LABELING PROCEDURES EMPLOYING CRystalline FLUORESCIN IソTHIOCYANATE

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ABSTRACT

GRIFFIN, CHARLES W. (Baltimore Biological Laboratory, Baltimore, Md.), THEODORE R. CARSKI, AND GEORGE S. WARNER. Labeling procedures employing crystalline fluorescein isothiocyanate. J. Bacteriol. 82:534–537. 1961.—The effect of altering the ratio of dye to protein in the labeling of three immune globulins with crystalline fluorescein isothiocyanate was the purpose of the study. Direct staining of group A streptococci and rabies-infected mouse brain, and indirect staining of Treponema pallidum (Nichols strain) comprised the three immunological systems selected for investigation. Maximal specific staining and minimal nonspecific background staining were demonstrated by the use of low dye-to-protein labeling ratios.

Numerous fluorescein-labeled antibody systems are under investigation using the labeling procedures described by Coons and Kaplan (1950) and modified by Riggs et al. (1958), and Marshall, Eveland, and Smith (1958). A labeling ratio of 1 part dye to 20 parts protein (0.05 mg dye per mg protein) was employed by these investigators. In the labeling of antiserum or fractions of antisera, difficulties with nonspecific background staining have been experienced by various workers. Also, variations in purity of fluorescein derivatives have contributed to problems in the preparation of satisfactory conjugates. A current review of the fluorescent antibody method by Beutner (1961) includes a discussion of the technical procedures, applications, and problems associated with the method.

The introduction of crystalline fluorescein isothiocyanate (FITC) of high chromatographic purity (Felton and McMillion, 1961) stimulated

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the present study. It appeared desirable to re-evaluate certain aspects of the labeling procedure, particularly the ratio of dye to protein. The present report describes the effect of altering the ratio of dye to protein on the staining characteristics of group A streptococcal globulin, antirabies globulin, and antihuman globulin.

MATERIALS AND METHODS

Conjugation of globulin fraction. Labeling procedures were performed with the globulin fractions of group A streptococcal antiserum, antirabies serum, and antihuman serum, which were obtained by conventional half-saturation with ammonium sulfate at 4°C. Precipitated globulin was dissolved in distilled water and dialyzed against 0.85% sodium chloride until the dialyzate was free of sulfate. Following dialysis, the globulin solution was diluted to approximately 2.5 g protein per 100 ml and adjusted to pH 9.5 by adding 0.5 M carbonate-bicarbonate buffer. Crystalline FITC was added directly to the chilled, stirred globulin solution (Marshall et al., 1958), but with variations in: (i) the ratio of dye to protein and (ii) the duration of conjugation. Dye-to-protein labeling ratios selected for investigation were 1:10 (0.10 mg dye per mg protein), 1:20 (0.05 mg dye per mg protein), 1:40 (0.025 mg dye per mg protein), and 1:80 (0.0125 mg dye per mg protein). Durations of conjugation selected were 30 min, 1 hr, 6 hr, and 23 hr. Immediately following conjugation, the labeled globulin was dialyzed with continuous agitation against 0.01 M phosphate-buffered saline (pH 7.4), then diluted to approximately 1.0 g protein per 100 ml and absorbed once on acetone-dried animal tissue powders where necessary.

Preparation and staining of smears. 1) Group A streptococcus and normal mouse brain impression smears:—Streptococcus smears were made with suspensions from the surface growth of slants and fixed in ethanol (Moody, Ellis, and Updyke,
1958). Impression smears of normal mouse brain were fixed in the same manner to provide an indication of nonspecific background staining. Staining was accomplished by covering both bacterial and normal mouse brain impression smears with serial dilutions of conjugates and placing them at 37°C for 30 min in a moist atmosphere. The slides were then rinsed in three changes of phosphate-buffered saline, dried, and mounted with buffered glycerol (pH 8.4).

2) Rabies-infected mouse brain smears:—The technical procedures for preparation and staining of smears of rabid mouse brain and proof of specificity of observed staining were as described previously (Goldwasser et al., 1959).

3) Treponema pallidum (Nichols strain) smears:—Smears were prepared and stained by the indirect procedure employing antihuman conjugates according to the methods of Deacon, Falcone, and Harris (1957) and Deacon, Freeman, and Harris (1960).

Evaluation of conjugates. Group A streptococcal conjugates were evaluated on the basis of: (i) specific staining titer against group A streptococci, (ii) nonspecific staining titer against normal mouse brain impression smears, and (iii) fluorescein-to-protein (F:P) ratios (Coons and Kaplan, 1950; Goldwasser and Shepard, 1958). Specificity of staining was proved by simultaneous titration of the conjugates in unlabeled group A streptococcal antiserum. Antirabies and antihuman conjugates were also evaluated on the basis of specific and nonspecific staining titer and F:P ratio.

Fluorescence microscopy. In the studies employing streptococcal and antihuman conjugates, microscopic observations were made with a Zeiss monocular microscope equipped with a UG 2 exciter filter and a GG 4 eyepiece barrier filter. An HBO 200 mercury burner with heat filter was used as the light source. The illuminating system employed during observation of the rabies specimens consisted of an HBO 200 mercury burner in a Reichert housing with built in heat filter, a Corning 5840 (one-half thickness) exciter filter and a Reichert 5082 UV excluding eyepiece filter. A monocular microscope equipped with a Fluorite 40X oil immersion objective was routinely used.

RESULTS

Direct staining of group A streptococci. Table 1 presents a summary of the results obtained with a series of conjugates prepared from the same lot of group A streptococcal antiserum. As shown in the table, in most instances staining titers of conjugates prepared in 30 min and 1 hr were as high as those prepared in 6 hr and 23 hr. Furthermore, labeling at a 1:40 ratio of dye to protein appeared to be as efficient as labeling at a 1:20 ratio. Labeling at a 1:10 ratio produced conjugates exhibiting maximal staining titers at all times studied. Diminished staining ability was demonstrated by conjugates prepared at a 1:80 ratio. The results obtained with mouse brain impression smears showed that globulin labeled at ratios of 1:10 and 1:20 evidenced the highest degree of nonspecific nuclear and cytoplasmic staining. Both nuclear

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<th>Dye-to-protein ratio</th>
<th>Time of conjugation</th>
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<th>Nonspecific background staining</th>
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* Highest dilution of conjugate exhibiting an estimated 4+ and 3 to 4+ fluorescent intensity; all conjugates absorbed once with rabbit liver powder before staining.
† Conjugate diluted in unlabeled group A streptococcal antiserum.
‡ Normal mouse brain impression smears; highest dilution of conjugate exhibiting at least a 1+ fluorescence.

TABLE 1. Staining characteristics and fluorescein-to-protein (F:P) values of group A streptococcal globulin labeled with crystalline fluorescein isothiocyanate at various dye-to-protein ratios
and general background tissue staining was reduced where globulin was labeled at 1:40 and 1:80 ratios. There was an increase in nuclear staining as the duration of conjugation increased at ratios of 1:10 and 1:20. All conjugates had been absorbed once with acetone-dried rabbit liver powder. It is seen from Table 1 that optimal conditions for labeling antistreptococcal A globulin were provided by a labeling ratio of 1:40 and a reaction duration of 1 hr or more. Maximal specific staining and minimal nonspecific background staining were provided by these conditions.

F:P ratios of conjugates were highest for globulin labeled at a 1:10 ratio and decreased as the ratio of dye to protein was decreased. Also, the F:P value became higher as the duration of conjugation was increased at each dye to protein level studied. It is interesting to note that at the 1:80 level, where F:P values were lowest, the staining titers were also lowest.

Direct staining of rabies virus antigen. A similar study of labeling ratios and reaction times was conducted using rabies virus as a model system. In this virus system, optimal conjugation was obtained using labeling ratios 1:40 or 1:80 with a reaction time of 6 hr or more. Nonspecific background staining was remarkably diminished when compared to that obtained when the widely used labeling ratio of 1:20 was employed. No decrease in brightness of specific staining was noted at 1:40 and 1:80 ratios. Conjugates labeled at the 1:80 ratio could be used after 2-fold dilution in normal rabbit brain suspension, whereas those labeled at the 1:40 ratio required one or two tissue powder absorptions prior to use. Four or five tissue powder absorptions were commonly required when rabies conjugates were prepared at the 1:20 ratio using crystalline FITC. Antirabies conjugate prepared at a ratio of 1:80 was further tested for stability. Samples were stored at temperatures of 4 C, -20 C, and -70 C and tested at intervals. At the end of 7 months, no decrease in the brightness of staining and no increase in the degree of nonspecific background staining was noted.

Indirect staining of T. pallidum (Nichols strain). A third, but more limited study was conducted using an indirect staining system consisting of T. pallidum (Nichols strain), human syphilitic serum, and fluorescein-labeled antihuman globulin. Optimal results were again obtained using labeling ratios of F:P of 1:40 and 1:80. The reaction time in this case was selected as 18 hr for convenience.

**DISCUSSION**

The significance of the results reported here appears to be that: (i) by the use of lower dye-to-protein labeling ratios, it is possible to reduce or remove nonspecific staining reactions where excess dye is a contributing factor to these reactions; and (ii) the use of FITC of known purity allows for more precision in conjugation procedures since it may be employed as a defined entity. Curtain (1958), in studies of electrophoresis of labeled globulin, has shown that globulin fractions most heavily labeled contribute most to nonspecific staining. Chadwick and Nairn (1960) have reported that conjugates prepared with fluorescein isocyanate and FITC contain unreacted fluorescent material which contributes both to specific and nonspecific staining; they suggest that the unreacted fluorescent material may be bound to protein by some means other than chemical combination. As Chadwick and Nairn point out, problems introduced by nonspecific staining will be relatively greater where low titer antiserum is employed for labeling.

With these factors in mind, a logical procedure for the preparation of satisfactory conjugates would seem to lie in the labeling of high titer antisera, or purified fractions thereof, at low dye-to-protein ratios. The most satisfactory labeling ratio would depend upon the particular immunological system under investigation.

**ADDENDUM**

After the present report was accepted for publication, an article by Goldstein, Slizys, and Chase (J. Exppl. Med. 114:89–110, 1961) appeared which describes a similar experience with crystalline FITC using another immunological system.

**LITERATURE CITED**


Coons, A. H., and M. H. Kaplan. 1960. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen


