Optimal Labeling of Antibody with Fluorescein Isothiocyanate.* (31196)

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Difficulty with nonspecific staining is often encountered in attempts to detect antigen in tissues by the fluorescent antibody method. One of the most important causes of nonspecificity is the presence in conjugates of serum proteins that are too heavily labeled with the fluorescent dye(1-5). Heavily labeled proteins acquire a relatively high negative charge which gives them a nonimmunological avidity for certain tissue components. Methods are available for removing the highly labeled serum proteins from conjugates. These methods, however, are not only time consuming but also often result in considerable loss of antibody.

McKinney *et al*(6) have reported an extensive study of the factors controlling antibody labeling with fluorescein isothiocyanate (FITC). They found conjugation of rabbit γ-globulin to be accurately controlled by the amount of FITC used, and to be essentially complete in 30 minutes if a reaction temperature of 25°C, a protein concentration of 2.5% and 0.05 M phosphate buffer (pH 9.5) were used.

A modification of the method of McKinney *et al*(6) was used to determine whether the optimal concentration of FITC to be used in conjugation was different for each serum. The results showed that there is an optimal concentration of FITC to use; if this concentration is predetermined conjugates with excellent staining properties can be prepared without any treatment after conjugation other than removal of the free FITC and dilution.

Materials and methods. Cells and virus. The source and cultivation of the FL line of human amnion cells used as the host for the reoviruses, the Lang strain of type 1, the D-5 Jones strain of type 2 and the Abney strain of type 3 reovirus have been described previously(7).

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Serum fractionation. The serums were fractionated with half saturated ammonium sulphate which had been neutralized with 1 N NaOH(8). The final precipitate was dissolved to one-half the original volume with 0.85% sodium chloride and was dialyzed against 0.85% sodium chloride until free of sulphate.

Conjugation procedure. An amount of crystalline FITC (Baltimore Biological Laboratory, Baltimore, Md.) in excess of 2.5 mg was dissolved in 0.1 M Na_2HPO_4 at a rate of 1.25 mg/ml. If the pH of the Na_2HPO_4 solution drops due to absorption of CO_2 from the air, difficulty may be encountered in getting the FITC into solution. Dissolution of the FITC can be facilitated by adding several drops of phosphate followed by dispersal of FITC clumps with a pipette or stirring rod.

One milliliter samples of the 5% globulin solution to be labeled were added to each of four 10 ml beakers. Eight-tenth, 0.6, 0.4 and 0.2 ml of the FITC solutions were added slowly with stirring to 4 beakers containing the globulin solutions. All solutions were brought to a total volume of 1.8 ml using 0.1 M Na_2HPO_4. The pH of each solution was adjusted to 9.5 with a Beckman glass electrode pH meter, Model G, using 0.04 N NaOH beginning with the solution that contained the least FITC. The volume of NaOH required to adjust the pH of the first solution was added to each of the other beakers; further pH adjustment of the latter 3 solutions was minimal. The volumes of the adjusted solutions were approximately 2 ml, and the respective solutions contained 20, 15, 10 and 5 mg of FITC/g of protein. Conjugation was continued for 30 minutes at room temperature.

All samples of globulin coupled with FITC were freed of uncombined FITC by passage through a G-50 Sephadex column equilibrated with buffered saline (0.01 M phosphate, 0.15

TABLE I. Specific and Nonspecific Staining Intensities* of Serum Globulins Labeled with Varying Concentrations of FITC.

mg FITC used per g of protein	Staining	Staining intensity at various dilutions of conjugate 1:1†	1:5	1:10	1:20	1:40	1:60
21	Specific	‡	4+	4+	3+	2+	2+
	Nonspecific	3+	2+	2+	1+	1+	—
18	Specific	4+	4+	4+	3+	2+	2+
	Nonspecific	2+	1+	—	—	—	—
15	Specific	4+	4+	4+	3+	2+	2+
	Nonspecific	2+	1+	—	—	—	—
12	Specific	4+	4+	4+	3+	2+	1+
	Nonspecific	2+	1+	—	—	—	—
9	Specific	4+	4+	3+	2+	2+	1+
	Nonspecific	2+	—	—	—	—	—
6	Specific	4+	4+	3+	2+	2+	1+
	Nonspecific	1+	—	—	—	—	—

* 4+, 3+, 2+, 1+ and — indicate staining intensities ranging from maximum to negative.
† 1:1 equals a protein concentration of 10 mg/ml.
‡ Nonspecific staining too intense to permit estimation of specific staining intensity. FL cell cultures infected with the Lang strain of type 1 reovirus were incubated for 24 hr and were fixed in acetone prior to staining.

M NaCl, pH 7.5) and were diluted to a protein concentration of 10 mg/ml. These solutions were further diluted and tested for specific and nonspecific staining as indicated in the Tables showing the results. Coverslip cultures containing approximately 10^3 infected cells and 4×10^5 uninfected cells were stained. The staining intensity was evaluated and graded on a scale from 4+ to —, indicating intensities ranging from maximum to negative.

The above conjugation procedure differs from the technique of McKinney *et al*(6). In this method 0.8 ml of FITC solution is added per milliliter of serum fraction containing 5% protein and the pH is adjusted to 9.5 with 0.04 N sodium hydroxide. In the procedure before modification, 0.2 ml of 0.15 M disodium phosphate and 0.2 ml of FITC solution in disodium phosphate were added per milliliter of serum fraction containing 5% protein. The pH was adjusted to 9.5 with trisodium phosphate and the volume was brought to 2 ml with 0.85% sodium chloride.

Results. The approach to antibody labeling used in this study was to perform preliminary conjugations with small samples of serum globulin to find the optimal concentration of FITC to use in a subsequent conjugation of a large volume of the globulin. Before this could be accomplished it was necessary to determine the range of FITC concentrations to use in the preliminary conjugation. A previous study(5) showed that sera labeled with 5 mg of FITC/g of protein acquire only minimal nonspecific staining. Therefore, samples of a globulin solution prepared from a serum obtained from a goat which had been immunized with viruses of the 3 reovirus types, were labeled with FITC concentrations ranging from 6 to 21 mg/g of protein. Evaluation of nonspecific and specific staining properties of these conjugates can be seen in Table I. There was some nonspecific staining even with a concentration of 6 mg of FITC/g of protein. The degree of nonspecific staining did not increase as much as expected as the concentration of FITC was increased up to 18 mg/g of protein. However, at 21 mg/g of protein the over labeling was marked and the nonspecific staining increased greatly. The conjugate labeled with 21 mg of FITC/g of protein would only stain with a 2+ intensity when it was diluted sufficiently to be rid of nonspecific staining, while the conjugate prepared with 18 mg of FITC stained with 4+ specific staining intensity.

Based upon the above results subsequent conjugations were carried out as described using FITC concentrations of 20, 15, 10 and 5 mg/g of globulin protein. Goat, rabbit and monkey reovirus immune serums; a monkey Yaba virus immune serum (courtesy of Dr.

TABLE II. Specific and Nonspecific Staining Intensities* of Goat, Rabbit and Monkey Globulins Labeled with Varying Concentrations of FITC.

	mg FITC used per g of protein	Staining	Staining intensity at various dilutions of conjugate 1:1†	1:5	1:10	1:20	1:40	1:60
Monkey globulin	30	Specific	‡	‡	3+	3+	2+	2+
		Nonspecific	4+	3+	2+	1+	—	—
	25	Specific	‡	4+	3+	2+	2+	2+
		Nonspecific	3+	2+	1+	—	—	—
	20	Specific	‡	3+	3+	2+	2+	1+
		Nonspecific	3+	1+	—	—	—	—
Rabbit globulin	30	Specific	‡	‡	‡	2+	1+	1+
		Nonspecific	4+	4+	3+	1+	1+	—
	25	Specific	‡	3+	3+	1+	1+	1+
		Nonspecific	3+	2+	2+	1+	1+	—
	20	Specific	‡	3+	3+	2+	1+	1+
		Nonspecific	3+	1+	—	—	—	—
Goat globulin	30	Specific	‡	‡	4+	3+	2+	2+
		Nonspecific	4+	3+	1+	1+	—	—
	25	Specific	‡	4+	3+	3+	2+	2+
		Nonspecific	3+	2+	1+	1+	—	—
	20	Specific	4+	4+	4+	3+	2+	2+
		Nonspecific	2+	1+	—	—	—	—

* 4+, 3+, 2+, 1+ and — indicate staining intensities ranging from maximum to negative.
† 1:1 equals a protein concentration of 10 mg/ml.
‡ Nonspecific staining too intense to permit estimation of specific staining intensity. FL cell cultures infected with types 1 (Lang strain) and 3 (Abney strain) of reovirus were incubated for 24 hr, while those infected with the D5 Jones strain of type 2 reovirus were incubated for 48 hr before they were fixed in acetone and stained. The goat, rabbit and monkey conjugates were used to stain Lang, D5 Jones and Abney infected cells respectively.

Dee Taylor); and a monkey respiratory syncytial virus antiserum (courtesy of Dr. Jack Schieble) were conjugated. In all cases the nonspecific staining titers were similar to those found in Table I. Nonspecific staining with all conjugates labeled with the highest concentration of FITC (20 mg/g of protein) was eliminated by diluting the conjugates to 1.0 mg of protein/ml. The results of specific staining varied with the different conjugates as would be expected with sera of varying antibody content.

Samples of goat, rabbit and monkey reovirus immune globulins were then labeled with 25 and 30 mg of FITC/g of protein to determine whether these concentrations of FITC were excessive for sera of different species of animals. Table II shows the results as well as those obtained with conjugates labeled with 20 mg of FITC/g of protein in earlier experiments. None of the conjugates prepared with 25 or 30 mg of FITC/g of protein stained specifically with an intensity greater than 2+ after the conjugates had been diluted sufficiently to eliminate nonspecific staining.

Discussion. McKinney *et al*(6) described a controlled method for labeling of antibody with FITC. Conjugates were tested on bacterial smears only, consequently nonspecific staining commonly encountered with cultured mammalian cells or sections of tissue was not discussed. The procedure described herein for optimal labeling antibodies to stain antigen in tissues is an obvious extension of the work of McKinney and coworkers and provides a relatively easy way to conjugate serum globulins to reduce nonspecific staining and to obtain maximal activity from conjugates.

The purpose of this study was to determine whether the optimal concentration of FITC to be used in conjugation was different for each immune serum, the assumption being that globulins of high antibody titer could be labeled more heavily since they could be diluted farther to eliminate nonspecific staining. The results obtained, however,

indicated that under the conditions used for conjugation, 20 mg of FITC/g of serum globulin was optimal for all conjugates. At concentrations of FITC lower than 20 mg the conjugates could not be diluted as far and at higher concentrations the nonspecific staining was excessive probably because antibody as well as nonantibody molecules were becoming overlabeled and were staining nonspecifically.

The intensity of specific staining of conjugates diluted sufficiently to rid them of nonspecific staining was greater when 20 mg of FITC/g of protein was used than in conjugates prepared with higher concentrations of FITC.

Twenty milligrams of FITC/g of protein are also recommended for labeling sera of low antibody titer since the nonspecific staining remained relatively low and such conjugates require passage through a DEAE cellulose column or some additional treatment, regardless of the FITC concentration used.

Some of the factors which control the rate of labeling of serum proteins by the method used in this study are the purity of the FITC, the ambient temperature, and the control of pH during the reaction. Since these conditions may vary, it is important to determine whether 20 mg of FITC/g of protein is optimal under conditions of the particular laboratory.

A factor involved in over labeling of serum proteins is excessive labeling in areas of high FITC concentration. In most methods of conjugation currently being used the pH is adjusted by adding carbonate-bicarbonate buffer to the serum proteins after which FITC is added slowly to reduce over labeling of proteins in localized areas of high FITC concentration. In the method described here the pH is adjusted after the FITC is mixed with the serum proteins. This eliminates the occurrence of "pockets" of high FITC concentration under conditions which allow for a rapid rate of conjugation, and consequently, contributes to more uniform labeling.

It should be pointed out, however, in methods where 0.5 M carbonate-bicarbonate buffer is used at a final concentration of 10% in the reaction mixture, that the final pH of the conjugate mixture is somewhat lower than the original pH of the buffer. This lowering of the pH is probably one of the main reasons that conjugation in some of the currently used methods occurs at a slow rate. If the pH falls sufficiently the rate of labeling may be slow, but on the other hand, the problem of localized labeling is also reduced.

Summary. A modification of a previously described procedure for controlled labeling of serum proteins with fluorescein isothiocyanate (FITC) is described. Specimens of serum globulin from several species of animals were conjugated with varying concentrations of FITC to determine whether each serum had an optimal FITC:protein ratio for conjugation. Twenty milligrams of FITC/g of protein were found to be near optimal for all sera conjugated under the conditions described. As this FITC concentration was exceeded, titers of nonspecific staining of FL cells increased faster than those of specific staining. The levels of nonspecific staining were low when conjugates were prepared with FITC concentrations of 20 mg or less. The advantage of this method of labeling is its rapidity and simplicity. With globulins of moderate to high antibody titer no special treatment of the conjugate is required to remove the highly labeled proteins.

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