A Fluorescent Test for Treponemal Antibodies. (23512)

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The value of fluorescent antibody procedures has been adequately demonstrated in a wide variety of studies(1,2,3). Until recently, these technics have not been applied in the field of venereal disease(4). It is the purpose of this report to describe and discuss the use of fluorescein labeled antiglobulin as an indicator of treponemal antibodies.

Materials and methods. Treponema pallidum, Nichols strain (T.p.) was used as an antigen and was obtained from rabbit testicular tissue by extraction as recommended for the *Treponema pallidum* Immobilization (TPI) test(5). Human serums were made available from laboratory stocks. Syphilitic rabbit serums were produced by intradermal inoculation of minimal infective doses of T.p. and bleeding from ear veins at weekly intervals. All serums were heated at 56°C for 30 minutes before being tested. Dilutions of serums and globulins were prepared in phosphate buffered saline pH 7.2 (BS). Production of goat antihuman serum (GAH) and goat antirabbit serum (GAR) followed the general procedure as outlined by Proom(6). Nanny goats of about 12 months of age were injected according to the following schedule: 1st intramuscular injections consisted of 5 ml potassium alum serum precipitate in each leg. The 2nd injection 2 weeks later consisted of 10 ml of precipitate into one leg. The 3rd injection, four weeks after the 1st, was 10 ml into one leg. Following a one-month rest, 10 ml whole serum was injected subcutaneously in the region of the back. One week following this injection, the animals were bled from the jugular vein. Globulins were prepared by repeated precipitation with half saturated ammonium sulfate and were dialyzed against BS. The protein content of this preparation was adjusted to a concentration of 1.5 g/% for subsequent fluorescein conjugation by the technics of Coons and Kaplan(7).* Finished conjugates were twice precipitated with ammonium sulfate and finally dialyzed against BS. Fluorescent microscopy was accomplished by use of Leitz and Reichert ultraviolet light assemblies and the appropriate filters. Microscopic observations were made using darkfield condensers and 4 mm. dry objective.

Test Procedure, Method A. T.p. suspensions demonstrating 2-10 organisms/H.P. microscopic field were used for slide preparations. Freshly extracted T.p. suspensions, or suspensions held in a refrigerator at 4°C without added preservative, were used with equal results. Smears were prepared on 3 x 1 inch microscope slides. A diamond point stylus was used to cut circles which would identify smear areas for later observations. A drop of T.p. suspension (antigen) consisting of approximately 0.01 ml was formed into a circular smear of about 10 mm diameter. Slides were air dried and gently fixed with heat. Approximately 0.03 ml of 1-5 dilution of serum (heated at 56°C for 30 minutes) was placed on each smear and spread in a circular fashion. Loss of moisture by evaporation was prevented by covering the slides with large Petri dish tops containing moistened filter paper. Smears were allowed to be in contact with serums for 30 minutes, at which time they were carefully rinsed with BS and were then allowed to stand in BS for 10 minutes. Excess moisture was drained from slides and smears were gently blotted. Fluorescein labeled GAH diluted 1-3, or GAR diluted 1-5 was placed on the smears in the same amount and manner described for serums. Slides were protected with a Petri plate as before for 30 minutes. At the end of this period, slides were washed and soaked for 10 minutes as previously described. Following this treatment, slides were carefully wiped free of moisture, but only the excess moisture was removed by shaking. A very small drop of mounting mixture consisting of one part

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TABLE I. Rabbit Experiment Illustrating Comparative Test Results.

Rabbit No.	No. TP inoculated	Days after inoculation	Serologic tests: Fluorescent antibody methods A	B	TPI	TPCF	VDRL slide
631	10^4	0	—	—	—	—	—
		14	—	—	—	—	—
		35	—	—	—	—	—
		42	—	—	—	—	—
		49	—	WR	—	R	—
		64	—	R	—	R	—
		112	—	R	—	R	—
630	10^3	0	—	—	—	—	—
		14	—	—	—	—	—
		35	—	—	—	WR	WR
		42	—	WR	—	WR	WR
		49	—	R	—	R	R
		64	WR	R	—	R	R
		112	R	R	WR	R	R
M12	10^5	0	—	—	—	—	—
		7	—	—	—	—	—
		14	—	WR	—	—	—
		21	—	R	—	R	R
		49	WR	R	R	R	R
		64	R	R	R	R	R

R = reactive; WR = weakly reactive; — = (NR) nonreactive.

buffered saline pH 7.2 and nine parts C.P. glycerine was placed on each smear, and the slide preparation was completed by placing a cover glass on the mounting medium. Controls consisted of mounted untreated T.p. smears, T.p. stained only with labeled GAH or GAR, and with known normal and syphilitic serums. Slides were examined immediately after preparation, but were stored at 4°C for several weeks for later reference.

Test Procedure, Method B. This method was in every respect similar to Method A except that slides were placed on a mechanical rotator at 160 rpm during the 30 minute periods that the treponemes were exposed to the serum dilutions and the fluorescein labeled globulin preparations.

Reading of test controls and serums. All smear preparations were examined by regular darkfield illumination and by means of filtered light from a high pressure mercury vapor bulb. Properly prepared untreated smears demonstrated well defined T.p. when visible illumination was used. The same field gave negative results when viewed with ultraviolet light (UV). Smears treated with labeled GAH or GAR only, or those treated with normal serums followed by treatment with fluorescein labeled preparations, demonstrated characteristics similar to those of untreated smears. Smears which had been treated with human or rabbit serums containing treponemal antibodies revealed fluorescent T.p. following the application of GAH or GAR. Positive reactions were observed to vary from strong to weak fluorescence. Results obtained by the fluorescent treponemal antibody test (FTAT) from syphilitic rabbits are illustrated in Table I. Findings obtained by means of other treponemal test procedures are also recorded for comparative purposes. The VDRL Slide test(8) results are listed as an example of reactions obtainable by a nontreponemal test. As will be observed, the reactions recorded for FTAT Method A are in only slight agreement with either FTAT Method B or the other test procedures. It will also be noted that more reactions were obtained by Method B than by Method A. Since rotation of slides is the only difference between these methods, it appears that this may account for the marked intensification of fluorescent tagging by this procedure.

Discussion. It is evident from the experimental results presented that the use of intact T.p. as an antigen and fluorescein labeled

TABLE II. Examples of Testing Discrepancies with Human Serums.

Serum No.	Serologic tests: FAST* method "B"	TPI	TPCF	VDRL slide
1456	R	R	R	R
1457	R	R	R	R
1459	R	R	R	WR
1468	—	—	WR	WR
1378	WR	R	WR	—
1477	R	—	R	—
1498	R	—	AC†	—
1501	R	R	AC	—
1503	R	—	AC	R
1512	R	R	WR	R
2013‡	R	incl.§	R	R
2028‡	—	—	AC	WR
2060‡	R	R	R	R
2062‡	R	R	R	R

R = reactive; WR = weakly reactive; — = (NR) nonreactive.

* Fluorescent Antibody Slide Test Method B.

† Anticomplementary.

‡ Serums demonstrated inconclusive (incl.) results in the TPI test before penicillinase treatment.

§ Control shows TP toxicity even after penicillinase treatment.

antiglobulin as an indicator of treponemal antibody coupling is a wholly feasible procedure. In the rabbit inoculation experiment, Table I, Method B appears to follow the course of immunologic response to T.p. infection in an acceptable manner. Method B and the TPCF test are apparently similar in respect to sensitivity, whereas Method A and the TPI procedure gave comparable results. The reactivity of Method B is further illustrated in Table II in which the results obtained by the 3 T.p. test procedures on human serums are compared. The serums in this series were selected to illustrate various combinations of reactivity found in a much larger group of serums tested. Reactivity in only one of the three T.p. tests is illustrated by serum No. 1468. Serums No. 1498 and 1503 were found to be reactive only by the FTAT Method B. Serums No. 2013, 2028, 2060 and 2062 were selected to illustrate that the FTAT Method B may demonstrate reactive results when the other T.p. tests have yielded inconclusive findings. It should be noted that specimens found to be anticomplementary in the TPCF test and inconclusive in the TPI test, due to penicillin or unknown toxic factors, may yield positive findings in the fluorescent antibody procedure.

In consideration of the practical application of the fluorescent treponemal antibody test Method B to syphilis serology, it must be emphasized that the specificity of the reaction has not yet been determined in terms of human disease. Such an evaluation is currently under consideration. It is evident, however, that syphilis infection in the rabbit gives rise to an antibody or antibodies that may be detected by fluorescent antibody testing. As previously mentioned, the fluorescent antibody testing procedure herein described compares favorably with the TPCF test in terms of sensitivity, and appears to be considerably more sensitive than the TPI procedure. If the reaction obtained by the fluorescent antibody testing method should prove to be adequately specific, several other important factors would recommend the method as a possible laboratory procedure. Among these may be included the following: the procedure is not as expensive as some T.p. tests, and materials can be made available from commercial sources; the actual reading of an individual test consumes approximately one minute and the entire test can be completed in about one hour; slides may be examined at a convenient time and filed for future reference under refrigeration (4°C) for several weeks. As previously stated, the validity of results obtained with the fluorescent antibody test in terms of human infection must await more complete documentation. Therefore, it is too early to speculate on the immunological significance of the antibodies detected by this means.

Summary. 1. A technic for the use of fluorescein labeled antiglobulin as an indicator of treponemal antibody is described. 2. The effect of rotation as an intensifier of fluorescent antibody reactions is determined. 3. Results obtained with fluorescent antibody tests on syphilitic rabbits are compared with results of other treponemal and nontreponemal tests. 4. Serological discrepancies obtained with fluorescent antibody and several other tests on human serums are presented.

Addendum. Since this article was offered for pub-

lication it has been found that extraction of treponeme smears with acetone for 15 minutes prior to use of serum reduces background fluorescence so is a preferred method.

1. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., *J. Immunol.*, 1942, v45, 159.
2. Goldman, Morris, *Am. J. Hyg.*, 1953, v58, 319.
3. Moody, Max D., Goldman, Morris, and Thomason, Berenice, *J. Bact.*, 1956, v72, 357.
4. Harris, Ad, Deacon, W. E., and Smith, C. A., *Bull. Conf. of State & Prov. P. H. Lab. Directors*, 1957, v15, 8.
5. Harris, Ad, Bossak, H. N., Olansky, S., *Pub. Health Lab.*, 1955, v13, 63.
6. Proom, H., *J. Path. Bact.*, 1943, v55, 419.
7. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.
8. Serologic Tests for Syphilis, 1955 Manual, PHS Publ., No. 411, Washington, D. C.

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Paroxysmal Nocturnal Hemoglobinuria. Evidence of Defect of Red Cell Stroma Manifested by Abnormalities of Lipids. (23513)

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Paroxysmal nocturnal hemoglobinuria (PNH) may be a manifestation or symptom of a chronic hemolytic anemia characterized by an abnormality of the red blood cell which renders it susceptible to hemolysis by the action of the "properdin system." The components of this system are normal constituents of the plasma namely properdin, Mg^{++} and cofactors resembling complement(1). The system apparently is not active at all against normal human red cells. A great amount of work has been done to study and define the plasma factors of the PNH hemolytic system, but the erythrocyte which carries the pathognomonic defect has received little attention except as an indicator of the reactions of the plasma factors. It has been accepted that the PNH erythrocyte is faultily constituted, but the nature of fault is unknown(2). The hemoglobin has a normal solubility and electrophoretic mobility. It has been suggested that the defect resides in the red cell stroma or in the metabolic machinery that maintains the stroma throughout the life span of the cell. The present study was undertaken to learn whether or not the stromal lipids of the PNH erythrocyte are different from those of normal red cells.

Materials and methods. The blood for these determinations was obtained from 2 patients with classical history and signs of PNH. Patient A has been known to have the disease since 1939. During the past 10 years he has gradually improved until at present he shows no evidence of hemolytic disease or anemia. His red cells, though giving the characteristic reactions *in vitro,* survive normally when transfused into the patient or a normal recipient. Patient B has a serious hemolytic disease, with anemia and hemoglobinuria, and 15 per cent of his red cells are reticulocytes. Control studies were done on the red cells of 14 normal adults. The lipid analyses were performed on the washed red cells. The details of the procedure are the subject of a separate report. In brief, the lipids were extracted by boiling ethanol-ether and the analysis was carried out under nitrogen. Total fatty acids were determined by weighing. Saturated fatty acids were precipitated as the lead salts and weighed (3,4). Polyethenoid fatty acids were measured by the alkali-isomerization technic of Herb and Riemenschneider(5,6). Oleic acid was computed to be the difference between the total fatty acids and the sum of the saturated and polyethenoid acids. Iodine number was determined by the Yasuda procedure(7). Cholesterol was determined by a standard method(8,9). Recovery experiments and parallel and segmental analyses on red blood cells from the same patient permit a high degree of confidence in the methods and results. The mean corpuscular area was determined by the photomicrography of fresh red cells in rouleaux by an original method which gives a high degree of accuracy. Mean corpuscular volume