United States Patent Office 3,708,572 Patented Jan. 2, 1973

3,708,572

INFECTIOUS MONONUCLEOSIS DIAGNOSTIC REAGENT AND METHOD

Frans Peetoom, Westwood, and Sandra Jean Kiddy, Pasadena, Calif., assignors to Baxter Laboratories, Inc., Morton Grove, Ill.

No Drawing. Filed Mar. 26, 1969, Ser. No. 810,827
Int. Cl. G01n 31/00, 33/16
U.S. Cl. 424—12 3 Claims

ABSTRACT OF THE DISCLOSURE

A reagent and method for the diagnosis of infectious mononucleosis employing horse erythrocytes which have been subjected to aldehyde fixation, specific sensitization with antiserum to Forssman-like antigens and then another fixation with said aldehyde.

This invention relates to a reagent and method for the diagnosis of infectious mononucleosis. More particularly, this invention relates to a reagent and method employing an agglutination reaction for the serological diagnosis of infectious mononucleosis in a one-step procedure that is specific for infectious mononucleosis.

Infectious mononucleosis is believed to be a viral disease although the infecting organism is not definitely known. The disease is accompanied by conspicuous hematologic findings and generally by a heterophilic titer against sheep erythrocytes of at least about 1:56.

It is known that many cases of infectious mononucleosis present great diagnostic difficulties. Over the years, various methods for the serological diagnosis of infectious mononucleosis have been suggested. Several agglutination tests have been devised heretofore which are based upon the phenomenon whereby the sera from patients with infectious mononucleosis will agglutinate the erythrocytes of various animal species such as the horse, ox and sheep. The agglutination is caused by the reaction between heterophile antibodies in the patients' sera and certain antigens in the erythrocytes from these animals.

Recently, Lee et al., Am. J. Clin. Path., vol. 49, pp. 3–18 (1968), reported that of the several different varieties of erythrocytes used in serological research on infectious mononucleosis, horse erythrocytes were found to be the most sensitive. These investigators also disclosed a spot test employing horse erythrocytes preserved with 3.8% soduim citrate. However, it is essential that this spot test be combined with a separate differential test for infectious mononucleosis employing guinea pig kidney in order to provide a complete diagnostic procedure that is specific for infectious mononucleosis.

Another recently described test procedure for infectious mononucleosis, which employs formalinized horse erythrocytes, is the rapid slide test of Hoff and Bauer, J. Am. Med. Ass'n., vol. 194, pp. 351–3 (1965). However, in experiments with this procedure, the desired specificity for infectious mononucleosis was not confirmed. Sinay et al., Am. J. Clin. Path., vol. 50, pp. 75–82 (1968).

The fixation of erythrocytes with aldehydes is described in U.S. Patent 3,096,250 and the use of aldehyde-fixed horse erythrocytes in the diagnosis of infectious mononucleosis is further described in South African Patent No. 66/4,497 and U.S. Patent 3,426,123.

It is an object of the present invention to provide a new and improved reagent and method for the diagnosis of infectious mononucleosis.

It is another object of the present invention to provide a reagent and method employing an agglutination reaction for the serological diagnosis of infectious mononucleosis

in a one-step procedure that is specific for infectious mononucleosis.

Other objects and advantages of the present invention will be apparent to the person skilled in the art after reading the specification herein and the appended claims.

The objects of this invention are achieved by providing a diagnostic reagent comprising especially treated horse erythrocytes for use in an agglutination reaction for the determination of increased heterophile antibodies in sera from patients suffering from infectious mononucleosis. In order to make the diagnostic reagent specific for infectious mononucleosis, it has been found necessary to especially treat the horse erythrocytes by a three-step procedure as follows:

First, by fixation with an organic aldehyde having from 2 to about 6 carbon atoms and containing from one to two —CHO groups, second, by specific sensitization with antiserum to Forssman-like antigens and third, by another fixation with said organic aldehyde.

Organic aldehydes which can be used in the practice of this invention are, for example, formaldehyde, malonic aldehyde, succinic aldehyde, glutaric aldehyde, adipic aldehyde, pyruvic aldehyde and similar such water-soluble aldehydes which are compatible with the horse erythrocytes. Glutaric aldehyde (glutaraldehyde) is the preferred aldehyde in accordance with this invention.

The aldehyde fixation in this procedure is accomplished by mixing the horse erythrocytes with an aqueous solution of from about 0.05% to about 2% aldehyde, and preferably about 1% aldehyde, to a final concentration of from about 0.1% to about 5% horse erythrocytes, and preferably about 2% horse erythrocytes.

The sensitizing with the antiserum to Forssman-like antigens is achieved by thoroughly mixing the initial aldehyde-fixed horse erythrocytes with the antiserum at about 1° C. to about 40° C., and preferably at about 5° C. At the preferred temperature of mixing of about 5° C., the mixing is carried out for about fifteen to about twenty-five hours, and preferably for about sixteen to about twenty hours. In general, as the temperature at which the mixing is carried out is increased, the time of mixing is proportionately decreased.

The proportions of horse erythrocytes and antiserum to Forssman-like antigens admixed in this procedure can vary within wide limits, depending in part upon the titer of the antiserum. In general, as the titer of the antiserum is increased, the volume required for sensitization of the erythrocytes is proportionately decreased. Preferably, about 10 ml. of a 40% horse erythrocytes suspension is mixed with about 80 ml. of antiserum in this procedure.

The antiserum used for coating the aldehyde-fixed erythrocytes is obtained from animals, preferably rabbits, which have been immunized by injection with Forssman-like antigens by any conventional immunization procedures. This antiserum can be antiserum to guinea pig kidney, horse erythrocytes or other tissues of these or other species of animals having similar Forssman-like antigens. As used herein, the term "Forssman-like antigen" is defined to mean those antigens which will not react with specific infectious mononucleosis antibodies. The preferred antigen is a guinea pig kidney antigen (GPK). GPK is preferably prepared by finely grinding or homogenizing a saline suspension of guinea pig kidney.

By employing the above three-step procedure, it has been found that all of the antigenic sites on the horse erythrocytes are blocked except antigenic sites that are specific for heterophile antibodies of infectious mononucleosis. Consequently, the treated horse erythrocytes can be used for the diagnosis of infectious mononucleosis without the problems of false positives or false negatives which occur with previously available methods for the

diagnosis of infectious mononucleosis. Moreover, it is unnecessary to conduct a separate differential test for infectious mononucleosis according to the present invention.

In the performance of the diagnostic test for infectious mononucleosis employing the diagnostic reagent of this invention, the diagnostic reagent contains from about 3% to about 10%, and preferably about 5%, of the treated horse erythrocytes (v./v.). Any diluent compatible with the treated erythrocytes can be used in this diagnostic reagent. A saline or other aqueous solution is preferred. If greater than about 5% cell concentration is used in making up the reagent, the reagent preferably is diluted to about 5% concentration before use. One drop of this reagent is then mixed with about two drops of the patient's serum in which complement has been inactivated, such as by heat-inactivation and the like. The reagent reacts positively (agglutination) within two minutes after its mixture with the inactivated sera from humans having infectious mononucleosis and negatively (no agglutination) with all other inactivated human sera, thereby providing a rapid one-step procedure that is specific for infectious mononucleosis.

The following example will further illustrate the invention defined herein although the invention is not limited to this specific example. All parts and percentages recited herein are on a weight basis unless otherwise specified.

EXAMPLE 1

In order to illustrate the diagnostic reagent and method for infectious mononucleosis of this invention, the following solutions are prepared and the following procedures are carried out:

(A) Preparation of solutions

(1) Prepare the following phosphate buffer in H_2O:

0.15 M $NaH_2PO_4 \cdot H_2O$—20.7 grams/liter (monobasic)
0.15 M Na_2HPO_4—21.3 grams/liter (dibasic)

Add the monobasic solution to the dibasic solution until the pH is 8.2.

(2) Prepare 0.15 M NaCl by adding 8.8 grams of NaCl to one liter of H_2O.

(3) Prepare the following buffer by mixing the above prepared reagents:

	Ml.
0.15 M phosphate buffer, pH 8.2	200
0.15 M NaCl	1800
Distilled H_2O	1000

(4) Prepare a 1% glutaraldehyde fixing solution using the above buffer in Step #3:

	Ml.
Buffer above	2304
25% glutaraldehyde solution	96

Store at 5° C. and use within 24 hours after preparation.

(B) Washing of horse cells

(1) Place 120 ml. of horse whole blood (Alsever's) in a centrifuge bottle.

(2) Centrifuge at 5° C. at 2500–4200×g for 15 minutes.

(3) Discard the supernatant and all of the buffy coat (white cells) and add at least 140 ml. of 0.15 M NaCl to the precipitate.

(4) Mix, centrifuge, and repeat the above wash with saline three times.

(5) After the last centrifugation, discard the supernatant.

(6) Remove 48 ml. of the packed cells and place in a 4 liter beaker.

(7) Add 72 ml. of 0.15 M NaCl (final cell concentration=40%).

(8) Place cells at 5° C. until ready for glutaraldehyde fixing which is done on the same day.

(C) First glutaraldehyde fixation of horse cells

(1) Place the beaker containing the horse cells on a slowly stirring magnetic mixer.

(2) Slowly add 2400 ml. of 1% glutaraldehyde solution to the cells (this will take about 30 to 45 minutes). Final cell concentration is 2%.

(3) After all of the glutaraldehyde solution is added, continue stirring slowly for 30 minutes.

(4) Immediately place in centrifuge bottles and spin at 1500×g in a centrifuge at room temperature (ca. 25° C.) for 15 minutes.

(5) Discard the supernatant and fill each bottle with 0.15 M NaCl.

(6) Resuspend cells well and repeat centrifugation.

(7) Wash in the above manner four additional times with saline.

(8) Wash five times with distilled H_2O.

(9) Resuspend cells in 72 ml. of 0.15 M NaCl (cell concentration=40%).

(D) Sensitization of fixed horse cells with rabbit antiserum

(1) Resuspend 40% horse cells well by stirring.

(2) Remove 10 ml. and place in a centrifuge tube.

(3) Centrifuge at 4200×g for 10 minutes and discard the supernatant.

(4) Add 80 ml. of antiserum obtained from rabbits which have been immunized by injection with Forssman-like antigens. (Prepared by admixing a suspension of finely ground guinea pig kidney antigen in saline, concentration 10 mg./ml., with an equal volume of Freund's adjuvant, administering to rabbits via initial intramuscular injection, followed by subcutaneous injections at 2 week intervals, 10 days before each bleeding, until the unabsorbed hemagglutinin titer is 1:224.)

(5) Resuspend well and place on a slowly stirring magnetic mixer at 5° C. for 16 to 20 hours.

(6) Centrifuge at 5° C. at 2500–4200×g for 15 minutes.

(7) Discard the supernatant and add 80 ml. of 0.15 M NaCl.

(8) Resuspend well and repeat the centrifugation.

(9) Discard the supernatant and add 6.0 ml. of 0.15 M NaCl (cell concentration=40%).

(E) Second glutaraldehyde fixation of horse cells

(1) Place the beaker containing the sensitized, fixed horse cells at 5° C. on a slowly stirring magnetic mixer.

(2) Add 190 ml. of a 1% glutaraldehyde solution slowly to the horse cells (this will take about 5 to 10 minutes).

(3) Continue stirring for 30 minutes.

(4) Immediately place in centrifuge bottles and centrifuge at 2500×g for 15 minutes at room temperature (ca. 25° C.).

(5) Discard the supernatant and wash the precipitate 10 times with saline.

(6) After the final wash, resuspend in 76 ml. saline (final cell concentration=5%).

The final reagent is preferably stored under refrigeration, such as from about 1° C. to about 10° C. and preferably from about 1° C. to about 5° C. This reagent can be made conveniently available for diagnostic purposes by packaging together with positive and negative control serums.

The above-prepared final reagent is suitable for use in the serological diagnosis of infectious mononucleosis in a one-step procedure in which one drop of the reagent is mixed with two drops of test serum in which complement has been inactivated. Agglutination occurs within about two minutes in a positive test and is absent in a negative test. Similar diagnostic results are obtained when succinic

aldehyde is substituted for glutaraldehyde in the above preparation of the diagnostic reagent.

Various other examples and modifications and adaptations of the foregoing examples will be apparent to the person skilled in the art after reading the invention defined herein and the appended claims without departing from the spirit and scope of the invention. All such further examples, variations and modifications are included within the scope of the invention as defined in the following claims.

What is claimed is:

1. The method of making a diagnostic reagent that is specific for infectious mononucleosis comprising thoroughly admixing washed horse erythrocytes with an aqueous solution of from about 0.05% to about 2% by volume of glutaraldehyde to a concentration of from about 0.1% to about 5% by volume of said erhythrocytes buffered at a pH of about 8.2, separating therefrom the thus-treated erythrocytes and thoroughly admixing said erythrocytes with guinea pig kidney antiserum to coat same therewith, separating therefrom the thus-treated erythrocytes and thoroughly admixing said erythrocytes with an aqueous solution of from about 0.05% to about 2% by volume of glutaraldehyde to a concentration of from about 0.1% to about 5% by volume of said erythrocytes buffered at a pH of about 8.2, and then separating therefrom and retaining the thus-treated erythrocytes.

2. A reagent for the diagnosis of infectious mononucleosis comprising the horse erythrocytes prepared by the method of claim 1.

3. In the performance of the serological diagnosis of infectious mononucleosis which employs an agglutination reaction between human serum and horse erythrocytes, the improvement comprising employing in said diagnostic method the horse erythrocytes prepared by the method of claim 1.

References Cited

UNITED STATES PATENTS

3,096,250	7/1963	Ingraham	424—12
3,426,123	2/1969	Hoff	424—12
3,594,466	7/1971	Guffroy	424—12

OTHER REFERENCES

Boyd: Fund. of Immunology, Interscience Pub., N.Y., 2nd Ed., 1947, pp. 139–142.

Rossier: Med. Hyg., vol. 743, 1966, reprint 4 pages.

Stuart: PSEMB, vol. 34, 1936, pp. 209–212.

STANLEY J. FRIEDMAN, Primary Examiner

A. P. FAGELSON, Assistant Examiner

U.S. Cl. X.R.

424—3, 11, 13