especially page 208.	20

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

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*& document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 26 June 1993	Date of mailing of the international search report JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer NANCY J. PARSONS Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02901

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF LABORATORY CLINICAL MEDICINE, Volume 92, Number 4, issued October 1978, J.E. RUTSTEIN et al, "Rheumatoid factor interference with the latex agglutination test for fibrin degradation products", pages 529-535, see especially pages 530-531.	14-17, 19, 21-30
Y	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 121, issued 1989, O.P. BORMER, "Interference of complement with the binding of carcinoembryonic antigen to solid-phase monoclonal antibodies", pages 85-93, see especially pages 87, 89, 92.	1-17, 21-30
Y	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 73, issued 1984, G. VANHAM, et al, "Influence of serum complement and rheumatoid factor on detection of immune complexes by the c1q and monoclonal rheumatoid factor solid-phase assay", pages 301-311, see especially pages 301-303, 310.	1-17, 21-30