Superiority of Fluorescein Isothiocyanate (Riggs) for Fluorescent-Antibody Technic with a Modification of its Application. (24222)

JOHN D. MARSHALL, WARREN C. EVELAND AND CHAUNCEY W. SMITH
(Introduced by Oscar Felsenfeld)
Armed Forces Institute of Pathology, Washington, D. C.

The fluorescent antibody technic of Coons and Kaplan(1) has been widely used for identification of microbiological agents(2-7). Until recently, fluorescein isocyanate was the only compound that could readily be conjugated to the antibody molecule and yield a product that was easily detectable with ultraviolet microscopy. The difficulties involved in preparation of fluorescein isocyanate and its subsequent conjugation have somewhat restricted the application of this technic. A further limiting factor has been the need for antisera of relatively high titer, since during conjugation a portion of the antibody globulin is denatured to such an extent that it is no longer reactive. To eliminate these disadvantages Goldman and Carver(8) modified the method of conjugation by use of a filter paper technic. Riggs(9) described an isothiocyanate derivative of fluorescein with a number of qualities that make it superior to fluorescein isocyanate. The purpose of this study was to determine the relative merits of each of the compounds employed in various methods of conjugation, and to seek possible improvements in the technic.

Material and methods. The following antisera were used throughout the study: *Pasteurella tularensis* and Venezuelan equine encephalomyelitis (VEE) received from the Army Chemical Center, Fort Detrick, Salmonella polyvalent and sheep antihuman furnished by Walter Reed Army Institute of Research, antirabbit from Cappel,* and *Cryptococcus neoformans* and *Saccharomyces cerevisiae* prepared in this laboratory. *Preparation of fluorescein compounds*: A single lot of fluorescein amine isomer No. 2, prepared by one of us (CWS), was used to make both the fluorescent isocyanate and the fluorescein isothiocyanate. The fluorescein isocyanate was synthesized by the method of Coons and Kaplan(1). A fluorescein isothiocyanate derivative was prepared by the method of Riggs (9): 2 ml thiophosgene was added to 5 ml dry acetone and placed on a rotary shaker. One gram of fluorescein amine was suspended in 5 ml dry acetone and the suspension was added drop by drop to the thiophosgene-acetone mixture. A dark yellow precipitate formed. The reaction was permitted to continue at room temperature with constant shaking for one hour. Then the mixture was placed in refrigeration at 4°C and allowed to stand overnight. The precipitate was collected under suction, washed with 4 to 5 volumes of Skellysolve B (petroleum naphtha)† and dried over calcium chloride. The yield was approximately 96%. The product was stored in a screw-capped vial at room temperature. *Conjugation of globulin fractions*: The ammonium sulphate precipitated globulin fraction of each antiserum was divided into 6 aliquots. Fluorescein isocyanate was used to label one of them by the method of Coons and Kaplan(1). A second aliquot was labeled with fluorescein isocyanate by the filter paper technic of Goldman and Carver(8). Fluorescein isothiocyanate was conjugated to a third aliquot by the procedure described by Riggs(9). A fourth portion of the globulin fraction was conjugated with fluorescein isothiocyanate, using the filter paper technic. A fifth sample was conjugated in the following manner: The globulin fraction was diluted with 0.15 M sodium chloride and carbonate-bicarbonate buffer, 0.5 M, pH 9, so that the final solution contained 10 mg protein per ml and 10% buffer solution. After the solution was cooled to 4°C, 0.05 mg fluorescein isothiocyanate was added for each milligram of protein. The mixture was transferred to a rotary shaker, placed in the 4°C cold room, and agitated for 12 to 18 hours to ensure uniform distribution of the dye. The conjugated globulin fraction was then transferred to a

* Cappel Laboratories, Inc., Westchester, Pa.

† Skelly Oil Co., Eldorado, Kan.

TABLE I. Comparison of Fluorescent Staining Titers of 7 Sera Conjugated by 5 Different Methods.

	Fluorescein isocyanate	Fluorescein isocyanate filter paper	Fluorescein isothio-cyanate	Fluorescein isothio-cyanate filter paper	Fluorescein isothiocya-nate powder
P. tularensis	5	20	80	160	160
Salmonella polyvalent	10	20	40	80	80
Sacc. cerevisiae	40	40	160	80	160
C. neoformans	*	10	20	20	40
V. E. E.	*	*	*	5	5
Antihuman	10	20	80	80	80
Antirabbit	40	80	160	160	160

Titers expressed as reciprocal of serum dilution. * Undiluted.

cellulose casing and dialyzed against 0.01 M phosphate-buffered saline solution, pH 7.0, until the dialysate was free of fluorescence when exposed to ultraviolet light. The final portion of globulin fraction was retained unconjugated for studying other types of antibody activity. *Fluorescent microscope*: A Leitz Ortholux microscope, equipped with a reflecting darkfield condenser, 4 mm dry objectives, 10 x widefield ocular and appropriate filters was used in conjunction with a 150-watt high pressure mercury vapor bulb mounted in a Leitz fluorescent lamp assembly.

Results. Smears were prepared from various antigens and fixed in absolute methanol for 10 minutes. After the smears were thoroughly air dried, they were covered with different dilutions of the labeled homologous antiglobulin and kept in a wet chamber at room temperature for 30 minutes. They were washed for 10 minutes in 0.01 M phosphate buffered saline, then mounted in 10% buffered glycerin. The smears thus prepared were examined under the ultraviolet microscope. The results (Table I), demonstrate that fluorescein isothiocyanate conjugated antisera yielded positive results in higher dilutions than the fluorescein isocyanate conjugates.

In the case of *P. tularensis,* the slides stained with undiluted antisera conjugated with fluorescein isothiocyanate fluoresced so brightly that a distorting halo was observed. This effect disappeared upon using higher dilutions. Conjugated antisera diluted to as much as 1:160 still stained the organisms so that the structure of the individual cells was easily recognized.

Similar results could not be obtained with the fluorescein isocyanate preparations when diluted more than 1:20. The reactions were specific, for no staining was observed when organisms other than the homologous species were tested.

The antihuman and antirabbit globulin preparations were tested by the indirect technic. Smears of *Salmonella typhi* were exposed for 30 minutes to the serum of an individual who had recently been immunized. The slides were washed for 30 minutes in phosphate-buffered saline to remove excess antiserum. The organisms coated with a layer of human antibody were then used as a homologous antigen. The conjugated antihuman sera were tested by the standard method. The antirabbit serum was tested in a similar manner using *Paracolobactrum aerogenoides* and the homologous rabbit antiserum.

Agglutination studies were performed with all antisera except the VEE and the antirabbit globulin. Those antisera that had been exposed to organic solvents during conjugation did not retain their complete titer when compared with the unconjugated fraction, while those conjugated by using either the filter paper technic or with the fluorescein isothiocyanate powder retained approximately the same titer as the control fraction.

Results are shown in Table II.

Discussion. Various methods for conjugation of fluorescein dyes have been tested. Fluorescein isothiocyanate has certain attributes which make it superior to fluorescein isocyanate for conjugation with immune sera. A more intense staining with fluorescein isothiocyanate conjugated antisera was demon-

TABLE II. Effect of Conjugation on Agglutination Titers of 5 Sera.

	Fluorescein isocyanate	Fluorescein isocyanate filter paper	Fluorescein isothiocyanate	Fluorescein isothiocyanate filter paper	Fluorescein isothiocyanate powder	Unconjugated
P. tularensis	160	320	160	640	640	640
Salmonella polyvalent	2	20	10	20	20	20
Sacc. cerevisiae	320	640	640	640	640	1280
C. neoformans	40	160	80	160	160	160
Antihuman	4	256	2	256	128	256

Titers expressed as reciprocal of serum dilution.

strated by the fact that they could be employed in much higher dilution. In some instances, the undiluted preparations were unsatisfactory because the fluorescence was so intense that distortion in the form of a halo was observed.

Our comparative study of the stability of various compounds confirmed Goldman's report(8) on the filter paper technic for storage of fluorescein isocyanate. The fluorescein isothiocyanate, on the other hand, has remained stable as a powder for a period in excess of 8 months. This obviates the necessity of applying the dye to filter paper for storage. One of the limiting factors in the use of the fluorescent antibody staining technic has been the necessity of maintaining highly purified organic reagents for use in conjugation. This requirement has been eliminated. Fluorescein isothiocyanate powder poured directly into a cooled, dilute, buffered antiserum yielded a product which was as good as, or superior to that prepared by any other method.

The ease with which serum may be conjugated by this method enables any laboratory to prepare and use it with a minimum of supplies, equipment, and personnel. The stability of the fluorescein isothiocyanate compound lends itself to centralized preparation. This compound and method will permit labeling of low titer antisera because little detectable denaturation of the protein occurs.

Summary. 1) The globulin fractions of antisera representing bacterial, viral, mycotic agents and antiglobulin fractions were labeled with 2 derivatives of fluorescein amine by 3 methods. 2) Fluorescein isothiocyanate was shown to be superior in stability, ease of conjugation, and degree of fluorescence. This direct method of adding the dye to a dilute buffered antiserum eliminates the need for organic reagents which may denature the protein.

1. Coons, A. H., Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

2. Moody, M. D., Goldman, M., Thomason, B. M., *J. Bact.*, 1956, v72, 357.

3. Moulton, J. E., Haworth, J. A., *Cornell Vet.*, 1957, v47, 524.

4. Eveland, W. C., Marshall, J. D., Jr., Silverstein, A. M., Johnson, F. B., Iverson, L., Winslow, D. J., *Am. J. Path.*, 1957, v33, 616.

5. Liu, C., Coffin, D. L., *Virology*, 1957, v3, 132.

6. Breitenfeld, P. M., Schafer, W., *ibid.*, 1957, v4, 328.

7. Goldman, M., Carver, R. K., *J. Exp. Med.*, 1957, v105, 549.

8. ———, *Science*, 1957, v126, No. 3278, 839.

9. Riggs, J. L., Masters Thesis, Univ. of Kansas, 1957.

Received June 24, 1958. P.S.E.B.M., 1958, v98.