

INHIBITION OF COMMON-ANTIGEN FLUORESCENCE IN GROUPING STREPTOCOCCI BY THE FLUORESCENT ANTIBODY METHOD¹

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The fluorescent antibody method of Coons and Kaplan (1950) has led to numerous studies of its practical application, many directed to the problem of rapid and accurate identification of microorganisms (Goldman, 1953, 1957; Moody, Goldman, and Thomason, 1956; Thomason et al., 1956, 1957; LaBrec, Formal, and Schneider, 1959; Winter, and Moody, 1959).

Moody, Ellis and Updyke (1958) showed that serological grouping of β -hemolytic streptococci was possible by specific fluorescent antibody staining of smears prepared from cultures. Rapidity, economy, and ease of performance were cited as advantages of this procedure over the conventional precipitin test. Because of its time-consuming nature, routine use of the latter has been impracticable in most laboratories except for special investigations despite evidence that infections with group A streptococci may be forerunners of rheumatic fever and its sequelae. Therefore, a method which permits rapid, accurate determination of the presence of group A hemolytic streptococci in routine throat specimens is of considerable importance.

This report deals with application of the fluorescent antibody technique to a large volume of routine cultures from presumed streptococcal infections submitted to a state health laboratory, with the problem of preparing and standardizing the reagents involved and with ancillary means of demonstrating, within 24 hr after receipt, the presence of group A streptococci in specimens when present in sufficient numbers to justify a logical assumption of etiological significance. Primarily it deals with rapid differentiation by inhibition of common antigen fluorescence among serological groups A, C, and G. The procedure

evolved from our studies has been employed successfully on a routine basis to report serological findings on the majority of specimens within 24 hr after receipt of throat swabs.

MATERIALS AND METHODS

Specimen collection and transport. Routine throat swabs from physicians were plunged into and left in a 1 per cent aqueous agar gel (nutrient-free) in a collection tube as provided in a mailing outfit successfully used in our laboratories for this purpose for more than 25 yr.

Bacteriological procedures. Upon receipt, swabs were streaked on glucose-free tryptose agar containing 7 to 8 per cent sheep blood. Incubation was anaerobic in an atmosphere of 90 per cent N₂ and 10 per cent CO₂ at 35 C. Plates were examined after 18 to 24 hr; then held for an additional 18 to 24 hr at room temperature and examined. β -Hemolytic streptococcus colonies were transferred to trypticase soy broth (about 12 ml) and to blood agar plates for reference purposes. Sediments of the broths were used for precipitinogen preparation and for fluorescent antibody studies.

In addition, during a portion of this study tubes containing 3 ml of Streptose broth (BBL) were inoculated with the swabs after inoculation of the blood agar plates. After 18 to 24 hr incubation at 35 C, sediments from these broth cultures were examined by the fluorescent antibody method whenever the corresponding blood agar plates showed colonies of β -hemolytic streptococci.

Antisera. Antisera for groups A, B, C, D, F, and G were prepared in rabbits by conventional methods utilizing stock cultures (SS132 (A); SS458 (B); SS188 (C); SS498(D); SS195 (F); SS13 (G)) furnished by the Communicable Disease Center, U. S. Public Health Service. These were used for precipitin tests and for preparation of the conjugates.

Preparation of globulin and conjugates. Globulin

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fractions were prepared by precipitation with ammonium sulphate. Conjugation with fluorescein isothiocyanate (BBL, lot no. 11059) was performed according to the procedure described by Marshall, Eveland, and Smith (1958). Adsorption of group A conjugates was done with equal volumes of packed group C cells at 35 C for 1 hr. Prepared conjugates were evaluated with recent isolates of groups A, B, C, F, and G, as determined by the precipitin technique of Lancefield (1928), as well as the stock strains used for antiserum production.

Precipitin tests. Initially, the hot hydrochloric acid method of Lancefield (1928) was used for preparing precipitinogens but the more rapid autoclave method of Rantz and Randall (1955) was substituted early in this study. Tests were performed by the capillary tube method of Swift, Wilson, and Lancefield (1943). All specimens subjected to the fluorescent antibody test were also tested by the precipitin test.

Preparation of smears. A "Flo-master" pen (Cushman-Denison Manufacturing Company, New York, New York) was used to make 2 circles approximately 1 cm in diameter on each alcohol washed slide. Sediments of 18 to 24 hr broth cultures were resuspended in buffered saline (pH 7.1 to 7.2) to an approximate McFarland 4 density. Cotton-tipped applicator sticks were used to transfer each suspension to each of the circles on a slide. Smears were allowed to air dry and then were heat fixed.

Fluorescent antibody staining. Staining was carried out with a 1:20 dilution of a group A conjugate in a moist chamber for 20 min. Slides were then immersed for 10 min in buffered saline, blotted with filter paper and mounted in buffered glycerol under a cover slip. The particular conjugate used to obtain the results reported was unadsorbed since it had been determined that adsorption of this particular lot was unnecessary under the conditions of use.

Inhibition technique. Inhibition of common antigen fluorescence was carried out sequentially using a predetermined optimal dilution of group C antiserum. A drop of diluted antiserum (e.g., 1:40) was applied to one of the encircled paired smears and blotted off after 5 min exposure. Group A conjugate was then applied to both smears and staining completed as described above. The comparative intensity of staining of the paired smears was then determined. If no

reduction of intensity was observed in the smear treated with group C antiserum, the specimen was adjudged to contain group A organisms.

Fluorescence microscopy. The optical system used for examination of mounts consisted of a Leitz Ortholux microscope fitted with dark-field condenser, 10× ocular and achromatic oil immersion objective (95×; na 1.0) with funnel stop; a lamp housing with Phillips CS 150 bulb; and appropriate ultraviolet filters.

Recording results. Intensity of fluorescent antibody staining was recorded as follows: 4+ = cells sharply outlined, bright green fluorescence; 3+ = cells sharply outlined, dull green fluorescence; 2+ = cells not sharply outlined, slight green fluorescence; + = cells not outlined, only faint green discernible in denser areas of smear.

RESULTS

Difficulties in obtaining group-specific fluorescent antibody staining. Strong cross reactions were reported by Moody et al. (1958) when group C strains—and to a lesser extent group G strains—were stained with group A unadsorbed conjugate although crosses were not observed with groups B, D, and F. Since those results were obtained with stock strains, we investigated the possibility that freshly isolated strains might respond differently. However, findings (table 1) paralleled those observed with stock strains. Strains of group C and G could not be differentiated effectively from those of group A when stained with unadsorbed group A conjugate. Furthermore, the staining of groups C and G could not be eliminated or reduced by dilution of the conjugate without rendering it ineffective against group A.

TABLE 1

Staining of streptococci with unadsorbed group A conjugate

Serological Group	No. Strains Tested	No. Strains Fluorescing in 1:20 Dilution	No. Strains Staining 4+ in Dilutions Indicated			
			1:20	1:40	1:80	1:160
A	10	10	10	10	2	0
B	10	0	0	0	0	0
C	10	10	5	5	0	0
D	1	0	0	0	0	0
F	6	0	0	0	0	0
G	7	7	2	2	0	0

Two lots of undiluted group A conjugate staining 4+ at 1:40 dilution were adsorbed with group C cells (strain SS188) for 1 hr at 35 C. Although adsorbed conjugates no longer stained group C and G cells, they failed to stain group A cells to more than a 2+ intensity in a 1:10 dilution. Both lots of the unadsorbed conjugate were prepared from the same lots of group A antiserum and of fluorescein isothiocyanate. Two additional group A conjugates retained their staining activity against group A after adsorption but also continued to cross with several group C strains. These difficulties in the production of satisfactory group-specific conjugates led to an exploration of the inhibition phenomenon as a means of differentiating group A cells from group C and G cells.

Development of technique for inhibition of common antigen fluorescence. One step inhibition suggested by Goldman (1956) has been used by others to prove specificity of reacting conjugates. Although this technique was considered as a possible solution to the problem of setting up a group A screen in the absence of a satisfactory adsorbed group A fluorescent antibody, we found that the two steps or sequential procedure could be used effectively in streptococcus fluorescent antibody studies. It was found that a 1:40 dilution of our group C unconjugated antiserum applied to smears for 5 min before staining did not affect the affinity of unadsorbed group A conjugate for group A cells, whereas this inhibiting step reduced the affinity of the conjugate for group C and group G cells so materially that satisfactory group differentiation could be made. This is shown in table 2. Inhibition by group G antiserum was not effective with group C cells. It will be noted that group C and group G cells pretreated with group C antiserum no longer showed significant cross reactions with the unadsorbed group A conjugate. This same pattern has prevailed throughout subsequent routine testing.

Routine application of technique for inhibition of common antigen fluorescence. During a recent 3-month period, 4,480 throat swabs were examined, of which 1,256 (28 per cent) yielded β -hemolytic streptococcus colonies on blood agar. A representative colony from each plate was transferred to trypticase soy broth and, after incubation, tested by precipitin test and by the inhibition of common antigen fluorescence

TABLE 2
Pretreatment with unlabeled group C antiserum on staining with group A conjugate

Group	Strain No.	Intensity of Staining of Cells Pretreated with:		
		Saline control	Normal rabbit serum control 1:40	Group C antiserum 1:40
A	A-1	4+	4+	4+
	A-2	4+	4+	4+
	A-3	4+	4+	4+
	A-4	4+	4+	4+
C	C-1	4+	3-4+	+
	C-2	3+	3-4+	+
	C-3	4+	4+	2+
	C-4	4+	4+	+
G	G-1	3+	2+	+
	G-2	2+	2+	0
	G-3	3+	+	0
	G-4	2+	2+	0

A-1, C-1, and G-1 were the strains used for antiserum production.

technique. In 17 instances, only an occasional β -hemolytic colony, not well isolated, was observed on the original plate and the streptococci present apparently were overgrown by contaminants in broth transfers, making it impossible to do precipitin tests. Table 3 shows results obtained on the 1,239 remaining isolants. The inhibition of common antigen fluorescence technique was fully as effective as the precipitin test in identifying group A streptococci.

In the 17 instances not included in the table, presence of group A cells was established in contaminated broth cultures by the inhibition of common antigen fluorescence technique. In other instances of contamination of first broth transfers, this technique had established the presence of group A cells several days before subcultures could be purified for confirmation by the precipitin test.

Precipitin tests with other group antisera also were performed on all isolates. Of the 305 isolates which were not group A by the precipitin test, 21 were group B; 16, group C; 41, group F; 56, group G. Since antisera against groups B and F were available in sufficient amounts for only part of this study, the remaining 170 were not subjected to complete study but were found not to

TABLE 3

Comparison of precipitin and inhibition of common antigen fluorescence (ICAF) results on streptococcus isolates

Group A	Precipitin Test		ICAF Test	
	No.	Per cent	No.	Per cent
Positive.....	934	76	934	76
Negative.....	305	24	305	24

be in groups A, C, or G. It is probable that most of these 170 belonged to groups B and F; a great many presented the colonial morphology representative of group F strains.

Use of the inhibition of common antigen fluorescence technique on primary cultures. To this point, it has been shown that the inhibition of common antigen fluorescence technique can be applied effectively to determine the presence of group A streptococci in throat cultures. However, the results were obtained on broth cultures isolated from primary plates, a procedure which delays definitive serological grouping until the second day after receipt of the specimen.

Direct examination of smears made from typical colonies on primary blood agar plates would shorten the reporting time considerably but such smears were found unsatisfactory because we could not obtain consistently even distribution of the organisms in mounts. Larger clumps of organisms adhere poorly to the slide; those which adhere often produce a low grade non-specific fluorescence. A possible explanation for this is that the conjugate was not rinsed completely from within the mass of organisms even when an antigen-antibody union had not occurred. Broth cultures are most suitable for fluorescent antibody work but direct inoculation of an enrichment broth with throat swabs will be fully effective only if competitive oral flora are selectively inhibited.

At this point, Streptosel broth (BBL), selective for streptococci, was tried. Examination by the inhibition of common antigen fluorescence technique of 18 to 24-hr Streptosel broth cultures was confined to those specimens on which blood agar plates inoculated in parallel showed β -hemolytic streptococcus colonies. These broth cultures were grouped by the inhibition of common antigen fluorescence technique in parallel with subcultures in trypticase soy broth from corre-

TABLE 4

Determination of group A streptococci from primary Streptosel broth cultures and from pure culture isolations by the inhibition of common antigen fluorescence technique

Group A Streptococci	Primary Streptosel Cultures	Trypticase Soy Subcultures from Blood Agar
Positive.....	503	644
Negative.....	364	223

sponding plates. Table 4 summarizes the results obtained on 867 such specimens. The most important observation is that of the 644 specimens in which group A streptococci were found, 503 (78 per cent) were grouped correctly in Streptosel broth within 18 to 24 hr after the specimen was received. Failure to find group A streptococci in 141 primary Streptosel broth cultures may have resulted chiefly from the effect of inhibitory agents in the broth on the small numbers of streptococci inoculated since, in all instances, fewer than 10 hemolytic colonies were present on the corresponding blood agar

Inhibition of growth of fluorescing staphylococci by Streptosel broth. Coagulase positive staphylococci, which stain with 2 to 4+ intensity with conjugates from rabbit sera, are inhibited in Streptosel broth. Subcultures on blood agar from Streptosel broth inoculated with specimens containing staphylococci and incubated 18 to 24 hr were repeatedly either negative or, at most, yielded only 1 to 3 staphylococcal colonies, whereas many were observed on the original primary blood agar plates. This was opportune since attempts to eliminate fluorescent antibody staining of these strains by the inhibition of common antigen fluorescence method were completely unsuccessful.

Coagulase positive staphylococci were found on primary blood agar plates from 25 (11.2 per cent) of 223 consecutive throat swab specimens indicating that their frequency can provide a major obstacle to the fluorescent antibody grouping of streptococci in throat swab broth cultures unless measures to inhibit their growth are taken.

DISCUSSION

Antigenic relationships among the β -hemolytic streptococci are brought out much more forcefully by fluorescent antibody serology than by

precipitin techniques. If the fluorescent antibody relationships are found to be based upon common group antigens, possibly strains of groups C and G may contain sufficient of the specific group A substance to justify re-evaluation of the prevailing opinion that only group A infections may lead to rheumatic sequelae in man.

Antigenic relationships among strains of groups A, C and G are so close that successful adsorption of cross reacting antibodies from group A conjugates seems achievable only to a limited degree and complete adsorption may be impossible without rendering homologous reactivity ineffective for fluorescent antibody work. We have reason to believe from preliminary data not presented that higher titered, more intensely staining group A conjugates present more of a problem in this respect than those of intermediate reactivity. With such conjugates, controlled adsorption with group C cells is indicated before use as the staining reagent in the inhibition of common antigen fluorescence technique.

It must be kept firmly in mind that the results reported herein were obtained with an optical system providing adequate but nevertheless only moderately intense fluorescence. Successful use of a system yielding more intense fluorescence would have required the use of different dilutions of antiserum and conjugate. Hence, a routine procedure should be instituted only after proper standardization of reagents under conditions imposed by the apparatus in routine use.

Nevertheless, the fluorescent antibody technique lends itself admirably to grouping of streptococci isolated from man when used on the sedimented growth in a medium selective for streptococci in conjunction with suitably standardized procedures which inhibit intergroup cross reactions. The technique we are now recommending consists of the following steps:

- 1) Inoculation of throat swabs into 3 ml streptosel broth.
- 2) After 18 to 24 hr incubation at 35 C, centrifuge cultures, prepare smears from sediments, and fix them by heat.
- 3) Apply a drop of a predetermined dilution of crude group C antiserum, let stand 5 min, and blot.
- 4) Apply a suitable group A conjugate in predetermined dilution, let stand in moist chamber for 20 min, rinse in buffered saline, mount in buffered glycerol, and examine for 3 to 4+

fluorescence with an appropriate optical system. It can be anticipated that controlled adsorption with group C cells will be required before a high titered group A conjugate can be used for this purpose.

5) Report intensely fluorescing cultures as group A; subject others to further fluorescent antibody studies as desirable.

Parallel primary streaking of swabs on sheep blood agar permits study for minimal numbers of streptococci which may not develop in the broth and for other organisms which may be of significance. Failure of small numbers of streptococci on throat swabs to grow in Streptosel broth would seem to be of minor importance since their etiological significance in small numbers is debatable.

The volume of routine work experienced since the start of this study has precluded investigation of alternative methods. In effect, we have arbitrarily chosen to screen first for group A. This does not indicate that initial screening for group C, or groups C and G, would not achieve similar accuracy.

The fact that the inhibition of common antigen fluorescence technique using group C antiserum effectively inhibits fluorescence of group G stained with group A conjugate, suggests that group G is more closely related to group C than to group A.

The intense fluorescence of coagulase positive staphylococci in conjugates of rabbit serum deserves further study. We have been more concerned to date with circumventing its interference than with studying its causes. We can report only that attempted inhibition by prior application of group C antiserum and normal rabbit serum has proved ineffective, whereas use of a suitable primary culture medium such as Streptosel broth will inhibit staphylococcal growth and eliminate the need for adsorption of conjugates with staphylococci. This solution would seem more practical than using both group A conjugate and normal rabbit serum conjugate to detect coagulase positive staphylococci which will stain with both conjugates while the group A streptococcus cells stain only with the group A conjugate. Furthermore, the latter procedure would not solve the problem of examination of mixed cultures by the fluorescent antibody method because both types of organisms may occur in a single specimen.

Successful use of Streptosel broth, however,

necessitates that it be exposed to no more heat than that recommended for its sterilization. Overheating renders it inhibitory for streptococci as well as staphylococci.

Further studies are in progress in which methods for preparation, evaluation, and standardization of antisera and conjugates will be investigated. These will involve extension of our studies to other serological groups of β -hemolytic streptococci.

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SUMMARY

Cross reactions for groups C and G in fluorescein-globulin conjugates prepared from group A streptococcal antisera could not be eliminated by dilution. Adsorption of conjugates reduced intensity of heterologous fluorescent antibody staining but did not completely eliminate crosses with all strains encountered. Adsorption, furthermore, materially reduced homologous activity and, therefore, did not present a completely satisfactory solution.

These observations led to the development of a technique for inhibition of common antigen fluorescence which has been used successfully to identify group A strains in large numbers of throat cultures on specimens mailed to a central state health laboratory. The application of this technique to smears from sediments of 18 to 24-hr Streptosel broth cultures inoculated with throat swabs permitted prompt reporting of group A streptococci. Parallel platings on sheep blood agar have revealed that these primary broth cultures fail only when streptococci are present in comparatively small numbers and, hence, may have been inhibited in the broth. An additional advantage of this broth for primary cultures is that it effectively inhibits growth of coagulase positive staphylococci which will fluoresce in any conjugate prepared from rabbit serum. The inhibition of common antigen fluorescence technique depends upon treatment of heat fixed smears

with crude group C antiserum in predetermined dilution prior to fluorescent antibody staining with a predetermined dilution of group A conjugate. The need for adsorption of a given group A conjugate with group C cells will depend upon the fluorescent antibody staining intensity of the conjugate under conditions imposed by the optical system with which it is used.

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