

[54] **RHEUMATOID AGGLUTINATION
TEST AND REAGENT**

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[58] **Field of Search**.....424/12, 8

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[57] **ABSTRACT**

A rheumatoid agglutination test reagent, which can be used for detecting rheumatoid factor in whole blood as well as serum, and which demonstrates excellent results with respect to its sensitivity, reproducibility in the detection, comprising a buffered saline of a pH 7.5 $\pm$ 1.5 wherein are contained proper amounts of γ -globulin coated resin particles formed by combining an ion-exchange resin with human γ -globulin in the said solution and one class of assistant selected from the group consisting of hemolytic agent and coloring agent which can color the said γ -globulin, method for preparation thereof and a diagnostic method of using some.

13 Claims, No Drawings

RHEUMATOID AGGLUTINATION TEST AND REAGENT

This invention relates to a rheumatoid agglutination test reagent which can be used for detecting rheumatoid factor in whole blood as well as serum, a technique not practiced heretofore, and which demonstrates excellent results with respect to its sensitivity, a reproducibility in the detection. This detecting method is superior to the conventional method in view of simplicity in testing. It also relates to the method of preparing the same as well as a diagnostic method of using the same. More particularly, the invention relates to a rheumatoid agglutination test reagent comprising a buffered saline of a pH 7.5 ± 1.5 wherein are contained a proper amount of γ -globulin coated resin particles formed by combining an ion-exchange resin with human γ -globulin in the said solution and one class of assistant selected from the group consisting of hemolytic agents and coloring agent which can color the said γ -globulin, and also preferably at least one other assistant selected from the group consisting of preservatives and stabilizers; and also to a method of preparing this reagent and a diagnostic method which uses the same.

It is known heretofore to diagnose rheumatism serologically by utilizing the γ -globulin coated particles formed from sheep's red blood corpuscle and a rabbit anti-sheep antibody. When these γ -globulin coated particles are mixed with the serum separated from man, agglutination takes place when the rheumatoid factor is present and does not occur in the absence of the said factor. This method of diagnosis is known as the Waaler-Rose Test (also referred to as simply the Rose Test). As a modification of this test, the Heller Modification which has improved on the accuracy of determination of the Rose Test is also known.

Further, also known are the method which use as the aforesaid γ -globulin coated particles those in which polystyrene latex is used instead of the red blood corpuscle and native or aggregated human γ -globulin is used instead of the aforesaid antibody (RA-Test) and the method which uses as the γ -globulin coated particles those in which particles of absorbents such as bentonite, kaolin and raw latex are utilized instead of the polystyrene latex in the aforesaid RA-Test.

The most important feature of the present invention is the utilization of an ion-exchange resin as an absorbent.

As hereinbefore noted, serum was used in all of the heretofore proposed methods of detecting the rheumatoid factor. This involved the disadvantage of separating the serum from the whole blood that was collected as well as the operation of diluting the serum. Hence, it could not be practiced readily, as it was necessary to have a fairly well equipped laboratory.

On the other hand, in the case of the aforesaid some γ -globulin coated particles consisting of polystyrene latex and γ -globulin, even though there was the advantage that the said latex as a compound which could be readily produced with fair reproducibility by a simple process, it had the disadvantage that due to the modification of the γ -globulin and nonspecific agglutination of the resin not only its stability was poor but also the reliability of its detection was inadequate, with the consequence that there was the disadvantage that in judging the results of the test a considerable degree of skill was required.

While the reason for the foregoing defect is not fully clear as yet, it is thought the principal reasons are that the binding between the particles of polystyrene latex and the γ -globulin is weak and further that the γ -globulin is modified to a degree in excess of that required for the test during the preservation of the reagent.

As a result of our research with a view to overcoming these numerous disadvantages and shortcomings, we found that as the γ -globulin coated particles those consisting of an ion-exchange resin and human γ -globulin could be used and that by the use of these in particular the complicated serum diluting operation could be dispensed with even when serum was to be used for the test. It was further found that a diagnostic reagent could be provided which could also be used for testing the whole blood. In addition, we found that this reagent not only demonstrated excellence with respect to its detective sensitivity and reliability of detection but also possessed excellent stability regardless of whether it was used for testing serum or whole blood. It was also found that the ion-exchange resin to be used for preparing these γ -globulin coated particles was readily available and also that the operation of preparing these particles was simple.

It is therefore an object of the present invention to provide a rheumatoid agglutination test reagent which has excellent stability and demonstrates superior detective sensitivity as well as superior reliability of detection. Another object is to provide a method of preparing such a reagent. A further object is to provide a method of diagnosis which uses this reagent.

Other objects and advantages of this invention will become apparent from the following description.

The γ -globulin coated particles which by being present in the rheumatoid agglutination test reagent of the present invention form agglutinations by reacting with the rheumatoid factor can be obtained by contacting an ion-exchange resin with human γ -globulin in a buffered saline.

As this buffered saline, useable is an aqueous saline solution whose pH has been adjusted to 7.5 ± 1.5 , preferably 6.5 - 8.5, and particularly preferably to 7.5 - 8.5, using an acid component such as glycine, tris(hydroxymethyl)aminomethane, potassium dihydrogenphosphate, boric acid, and hydrochloric acid, and a basic component such as an alkali hydroxide as sodium hydroxide and potassium hydroxide, an alkali carbonate as sodium carbonate and potassium carbonate, and others such as borax and dipotassium hydrogenphosphate. Especially preferred is the buffered solution in which glycine and sodium hydroxide have been used.

The γ -globulin coated particles which have been formed can be separated from the buffered saline in which they were formed to be suspended in a separately prepared buffered saline, after which one of either a hemolytic agent or a coloring agent which can color the γ -globulin may be added. Alternatively, the hemolytic agent or coloring agent can be added to the buffered solution in which the γ -globulin coated particles have been formed, without separation of the particles therefrom. Hence, the pH of the buffered saline need not necessarily be adjusted to the pH specified in forming the foregoing particles, and the adjustment of the pH to 7.5 ± 1.5 , preferably 6.5 - 8.5, and particularly preferably to 7.5 - 8.5 need only be carried out so as to attain these pH values in the final product.

In combining the ion-exchange resin with the human γ -globulin in the aforesaid buffered saline, the addition of the two components to the buffered solution may be made at the same time, or the ion-exchange resin may be added first followed by the addition of the human γ -globulin, or the addition of the two may be in reverse order, i.e., adding the human γ -globulin first followed by adding the ion-exchange resin. Again, it is also possible to add the two components separately to two buffered solutions, which are then mixed.

There is no particular restriction as to the temperature of the reaction at which the γ -globulin coated particles are formed, it being possible to carry out this reaction under conditions of application of heat or cooling. For example, the reaction can be carried out while cooling in an ice waterbath, or it can be carried out while being heated. It is usually preferred however to operate at a temperature of $0^\circ - 10^\circ \text{C}$.

As examples of the ion-exchange resins to be used in forming the γ -globulin coated particles, included are those selected from the group consisting of the strongly acidic ion-exchange resins, the strongly basic ion-exchange resins and the weakly basic ion-exchange resins. Particularly preferred for accomplishing a satisfactory combination with the human γ -globulin are the weakly basic ion-exchange resins.

The particle size of these ion-exchange resins is preferably of an average particle diameter of $0.5 - 3$ microns, and from the standpoint of attaining an improved stability for the product reagent it is preferred that particles coming within the foregoing particle diameter range account for 75 percent by weight, preferably 85 percent by weight, and particularly preferable at least 90 percent by weight of the total particles. The ion exchange resins that may be employed include the strongly acidic ion-exchange resins, such as sulfonated styrene-divinylbenzene and sulfonated phenolic resins; the strongly basic ion-exchange resins, generally quaternary ammonium styrene polymers; and the weakly basic ion-exchange resins, such as the amine derivatives of crosslinked polystyrene, crosslinked acrylic and phenol-formaldehyde types.

As specific examples of these ion-exchange resins, mention can be made of the strongly acidic ion-exchange resins such as Amberlite IR-120 (Rohm & Haas Co.), Diaion SK-1 (Mitsubishi Chemical Co.), Dowex 50W (Dow Chemical Co.); and Duolite C-20 (Diamond Alkali Co.), C-3, C-10; the strongly basic ion-exchange resin such as Amberlite IRA-400, 900, Diaion SA-10A, 20A, Dowex 1, 2; and Duolite A-101, A-40; and the weakly basic ion-exchange resins such as Amberlite, IR-45, IR-4B, XE-168, IRA-68, IRA-93; Duolite A-2, A-30T, A-30B, A-4, A-41 (contains quaternary ammonium and tertiary amine groups); and Dowex 4, 44.

According to the present invention, the γ -globulin coated particles consisting of the aforesaid ion-exchange resin and human γ -globulin are used with or without first being separated from the buffered saline in which they were formed. In the case they have been separated, they are added to a similar buffered saline such as described. To the buffered solution thus containing the γ -globulin coated particles are then incorporated as assistant either a hemolytic agent or a coloring agent which can color the said γ -globulin. Still more preferably, at least one other assistant selected from

the group consisting of the preservatives and stabilizers can be incorporated.

A single class or a plurality of classes of the hemolytic agents, coloring agents, preservatives and stabilizers can be incorporated. Further, there are no restrictions as to the order in which the assistants are added. They can be added in any order or they can all be added at once.

As the hemolytic agent, useable are such as the saponin, alkaloids, salts of bile acid, urea, alkylamines and surfactants. As the foregoing surfactants useable are the anionic surfactants such as sodium oleate, sodium lauryl sulfate, sodium cetyl sulfate, sodium stearyl sulfate, sodium alkylbenzene sulfonate and dialkyl sulfosuccinate; the cationic surfactants such as alkyltrimethylammonium chloride, benzyldimethylalkylammonium bromide and cetylpyridinium chloride; the nonionic surfactants such as polyoxyethylene-sorbitanmonolaurate, polyoxyethylene-sorbitanmonooleate, polyoxyethylene stearate, polyoxyethylene lauryl alcohol, and polyglycol ether of alkylphenol; the amphoteric surfactants of the fatty acid imidazoline, Miranol, type; and the polyethylene glycol type activators (200, 400, 600 and 800). Of these hemolytic agents, particularly advantageous are the saponin, dodecylamine acetate, which is an alkylamine, or the Miranol type activator.

On the other hand, the coloring agent may be any which is capable of coloring human γ -globulin. Included are, for example, as red, Ponceau R (C.I. 16150), Ponceau 3R (C.I. 16155) and Rose Bengal (C.I. 45440), as green, Naphthal Green B and Brilliant Green (C.I. 42040), and as blue, Evans Blue, Brilliant Blue FCF (C.I. 42090), Patent Blue (C.I. 42051) and Trypan Blue (C.I. 23850).

As the preservatives, mention can be made of sodium azide, the phenols such as cresols, chlorothymol, thymol and paraoxybenzoic acid esters, and the mercuric phenyls such as mercuric phenyl acetate, mercuric phenyl sulfate and mercuric phenyl borate.

Again, a stabilizer can be added for further enhancement of the stability of the final product. This is especially true in the case of the reagent containing the γ -globulin coated particles and a hemolytic agent, which is used for directly testing whole blood. Since in this case the elution of hemoglobin and other components take place when the reagent is added to cause, on occasions, the agglutination between the γ -globulin coated particles and factors other than the rheumatoid factor, the use of a stabilizer is to be particularly recommended. As the stabilizers also tend to assist the stability of the γ -globulin, they can also be utilized in the case of a reagent containing the γ -globulin coated particles and a coloring agent, which is to be used for testing serum.

As such stabilizers, included are the serum albumins, peptones, gelatin and such amino acids as acetyl tryptophan.

In the invention reagent, the amounts in which the γ -globulin coated particles, hemolytic agent, coloring agent, preservative and stabilizer are contained in the buffered saline can be suitably varied depending upon the classes of the agents and their combination. Usually, the following ranges are used based on the reagent; namely, $0.5 - 2$ percent by weight, and

preferably 1 - 1.5 percent by weight, of the γ -globulin coated particles; 0.5 - 2 percent by weight, and preferably 0.1 - 0.5 percent by weight, of the hemolytic agent; 0.005 - 0.15 percent by weight, and preferably 0.01 - 0.1 percent by weight, of the coloring agent; 0.005 - 0.5 percent by weight, and preferably 0.1 - 0.2 percent by weight, of the preservative; and 0.005 - 1.0 percent by weight, and preferably 0.01 - 0.5 percent by weight, of the stabilizer.

In diagnosing rheumatism using the invention reagent, when the reagent used is that of a pH 7.5 ± 1 and containing the γ -globulin coated particles and a coloring agent in a buffered saline, which may also contain a preservative and/or a stabilizer, serum is separated from the whole blood and this serum is submitted to the test directly. The complicated procedure of diluting the serum as in the conventional technique becomes unnecessary in this case. On the other hand, when the reagent used is one of a pH 7.5 ± 1 and containing the γ -globulin coated particles and a hemolytic agent in a buffered saline, which may also contain a preservative and/or a stabilizer, the whole blood can be used for the testing. The test can be carried out very readily and promptly as in the case of the determination of blood type. That is to say, when fresh blood taken from the ear or finger is mixed with a drop of the reagent, the characteristics of the rheumatoid agglutination are demonstrated as in the case of the conventional method which uses serum. Further, in view of the color tone of hemoglobins, the determination can be accurately made without the necessity of prior coloring with a coloring agent.

Two typical examples of preferred recipes of the rheumatoid agglutination test reagent of the present invention are given below.

(1) When whole blood is to be tested.

A glycine-buffered saline of a pH 8 - 9 containing the following:

	Grams per 100 ml of solution
γ -globulin coated particles consisting of a weakly basic ion-exchange resin and human γ -globulin	1 - 2.0
Saponin (hemolytic agent)	0.3 - 0.7
Sodium azide (preservative)	0.1 - 0.15
Serum albumin (stabilizer)	0.27 - 0.34
Acetyl tryptophan (stabilizer)	0.01 - 0.015

One example of this type is as follows:

	Content (gram) per 100 ml of glycine buffered saline
γ -globulin coated particles consisting of human γ -globulin and weakly basic ion-exchange resin Amberlite IR-45 (average particle diameter 2.1 μ , 90% being of particle diameters 1 - 3 μ)	1.50
Saponin	0.50
Sodium azide	0.10
Serum albumin of cow	0.30
Acetyl tryptophan	0.012

(2) When serum is to be tested.

γ -globulin coated particles consist- ing of a weakly basic ion-exchange resin and human γ -globulin	0.5 - 1.0%
Sodium azide (preservative)	0.1 - 0.15 %
Serum albumin (stabilizer)	0.27 - 0.34 %

Trypan Blue (coloring agent) 0.01 - 0.015 %
An example of such a type is given below:

	Content (gram) per 100 ml of glycine buffered saline
γ -globulin coated particles consisting of human γ -globulin and weakly basic ion-exchange resin Amberlite IR-45 (average particle diameter 2.1 μ , 90 % being of particle diameters 1 - 3 μ)	1.50
Evans blue	0.01
Sodium azide	0.10
Serum albumin of cow	0.30
Acetyl tryptophan	0.012

For a better understanding of the present invention, the following examples are given which illustrate several modes of practicing the present invention.

EXAMPLE 1

Preparation of a reagent to be used with whole blood.

Two grams of an ion-exchange resin Dowex 44 (OH type, product of Dow Chemical Co.) crushed and rendered into uniform fine particles of diameters 1 - 2 microns are suspended in 14 ml of glycine buffered saline (pH 8.2, N/10 glycine and N/20 sodium hydroxide). Commercially available human γ -globulin is diluted with the aforesaid aqueous solution to a concentration of 2 percent. After heating this latter aqueous solution for 10 - 15 minutes at $60^\circ - 65^\circ \text{C}$., 1.5 ml thereof is added to 1.5 ml of the aforesaid suspension, following which the reaction is carried out for 2 hours in ice water with stirring. Next, the resin (resulting γ -globulin coated particles) is centrifuged and settled, followed by thorough washing and thereafter adding of 8 ml of the aforesaid glycine buffered saline. To this suspension are then added 40 mg each of nonionic polyoxyethylene lauryl ether (hemolytic agent) and albumin (stabilizer) and further 0.8 mg of thimerosal (preservative) to obtain the product (pH = 8.5). This reagent is preserved at $2^\circ - 10^\circ \text{C}$. and is shaken well before using.

EXAMPLE 2

Preparation of a reagent to be used with whole blood.

To a suspension processed as in Example 1 except that 2 grams of finely divided ion-exchange resin Amberlite IR-45 (NH_2 type, product of Rohm & Haas Co.) are used, are added 40 mg each of saponin (hemolytic agent) and albumin (stabilizer) and also 10 mg of sodium azide (preservative) to obtain the product (pH = 8.5).

EXAMPLE 3

Preparation of a reagent to be used with whole blood.

To a suspension processed as in Example 1 except that 2 grams of finely divided ion-exchange resin Amberlite IR-4B (OH type) are used, are added 40 mg each of laurylamine (hemolytic agent) and albumin (stabilizer) and also 0.8 mg of chlorothymol (preservative) to obtain the product (pH = 8.2).

EXAMPLE 4

Preparation of a reagent to be used with whole blood.

To a suspension as in Example 1 except that 2 grams of finely divided ion-exchange resin Amberlite IRA-411 (chloro type) are used, are added 40 mg each of saponin (hemolytic agent) and albumin (stabilizer) and also 0.8 mg of mercuric phenyl sulfate (preservative) to obtain the product (pH = 8.2).

EXAMPLE 5

Preparation of a reagent to be used with whole blood.

To a suspension processed as in Example 1 except that finely divided ion-exchange resin Amberlite IRC-50 (H type) is used, are added 40 mg each of Miranol C2M (hemolytic agent) and albumin (stabilizer) and also 0.8 mg of thimerosal (preservative) to obtain the product (pH = 8.8).

EXAMPLE 6

Preparation of a reagent to be used with serum.

Two grams of an ion-exchange resin Dowex 44 (or 3) (OH type) comminuted and rendered into uniformly finely divided particles of diameters 1 - 2 microns are suspended in 13 ml of glycine buffered saline (pH 8.2, N/10 glycine, N/20 caustic soda). Separately, commercially available human γ -globulin is dissolved in the same buffered solution to a concentration of 2 percent and heated for 10 - 15 minutes at 60° - 65° C. 1.5 ml of this latter solution is added to 1.5 ml of the foregoing suspension, after which the mixture is reacted for 2 hours in ice water with stirring. This is followed by adding 1 percent Brilliant Green solution (coloring agent), centrifuging and settling the colored resin (γ -globulin coated particles), thorough washing and thereafter adding 20 ml of glycine buffered saline containing 0.1 percent sodium azide (preservative) to obtain the product (pH = 7.5). This reagent is preserved at 2° - 10° C. and is thoroughly shaken before using.

EXAMPLE 7

Preparation of a reagent to be used with serum.

0.5 Gram of finely divided ion-exchange resin Amberlite IR-120 (H type, product of Rohm & Haas Co.) is suspended in 10 ml of glycine buffered saline (pH 8.2) containing 0.01 percent thimerosal (preservative). To this suspension are added 10 ml of the same 2 percent human γ -globulin solution as that described in Example 6, after which the mixture is heated at 55° C. for 15 minutes. This is followed by adding 0.1 percent Evans Blue solution (coloring agent) to dye the mixture, followed by the addition of mercuric phenyl acetate (preservative) and dilution of the mixture with 80 ml of the aforesaid glycine buffered saline to obtain the product (pH = 8.2).

EXAMPLE 8

Preparation of a reagent to be used with serum.

Weight %

γ -globulin coated particles consisting of human γ -globulin and the same ion-exchange resin as used in Example 7 0.5 - 1.0

Thimerosal
Trypan Blue
Albumin

0.01
0.01
0.27 - 0.34

Starting materials of the foregoing composition are used and a reagent is prepared as in Example 6, ensuring that the maximum concentrations of the ingredients in a glycine buffered saline (pH 6.5 - 8.5) are in the foregoing properties.

EXAMPLE 9

Preparation of a reagent to be used with serum.

0.5 Grams of finely divided ion-exchange resin Dowex 44 is suspended in 10 ml of glycine buffered saline (pH = 8.2). Ten ml of a separately prepared 2 percent human γ -globulin solution heated in advance at 60° - 65° C. for 10 - 15 minutes are then reacted in the cold state with the foregoing suspension. This is followed by the addition of 0.1 percent Evans Blue solution (coloring agent) and chloroethymol (preservative) and treating as in Example 7 to obtain the product (pH = 8.2).

We claim:

1. A rheumatoid agglutination test reagent for detecting rheumatoid factor in whole blood comprising a buffered saline solution having a pH of 6 to 9 containing:

a. 0.5 - 2 percent by weight of weakly basic ion exchange particles coated with human γ -globulin; and

b. 0.05 - 2 percent by weight of a hemolytic agent.

2. The reagent of claim 1 wherein said hemolytic agent is selected from the group consisting of saponin, nonion polyoxyethylene lauryl ether and laurylamine.

3. The reagent of claim 1 wherein at least 75 percent by weight of said weakly basic ion-exchange resin particles have an average particle diameter of from about 0.5 to about 3 microns.

4. The reagent of claim 1 wherein said reagent further contains:

d. 0.005 - 0.5 percent by weight of a preservative selected from the group consisting of sodium azide, cresol, chlorothymol, thymol, para-ox-ybenzoic acid esters, thimerosal, mercuric phenyl acetate, mercuric phenyl sulfate and mercuric phenyl borate.

5. The reagent of claim 1 wherein said reagent further contains:

e. 0.005 - 1.0 percent by weight of a stabilizer selected from the group consisting of acetyl tryptophan, albumin, peptone and gelatin.

6. A method of preparing a rheumatoid agglutination test reagent when comprises contacting weakly basic ion-exchange resin particles with human γ -globulin in a buffered saline solution having a pH of from about 6 to about 9 so as to form weakly basic ion-exchange particles coated with said human γ -globulin and subsequently adding thereto a hemolytic agent the resulting reagent comprising 0.05 - 2 percent by weight of each of said hemolytic agent and globulin coated particles.

7. The method of claim 6 wherein said hemolytic agent is selected from the group consisting of saponin, nonionic polyoxyethylene lauryl ether and laurylamine.

8. The method of claim 6 wherein at least 75 percent by weight of said weakly basic ion-exchange resin parti-

cles have an average diameter of from about 0.5 to about 3 microns.

9. A method of diagnosing rheumatoid factor which comprises mixing human whole blood with an effective agglutinating amount of a test reagent which comprises a buffered saline solution of a pH of from about 6 to about 9 containing:

- a. 0.5 - 2 percent by weight of weakly basic ion-exchange particles coated with human γ -globulin; and
- b. 0.05 - 2 percent by weight of hemolytic agent.

10. The method of claim 9 wherein said hemolytic agent is selected from the group consisting of saponin, nonionic polyoxyethylene lauryl ether and laurylamine.

11. The method of claim 9 wherein at least 75 percent by weight of said weakly basic ion-exchange resin

particles have an average particle diameter of from about 0.5 to about 3 microns.

12. The method of claim 9 wherein said test reagent further contains:

- d. 0.005 - 0.5 percent by weight of a preservative selected from the group consisting of sodium azide, cresol, chlorothymol, thymol, para-ox-ybenzoic acid esters thimerosal, mercuric phenyl acetate, mercuric phenyl sulfate and mercuric phenyl borate.

13. The method of claim 9 wherein said test reagent further contains:

- e. 0.005 - 1.0 percent by weight of a stabilizer selected from the group consisting of acetyl tryptophan, albumin, peptone and gelatin.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,689,632

Dated September 5, 1972

Inventor(s) YUTAKA MIZUSHIMA and HIDETAKA NAGAI

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Claim 2, Column 8, line 35, change "nonion" to --nonionic--

Claim 4, Column 8, line 42, change "d." to --c.--

Claim 5, Column 8, line 51, change "e." to --d.--

Claim 12, Column 10, line 5, change "d." to --c.--

Claim 13, Column 10, line 13, change "e." to --d.--

Signed and sealed this 30th day of January 1973.

(SEAL)

Attest:

EDWARD M. FLETCHER, JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents