

[54] **FLUORESCENT LABELED ANTIBODY REAGENT**

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[57]

ABSTRACT

A lyophilized stable labeled antibody reagent that
comprises a non-reducing polysaccharide, a normal
serum and a labeled antibody, particularly an antibody
labeled with a fluorescent chemical is provided that is
exceptionally specific to a given antigen. Specificity is
such that Group A, β -hemolytic Streptococci, can
readily be identified in the presence of Group C or
Group G Streptococci or *Staphylococcus aureus*. The
reagent is stable in the lyophilized state at tempera-
tures of up to 37°C. for a period of months and in the
reconstituted state at 2° to 8° C. for a period of weeks.

7 Claims, No Drawings

FLUORESCENT LABELED ANTIBODY REAGENT

This invention relates to new and novel compositions of labeled antibodies which are useful for detecting the presence of and identifying various micro-organisms including viruses, both DNA and RNA; bacteria, fungi, protozoans, helminths, and for the determination of various materials such as neoplastic cells, urokinase, albumen concentrations, and the like, which are present in tissues.

Labeled antibodies have been used previously for identifying various antigens. The basis for their action is that each antibody has a counterpart in some antigen. An antibody specific to an antigen will become attached to that antigen whenever encountered by the antibody. If an antibody that is known to be specific for a particular antigen within a group of antibodies is isolated from the globulin portion of serum or plasma of a host animal which was stimulated to produce that antibody, it can then be labeled or tagged by known means by conjugating the antibody with a labeling agent. Such labeling agents include physically detectable substances such as fluorescent chemicals, radioactive isotopes and the like.

Then, when used diagnostically, if the counterpart antigen is present in some prepared sample, the labeled antibody will attach itself to that antigen. The presence of the antigen can then be confirmed through detection of the labeling agent in the sample. Thus, in the instance of a fluorescent labeling agent, a microscopic examination under illumination which causes the labeling agent to fluoresce, reveals the presence of any antibody that has been attached to a counterpart antigen.

For diagnostic purposes, a labeled antibody should be made quite specific. For highest specificity, a labeled antibody should be so monospecific that it will react only with those antigens whose detection is desired, but without cross reaction with other closely related antigens which may have quite dissimilar or insignificant clinical consequences.

At the present time, there exist methods that are capable of producing such monospecific antibodies, but these highly specific labeled antibodies have proven quite unstable. Efforts to produce labeled antibodies that are both monospecific and stable have been thus far unsatisfactory.

Not only is it desirable to develop stabilized compositions of monospecific antibodies in general, but there is a particular need for a specific, stable, labeled antibody that can be used diagnostically to determine the presence of the A Group of β -hemolytic Streptococci. Identification of Group A β -hemolytic Streptococci is especially important because of its involvement in such diseases as scarlet fever, rheumatic heart disease and glomerulonephritis. A complaint of sore throat and fever, although symptoms of infection with β -hemolytic Streptococci, are also symptomatic of many other less serious infections of minor consequence. If the infection would be Streptococcal in nature and not controlled, complications can result in susceptible individuals.

Accordingly, a primary object of this invention is to provide an improved labeled antibody reagent having superior sensitivity that assures clear and distinct readings that enable one to detect the presence of organisms at a count as low as 500 per milliliter.

An additional object of this invention is to provide a labeled antibody reagent that resuspends to give a clear solution without requiring centrifugation to remove particulate matter prior to use.

A still further object of this invention is to provide a labeled antibody reagent that is highly specific giving consistent 4+ readings.

And yet a further object is to provide a labeled antibody reagent that is stable in the lyophilized state for a period of months at -20 to $+37^{\circ}$ C., and furthermore, when reconstituted, results in an operative solution which can be stored at from about $2-8^{\circ}$ C. for weeks without loss of sensitivity, and also which operative solution can be refrozen without significant loss of potency for a period of several months.

In carrying out this invention, a highly specific, preferably, a monospecific antibody is first obtained by recognized methods that are known in the art. This monospecific antibody is then conjugated with a labeling agent. Fluorescein isothiocyanate (FITC) is the preferred labeling agent particularly because of its excellent fluorescent characteristics including both brightness and color which make it stand out against a background. When FITC is used as the labeling agent, the ratio of FITC to the protein of the antibody on a weight basis should be in the range of from 5 to 20 percent and preferably 4 to 12 percent. The conjugated labeled antibody is then blended with normal serum preferably obtained from the same species used to produce the specific antibody. The addition of the normal serum has the beneficial effect of further reducing or eliminating whatever residual non-specificity of the labeled antibody may remain. The stability of the now highly specific labeled antibody is further increased by the addition of a non-reducing polysaccharide, a disaccharide of the polysaccharide sucrose being preferred. To enhance shelf life, a preservative such as thimerosal can be added if desired.

The mixture comprising a phosphate saline buffered blend of labeled antibody, normal serum, and disaccharide is lyophilized and sealed under vacuum. While the volume of the solution that is to be lyophilized is not critical, it has been found that best results are obtained when the volume is from one-half to one-twentieth of the volume of reconstituted solution.

The following specific example will further illustrate the preparations and use of the novel reagent of this invention.

Group A Streptococci, type 12, were grown in 56 liters of Todd-Hewitt broth, harvested and washed five times with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.0 (PBS). The wet weight of the packed cells was 318 grams. To 5 grams of washed cells were added 40 ml. of pepsin solution (1.0 mg/ml) in 0.01N HCl. The pH of the solution was adjusted to 2.0 using 1N HCl. The suspension was incubated in a 37° C. water bath for 2 hours with magnetic stir bar agitation. The suspension was then neutralized with 1 N NaOH and concentrated by centrifugation. The concentrated suspension was washed three times with 0.15 M NaCl and finally resuspended in 30 ml. of 0.15 M NaCl. To 10 ml. of this suspension in a vaccine bottle was added 70 ml. of 0.15 M NaCl and 0.8 ml. of 1 percent thimerosal. After proper sterility checks, this vaccine was used to inject New Zealand White Rabbits and produce immune sera to the group specific polysaccharide of Group A Streptococci.

Group A Streptococci, type 12, were grown in 56 liters of Todd-Hewitt broth, harvested and washed three times with PBS and two times with distilled water. The wet weight of the packed cells was 360 grams. The collected cells were diluted with distilled water to give a total volume of 1,200 ml. To a 6 oz. Teflon screw cap bottle were added 80 grams of sterile dried glass beads (Superbrite, type 120, 0.0045 inches av. diameter), 3M Co., 40 ml. of bacterial suspension, and 0.05 ml. of octyl alcohol. The capped bottle was shaken for 3 minutes at 4,000 oscillations per minute with sufficient CO₂ delivered to the chamber to prevent heating. The broken cells were separated from the glass beads by filtration through a coarse sintered glass filter. The filtrate of broken cells was collected by centrifugation, resuspended in M/30 phosphate buffer, pH 8, and washed twice with this buffer. The washed cells were resuspended in the pH 8 buffer and treated with trypsin by incubation with trypsin (0.01% w/v) at 37° C. for 2-3 hours. The trypsin treated cell walls were washed three times with phosphate buffer and then lyophilized. The dried cell walls were extracted with 300 ml. formamide by heating at 148-158° C. for 40 minutes. The formamide extract was clarified by centrifugation and any solubilized proteinaceous material precipitated from the supernatant by the addition of 2.5 volumes of 95 percent ethanol containing 1% HCl. To the clarified supernatant was added an equal volume of cold acetone acidified with 1% HCl and the precipitated group specific polysaccharide collected by centrifugation. The precipitated polysaccharide was washed successively with 95 percent ethanol and ethyl ether and the granular polysaccharide collected. This polysaccharide was analyzed and shown to contain 75-80 percent rhamnose, 10-20 percent N-acetyl glucosamine, 2 percent protein (Lowry) believed due to amino acids (\approx 2 percent).

The rabbit sera used was first heated for 30 minutes at 56° to decomplement it and centrifuged to remove any particulate material. While gently mixing a quantity of rabbit serum such as 10 ml., 2 mg. of the afore-described formamide extracted group specific polysaccharide was added in a volume of 1 ml. The preparation was mixed, incubated at room temperature for 10-15 minutes and then incubated overnight at 0-4° C. The antigen-antibody complex was collected by centrifugation and washed four times with 0.15 NaCl by resuspending the pelleted complex in the saline and then repelleting the complex by centrifugation. The washed complex was dissociated by dispersing the pellet in 0.05 M glycine-HCl, NaCl buffer, pH 2.65 and adjusting the pH to 2 with HCl.

The monospecific antibody was separated from the polysaccharide by loading it on a 5.0 x 31 cm Sephadex-G-100 column equilibrated with glycine buffer. The monospecific antibody was eluted with glycine buffer into collection tubes containing Trisglycine buffer of Ph 8.4 to neutralize the antibody solution. The monospecific antibody was then concentrated by pressure dialysis and washed with normal saline (0.85 percent solution of NaCl).

The monospecific antibody was then conjugated with FITC at a protein to fluorescein (w/w) ratio of 5:1. The excess non-conjugated FITC was removed by passing the mixture through a Sephadex-G-50 column equilibrated with pH 7.2 phosphate buffered saline and

eluted with phosphate buffered saline. The eluted conjugated specific antibody is then collected.

The specific antibody was then blended with phosphate buffered saline (0.15 Molal NaCl; 0.0067 Molal Na₂HPO₄ · 7H₂O; and 0.0029 Molal NaH₂PO₄ · H₂O).

To the phosphate buffered saline is then added normal rabbit serum, and sucrose together with thimerosal. The final solution had the following concentration:

Antibody	0.1 grams protein/liter solution
Normal serum	35 grams protein/liter solution
Sucrose	55 grams/liter solution
Thimerosal	0.01 gram/liter solution

This labeled antibody reagent was itself one-tenth the volume of the preferred volume of an operative solution after having been reconstituted with a tissue isotonic solution preferably a phosphate buffered saline. When reconstituted, it is desired that the concentration of antibody in the operative solution should be 0.01 gram of protein/liter.

The labeled antibody reagent is then lyophilized for 17-18 hours at 5 μ pressure at 5° C. After lyophilization is complete, the vials are sealed. Experience has shown that in the lyophilized state the composition is stable in excess of 12 months of 37° C.

The operative solution is used in the same manner as are presently known labeled antibody solutions. For example, such techniques are fully set forth in *Fluorescent Antibody Methods*, M. Goldman, Academic Press (1968); and Cherry, W.B., Goldman, M., Carski, T.R. and Moody, M.D., Public Health Service Publication No. 729, "Fluorescent Antibody Techniques in the Diagnosis of Communicable Diseases," U.S. Government Printing Office, Washington, D.C. 1960.

Although the solution set forth in the previous example is the preferred embodiment, the labeled antibody reagent of this invention can comprise a isotonic solution, and aqueous phosphate buffered saline being suitable, having from 1.0 to 0.05g. of antibody protein per liter of solution; 20 to 50 grams protein per liter of solution of normal serum; 8 to 75 grams per liter of a polysaccharide, e.g., sucrose fructose and a small amount of a preservative such as to render the solution bacteriostatic.

In the lyophilized state, the lyophilized material should comprise at least 0.05 percent monospecific labeled antibody, 10 to 75 percent of a polysaccharide and 20 to 55 percent of normal serum.

I claim:

1. A stable fluorescent labeled antibody reagent specific to Group A streptococci comprising an isotonic solution and

- a. a monospecific antibody specific to Group A streptococci conjugated with a fluorescent labeling agent on a weight basis of 5 to 20 percent of said labeling agent based on the protein content of said antibody, the conjugated labeled antibody having a concentration of 1 to 0.05 grams protein derived therefrom per liter of solution;
- b. a normal serum comprising from about 20 to about 50 grams protein derived therefrom per liter of solution, said normal serum being derived from the same host species as that of the monospecific antibody;
- c. a polysaccharide selected from the group consisting of sucrose and fructose for stabilizing said reagent.

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- gent comprising 8 to 75 grams per liter of solution; and
- d. a sufficient quantity of a preservative to render said reagent bacteriostatic.
2. The reagent of claim 1 in which said labeling agent is fluorescein isothiocyanate.
3. The reagent of claim 1 in which said polysaccharide is sucrose.
4. The reagent of claim 1 in which said preservative is thimerosal.
5. The reagent of claim 1 in which said isotonic solution is phosphate buffered saline.
6. A reagent solution suitable for lyophilization comprising an admixture of at least 0.05 percent monospecific fluorescent labeled antibody for the detection

of Group A streptococci, 10 to 75 percent polysaccharide selected from the group consisting of sucrose and fructose, 20 to 55 percent normal serum derived from the same host species as that of the monospecific antibody; and a quantity sufficient of a preservative, said admixture combined with an isotonic carrier.

7. A lyophilized reagent comprising at least 0.05 percent of a monospecific fluorescent labeled antibody, specific to Group A streptococci, 10 to 75 percent of a polysaccharide selected from the group consisting of sucrose and fructose, and 20 to 55 percent of a normal serum derived from the same host species as that of the monospecific antibody.

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