

Use of Immunoglobulin Coupled to Agarose Beads for Examining the Specificity of Conjugates

ROY W. SCALES,* NORMAN F. JACOBS, JR., AND ROMULUS SKAGGS

Center for Disease Control, Atlanta, Georgia 30333

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Preparations of purified immunoglobulins, light chains, and unrelated proteins (used as controls) were covalently linked to agarose beads and used to study the specificity of fluorescein isothiocyanate conjugates. The data demonstrate how these beads can be used to detect immunological and non-immunological reactivity in conjugates. Commercial conjugates, conjugates prepared in this laboratory, and fluorescein-labeled normal immunoglobulins demonstrated high reactivity with control beads unless chromatographed on diethylaminoethyl-cellulose to select for the proper fluorescein-protein ratio. Undesirable immunological reactivity could be demonstrated in commercial conjugates and was shown to be due to anti-light-chain antibody.

Both direct and indirect approaches of the fluorescent antibody technique have found wide application in the detection of antigens and antibodies. Methods that have generally been used for evaluating fluorescent antibody reagents include gel diffusion, immunoelectrophoresis, and other forms of the precipitation reaction. Ideally, conjugates should be tested and evaluated within the system for which they were designed, as recommended by others (2, 6).

Fluorescent microscopy has been used for evaluating class-specific conjugates in relatively few published studies. By covalently coupling antigens (or antibodies) to a solid phase, such sensitivity and specificity testing can be performed. The cyanogen bromide technique (1) for coupling antigens and antibodies to agarose beads is gentle and reproducible. Recently, Van Dalen et al. (11) reported such an approach for studying antigen-antibody reactions in immunofluorescence. This report describes a method for preparing and evaluating immunoglobulin (Ig) class-specific conjugates. Both immunological and nonimmunological cross-reactivities were identified and removed from commercial and laboratory-prepared conjugates.

MATERIALS AND METHODS

Antigens and antisera. Anti-human IgG was prepared by immunizing New Zealand white rabbits with IgG which had been purified on diethylaminoethyl (DEAE)-cellulose and was then mixed with Freund incomplete adjuvant. This antiserum was then passed through a light-chain immunoadsorbent column prepared as described below. Anti-human IgA was prepared by immunizing rabbits with secretory IgA isolated from colostrum (10). This anti-

serum was absorbed with human IgG and cord serum immunoadsorbents. Anti-human IgM was prepared by immunizing rabbits with IgM purified according to the method of Chaplin et al. (5). This antiserum was absorbed with secretory IgA and cord serum immunoadsorbents. Light chains were prepared from purified IgG as described by Fleischman et al. (8) and recycled twice on Sephadex G-100 1 M acetic acid.

Coupling of immunoglobulins to agarose beads. These methods were essentially those of Cuatrecasas (7). Sepharose 4B was activated with cyanogen bromide (100 mg/ml of packed beads) at pH 11.0 for 6 to 8 min; it was washed thoroughly with cold distilled water and then with 0.5 M sodium bicarbonate, pH 8.0. Proteins were coupled at a concentration of 5 to 10 mg/ml in bicarbonate buffer overnight at 4 C. Unreacted sites on the beads were blocked with 100 ml of 1 M ethanolamine, pH 8.0, overnight at 4 C. These beads were thoroughly washed with 0.1 M glycine-hydrochloride, pH 2.5, and with 0.01 M tris(hydroxymethyl)aminomethane(Tris)-buffered saline, pH 7.2. The beads were then ready for use as an immunoadsorbent or as the test particle in fluorescent microscopy.

Preparation of conjugates. The IgG fractions from the above-mentioned rabbit antisera were used for labeling with fluorescein isothiocyanate. Conjugation was performed at room temperature for 6 h according to the method of Goldstein et al. (9). The reaction mixture was then chromatographed through Sephadex G-25 equilibrated with 0.05 M Tris-hydrochloride, 0.15 M NaCl, pH 8.0, to remove free fluorescein isothiocyanate and other reaction by-products. The voided peak (labeled Ig) was then chromatographed on DEAE-cellulose equilibrated with the same buffer. The material eluting with this buffer (0.05 M Tris-hydrochloride, 0.15 M NaCl, pH 8.0) was used in these studies (3). Commercial conjugates (anti-IgG, -IgM, and -IgA) from one company were also evaluated and processed in this manner.

Beads with bovine serum albumin or guinea pig light chains coupled to them served as controls. All conjugates were concentrated to original volume.

Test procedure and fluorescent microscopy. For testing, 0.2 ml of bead suspension (50%) and 0.2 ml of conjugate were mixed and shaken every five min for the next 15 min; next, they were centrifuged and washed 5× with 0.1 M phosphate buffer, pH 8.0. One drop of the suspension was placed on a slide to which was added one drop of mounting fluid (pH 8.0-buffered glycerin). Fluorescent microscopy was performed with a Zeiss microscope with an Osram HBO 200-W4 light source, a Zeiss BG-12 exciter filter, and a Zeiss no. 50 barrier filter. Intensity of fluorescence was graded 0 to 4+, wherein 0 = negative fluorescence, 4+ = brilliant green fluorescence, and 1+, 2+, and 3+ = intermediate levels of fluorescence.

RESULTS

Sources of non-immunological staining reactivity. Initial efforts in this investigation indicated that two different types of non-immunological staining reactivity were occurring. In a preliminary experiment it was shown that a preparation of control beads had not been effectively blocked with ethanolamine. Upon re-incubation with an extreme excess of ethanolamine, these beads remained negative when exposed to the conjugate. Although experiments were not performed to define the minimal amount of blocking agent required, we routinely used a 25-fold molar excess (with respect to CNBr used) and allowed the reaction to proceed overnight at 4 C.

A second type of non-immunological reactivity was observed with control beads containing bovine serum albumin or guinea pig light chains. These beads stained intensely with all conjugates and with conjugated normal goat IgG (Table 1). However, this reactivity could be eliminated by DEAE-cellulose chromatography of the conjugates. Native beads were not stained by any of the pretreated conjugates. The negative values (Table 1) of the bovine serum albumin and guinea pig light chain beads were obtained by using conjugates eluted from DEAE-cellulose at 0.15 M NaCl, 0.05 M Tris-hydrochloride, pH 8.0. This corresponded to a fluorescein-protein ratio of approximately 1.

Evaluation of conjugates: immunoelectrophoresis versus fluorescent microscopy. Figure 1 shows an example of results obtained when commercial conjugates were tested by immunoelectrophoresis and fluorescent antibody and clearly demonstrates the major reason for undertaking these experiments. These conjugates had been processed on DEAE-cellulose to eliminate the non-immunological reactivity described above. Each of the conjugates shows

TABLE 1. Results of testing conjugates with purified Ig on agarose beads^a

Ig on beads	Conjugate	Treatment of conjugates		
		None	DEAE chromatography	DEAE plus light-chain IA
IgG	Anti-IgG	4+	4+	4+
	Anti-IgM	4+	4+	Neg
	Anti-IgA	4+	4+	Neg
IgM	Anti-IgG	4+	1+	Neg
	Anti-IgM	4+	4+	4+
	Anti-IgA	4+	3+	Neg
IgA	Anti-IgG	4+	3+	Neg
	Anti-IgM	4+	4+	Neg
	Anti-IgA	4+	4+	4+
Light chains	Anti-IgG	4+	4+	Neg
	Anti-IgM	4+	2+	Neg
	Anti-IgA	4+	2+	Neg
BSA or guinea pig light chains	Anti-IgG	4+	Neg	
	Anti-IgM	4+	Neg	
	Anti-IgA	4+	Neg	
	Normal goat IgG	4+	Neg	

^a Abbreviations: IA, immunoabsorbent; Neg, negative; BSA, bovine serum albumin.

only a single precipitin line with its corresponding antigen (Ig). However, each conjugate showed varying degrees of cross-reactivity in fluorescent microscopy when reacted with beads containing a different purified Ig. Most of these latter reactions were of high intensity.

The data in Table 1 are representative of numerous batches of conjugates prepared in our laboratory and of conjugates prepared by one commercial company. The immunological cross-reactivity seen in fluorescent microscopy was due to antibodies directed toward light chains. This reactivity could be eliminated by passing the conjugates through a light-chain immunoabsorbent column.

DISCUSSION

We undertook this study to develop a qualitatively defined system for evaluating the specificity of fluorescent conjugates. The data show that immunoelectrophoresis alone is inadequate for such evaluation. By using purified immunoglobulins covalently coupled to agarose beads, we were able not only to determine the specificity of particular conjugates, but also to render the conjugates monospecific with appropriate absorptions. Although we did not quantitate the amount of material that could be detected, such a system can be used for quantification (2, 11).

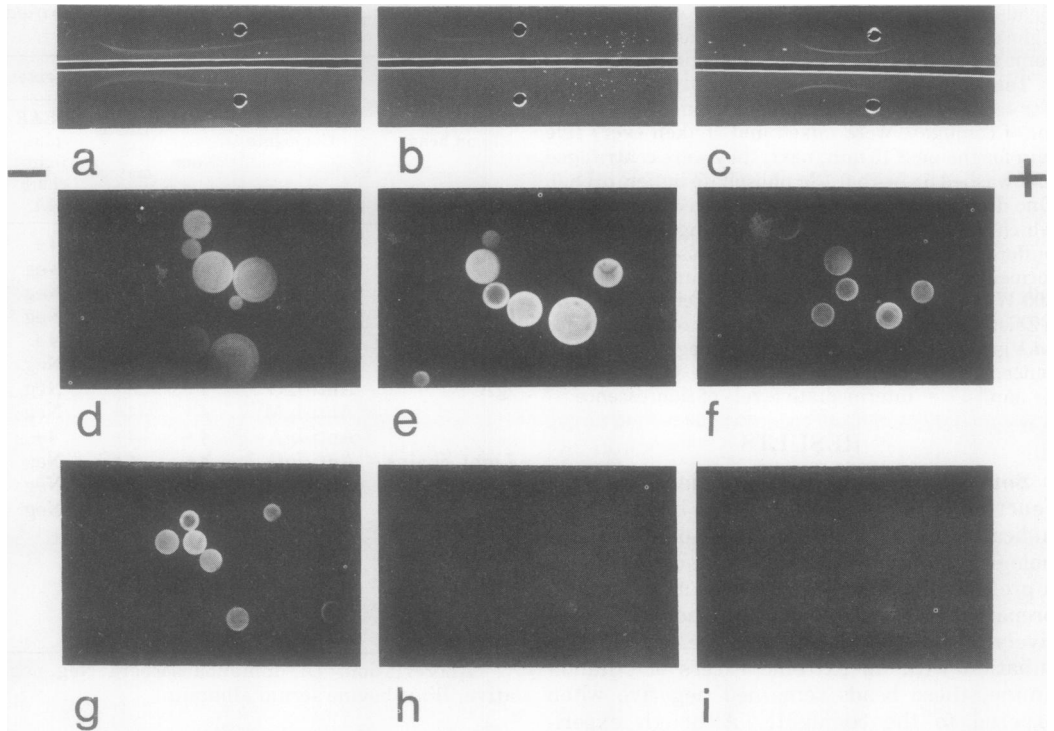


FIG. 1. Immunelectrophoresis and fluorescent microscopy of conjugates after DEAE-cellulose chromatography. Normal human serum in each top well: (a) anti-IgG; (b) anti-IgM; (c) anti-IgA; (d) IgG beads + anti-IgG; (e) IgG beads + anti-IgM; (f) IgA beads + anti-IgG; (g) IgA beads + anti-IgM; (h) IgM beads + anti-IgA post light-chain immunoabsorbent; (i) IgA beads + anti-IgG post light-chain immunoabsorbent.

This study identifies two forms of unwanted conjugate reactivity. A non-immunological heavy staining by conjugates occurred when unfractionated conjugates and conjugated normal goat IgG were reacted with beads to which a heterologous protein such as bovine serum albumin or guinea pig light chains had been coupled. This problem was overcome by fractionating the conjugates to obtain antibody labeled with fluorescein in fluorescein-to-protein ratio of approximately 1. When the conjugates were passed through a DEAE-cellulose column, the molecules eluting at 0.15 M no longer exhibited this undesired staining. None of the conjugates ever stained native Sepharose 4B (or Sephadex G-25 used in earlier experiments). If the nonspecific immunofluorescence had been due to a high affinity for carbohydrate moieties, this reactivity would have been removed at the G-25 filtration step prior to DEAE chromatography. Thus, we feel that the data indicate that the loss of nonspecific fluorescence is due to the elimination of heavily labeled Ig that can react with charged moieties (protein) coupled to the beads. However, these fractionated conjugates exhibited immunological cross-reactivity (Ta-

ble 1). After absorption with a human light-chain preparation, the conjugates reacted only with the appropriate Ig.

Fluorescent microscopy has been used to evaluate conjugates in several previous studies. Van Dalen et al. (11) found that fluorescence could be measured quantitatively by using agarose beads; however, they did not evaluate the specificity of unknown conjugates or attempt to purify conjugates by immunoabsorption. The high blank (control) values they reported when ethanolamine was used were not observed in our study, perhaps because we used a high concentration of ethanolamine. Bergquist and Kreisler (2) prepared polymerized Ig microspheres to use as antigens for assessing conjugate reactivity, but no attempt was made to use this system for purification. In a recent study, Case et al. (4) reported using embedded sections of agarose beads for evaluating fluorescent conjugates. For negative controls, they used agarose beads which had been activated and blocked without having any protein coupled to them. Unwanted specificity was noted in almost all conjugates tested and was removed by absorption with whole Ig. However, our results

show that unwanted reactivity may be caused by non-immunological staining due to an excessively high fluorescein-to-protein ratio of the conjugate. This reaction does not occur with beads that do not have any protein coupled to them; the reactivity can be removed by absorption of the conjugate with a protein-containing bead. Therefore, it is not clear whether the undesired reactivity reported in their study represented true immunological cross-reactions and, if so, whether it was due to light-chain or heavy-chain cross-reactivity.

Fluorescent conjugates offer a powerful tool for the identification for class-specific antibodies, but to accomplish this the conjugate must be monospecific. The procedures described here will allow recognition of unwanted staining, characterization of its nature, and purification of conjugates.

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