Papain-Treated Globulins in Specific and Cross-Reacting Immunofluorescent Staining¹

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ABSTRACT

JONES, WALLIS L. (Communicable Disease Center, Atlanta, Ga.), AND J. W. FOSTER. Papain-treated globulins in specific and cross-reacting immunofluorescent staining. *J. Bacteriol.* **91**:984-986. 1966.—Cross-reaction staining with preimmune and specific fluorescein conjugates to *Corynebacterium diphtheriae* was studied by use of papain-treated antisera. Enzyme-treated antisera were labeled and chromatographically separated into three fractions. Fractions were tested by both direct and inhibition fluorescent-antibody procedures. The cross-reaction staining was found in all three fractions, whereas specific staining was found in only two fractions.

Fluorescent-antibody (FA) reagents have been prepared and used to identify *Corynebacterium diphtheriae* in pure and mixed cultures. The organisms were detected regardless of their type or their ability to produce toxin. Conjugates used in this procedure, both specific and normal, often contain antibodies or antibodylike substances that react with certain streptococci and *Staphylococcus aureus*. These reactions may be a source of error in interpretation of the tests. Measures have been taken to alleviate these problems by inhibition procedures, counterstaining technique, sorbing conjugate with heterologous bacteria, diethylaminoethyl cellulose chromatography (1), and improving labeling techniques (4).

Certain undesirable staining reactions still remain, however, with any of the mentioned techniques, and specific staining may be reduced so that it is of little value as a diagnostic aid.

The purpose of this work was to determine whether a fraction responsible for the undesired staining given by *C. diphtheriae* specific antiserum and by normal serum could be detected. Rabbit γ -globulin treated with papain (7, 8) was used to prepare labeled and unlabeled reagents for this study. Each fraction collected was analyzed by direct FA and inhibition of fluorescent reactions.

MATERIALS AND METHODS

Strains. The six strains of *C. diphtheriae* used in the development of the original FA test were used as

representative strains of the diphtheria organism (6). Staphylococcal and streptococcal strains that had been found previously to cross-stain with both specific and normal conjugates were also employed.

Production of fluorescein conjugates. Antisera to the O and K antigens of *C. diphtheriae* were produced in rabbits as previously described by Moody and Jones (6). Globulins were separated from the rabbit sera by the addition of ammonium sulfate to a final concentration of 1.95 M and were labeled at 25 C with fluorescein isothiocyanate (4).

Preparation of papain enzyme. The enzyme was prepared by a modification of a procedure reported by Kimmel and Smith (3). Crude papaya latex (18 g) was mixed with washed sand (15 g) and ground in a mortar with 20 ml of 0.04 M cysteine (6.3 g of cysteine HCl in 1,000 ml of 0.054 N NaOH).

Commercial papain (Nutritional Biochemicals Corp., Cleveland, Ohio) and the prepared papain were each recrystallized by dissolving a minimal amount in water at room temperature. A 10-ml amount of saturated NaCl per 300 ml of protein solution was added slowly while stirring; then the mixture was allowed to stand overnight in the cold, and was centrifuged the next day. Crystallization was repeated 2 days later, and crystals were collected. The twice-recrystallized papain was then used to prepare mercuripapain as follows.

Twice-recrystallized papain was dissolved in 70% ethyl alcohol containing 0.006 M HgCl_2 to make a 1.5% solution. This solution was refrigerated for 5 days, and the crystals thus formed were removed by centrifugation.

Papain addition and fractionation. A 1-mg amount of mercuripapain in 0.3 ml of buffer (pH 7.0; 0.1 M potassium phosphate, 0.01 M cysteine, and 0.002 M ethylenediaminetetraacetate) was added to each 10-ml portion of 1.0 g/100 ml rabbit globulin (7, 8).

¹ Part of this work is taken from a thesis presented by the senior author in partial fulfillment of the requirements for the M.S. degree, University of Georgia.

This solution was incubated for 16 hr at 37 C. The digested protein solution was dialyzed against normal saline. Portions of these digested globulins were labeled with fluorescein isothiocyanate.

Carboxymethylcellulose chromatography. Labeled and unlabeled papain-digested globulins were separated by column chromatography. Carboxymethylcellulose columns were prepared by packing a glass column, 25 mm in diameter, with carboxymethylcellulose in 0.01 M acetate buffer, pH 5.5 (7, 8).

A 40-ml amount of each conjugate and of each unlabeled papain digest was chromatographed by use of acetate buffer in a gradient from 0.01 to 0.9 M. Three fractions were collected and pervaporated to 0.4 g/100 ml protein. The pervaporated conjugates were dialyzed against phosphate-buffered saline to restore the proper molarity and pH.

Fluorescent microscopy studies. Fluorescent studies were done with each chromatographically separated fraction of the papain-digested and fluorescein-labeled globulin, with digested and labeled but unseparated globulins, and with undigested, unseparated conjugate. Strains of *C. diphtheriae*, *S. aureus*, and streptococci were used as slide antigens.

Smears were made from cells grown in Heart Infusion Broth plus 0.05% glucose (HIB) overnight. Smears were fixed with 95% ethyl alcohol and were stained for 30 min with the conjugates, rinsed in 0.01 M phosphate-buffered saline (PBS) for 10 min, and mounted in the usual manner. Examination of smears was accomplished with a fluorescence assembly by use of an Osram HBO-200 mercury vapor lamp and Schott BG-12 exciter filter and OG-1 barrier filter. The one-step FA inhibition test (5) was applied with the use of each chromatographic fraction of the papain-digested globulin, the unseparated papain-digested globulin, and the untreated globulin. The labeled conjugate used in the inhibition test was untreated with papain. The unlabeled protein solution was added to the labeled conjugate, and the mixture was allowed to react for 30 min on the smear. Washing, mounting, and examination procedures were the

same as those used for the direct fluorescent examination.

RESULTS

Fluorescent staining reactions. Conjugates were titrated and applied to smears. The observed intensity of fluorescence of these smears is seen in Table 1. Cross-reactions as well as specific fluorescent staining were observed at titers of 1:128 when staining with either fraction I or II. However, in fraction III the *C. diphtheriae* strains had titers only to 1:8, whereas cross-staining with the other strains was still intense at 1:128.

Table 2 shows staining reactions with fractions comparable to the ones above but prepared from preimmune serum conjugates and diluted 1:8. *C. diphtheriae* strain 7 and the cocci showed moderate staining.

When the inhibition method was used, unlabeled fractions I and II inhibited effect on fluorescent reactions. Table 3 demonstrates a slight-to-moderate degree of fluorescence with specific cells and a marked degree with the cross-reacting strains.

Analytical ultracentrifuge studies were carried out on fraction II and on globulins that were treated with papain but not separated by chromatography. Analysis of the unseparated mixture of fractions should show whether any components were present other than the three collected. The results given in uncorrected Svedberg units are: papain-treated (not separated), 2.8S; fraction II, 2.7S.

DISCUSSION

This study demonstrates that papain-digested globulins can be labeled with fluorescein isothio-

TABLE 1. Fluorescent intensities of specific conjugates by the direct FA technique*

Organism	Fraction I and fraction II					Fraction III				
	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
<i>Corynebacterium diphtheriae</i>										
4.....	4	3	2	2	1	N	N	N	N	N
5.....	4	4	3	3	2	N	N	N	N	N
6.....	4	4	3	3	2	N	N	N	N	N
7.....	4	4	3	3	2	2	1	N	N	N
8.....	4	4	4	2	2	N	N	N	N	N
9.....	4	3	2	1	N	N	N	N	N	N
<i>Streptococcus</i> C-1.....	4	4	4	4	3	4	4	4	4	3
<i>Streptococcus</i> C-4.....	4	4	3	3	3	4	4	4	3	3
<i>Streptococcus</i> G-9.....	4	4	3	3	3	4	4	3	3	3
<i>Staphylococcus aureus</i>	4	4	4	4	4	4	4	4	4	4

* Key to fluorescence intensity: N, no fluorescence; 1, slight fluorescence, not definitely green; 2, low fluorescence, yellow-green; 3, moderate fluorescence, bright yellow-green; 4, maximal fluorescence, brilliant yellow-green.

TABLE 2. *Fluorescent intensities of preimmune conjugates by the direct FA technique**

Organisms	Un-treated conjugate (1:8)	Treated, unseparated (1:8)	Fractions		
			I 1:8	II 1:8	III 1:8
<i>Corynebacterium diphtheriae</i>					
4.....	N	N	N	N	N
5.....	N	N	N	N	N
6.....	N	N	N	N	N
7.....	2	2	2	2	N
8.....	N	N	N	N	N
9.....	N	N	N	N	N
<i>Streptococcus</i> C-1....	3	2	2	2	2
<i>Streptococcus</i> C-4....	3	2	1	1	1
<i>Streptococcus</i> G-9....	3	2	1	2	2
<i>Staphylococcus aureus</i>	4	4	1	2	2

* Key to fluorescence: N, no fluorescence; 1, slight fluorescence, not definitely green; 2, low fluorescence, yellow-green; 3, moderate fluorescence, bright yellow-green; 4, maximal fluorescence, brilliant yellow-green.

TABLE 3. *Inhibition of fluorescent staining by use of unlabeled specific globulin fractions**

Organism	Un-treated globulin	Papain-treated, unseparated	Fractions		
			I	II	III
<i>Corynebacterium diphtheriae</i>					
4.....	1	2	1	1	3
5.....	1	1	2	1	2
6.....	1	2	2	2	3
7.....	1	2	2	2	2
8.....	1	1	2	1	2
9.....	1	1	1	1	3
<i>Streptococcus</i> C-1....	N	N	N	N	1
<i>Streptococcus</i> C-4....	N	N	N	N	1
<i>Streptococcus</i> G-9....	N	N	1	N	1
<i>Staphylococcus aureus</i>	N	N	1	1	2

* Key to fluorescence intensity: N, complete inhibition; 1, marked inhibition; 2, moderate inhibition; 3, slight inhibition.

cyanate and separated by carboxymethylcellulose chromatography into three active fractions. Two of these fractions, I and II, reacted with diphtheria organisms at or near the same titer as untreated conjugate. These fractions also reacted with the cross-reacting strains. Fraction III reacted only with the cross-reacting strains in contrast to un-

treated conjugate. Since the cross-reaction was seen in all three fractions, perhaps more than one factor is involved in specific versus cross-reaction staining.

No fraction was shown to stain only the specific organism for which the antisera were originally prepared. Any fraction that stained *C. diphtheriae* also stained the aforementioned coccal strains.

Although this study did not yield a more specific FA reagent for direct staining, it would appear that the fractions obtained are similar to those reported by Porter, and such reagents may be of value in reducing or eliminating cross-staining reactions involved in other FA procedures. This technique may be of value in reducing non-specific staining in tissues when tissue sections are examined by FA techniques. Fractions I and II from papain-treated antiglobulin can be pooled and used to stain specific organisms.

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