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Immunofluorescent Identification of Type 12 Group A Streptococci

WILLIAM F. VINCENT, KATHLEEN J. LISIEWSKI, AND WILLIAM W. ULLMANN

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201, and Laboratory Division, Connecticut State Department of Health, Hartford, Connecticut

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The fluorescent antibody (FA) conjugate prepared by labeling streptococcal M type 12 antibody with fluorescein isothiocyanate was found to exhibit considerable nonspecific FA staining with other group A M-serotypes. The cross-reactions could be reduced sufficiently or eliminated by the addition of adsorbed homologous blocking serum (AHB) but not by preimmune serum. The AHB was prepared by adsorbing type 12 antiserum with untreated homologous cells. Comparative staining with unblocked and AHB-blocked FA conjugates enabled type 12 streptococci from clinical specimens to be rapidly and accurately identified.

The M proteins of group A streptococci have been implicated with several postinfectious sequelae such as glomerulonephritis and are believed to be requisites for virulence. Consequently, diagnostic examinations of streptococcal isolates should include M serotyping to alert the physician to potential sequelae. Several M serotypes may be prevalent in a community at any one time (6), further necessitating specific knowledge of M serotypes for critical epidemiological studies of streptococcal outbreaks.

M serotyping is usually performed by the microcapillary precipitin test (7). This method is laborious, consumes relatively large amounts of reagents, and is not generally practical for large-scale surveys. Fluorescent-antibody (FA) staining, on the other hand, is rapid, requires far less antiserum per determination, and is routinely used by many public health laboratories for the identification of group A streptococci (3, 5). However, this technique has not proven useful for the determination of M serotypes (W. F. Vincent and E. K. Borman, *unpublished data*) with the exception of type 1 (1).

This study reports the successful application of FA staining techniques to the serotyping of type 12 streptococci and provides a suitable method for large-scale surveys of streptococcal isolates.

MATERIALS AND METHODS

Strains and culture medium. Streptococcal strains. representing 38 of the 47 known serotypes, were obtained from the Center for Disease Control, Atlanta. Ga., or were taken from the culture collection of the Connecticut State Health Laboratory. Diagnostic isolates for typing were obtained through J. R. Redys of the Connecticut State Department of Health. All cultures were grown in Todd-Hewitt broth (Difco) supplemented with 2% yeast extract (8).

Antisera. *Streptococcus pyogenes* type 12 (Diag 65186) was subjected to three mouse passages before use as an immunogen. Vaccines were prepared by heat-killing whole cells and were injected into 3- to 5-kg New Zealand white rabbits by the procedures of McCarty and Lancefield (2).

Type-specific antiserum was prepared by adsorption with cells of the homologous immunizing strain which had been treated with trypsin to remove the M antigen. The cells from 2 liters of an 18-hr culture were collected by centrifugation and washed three times with 200-ml samples of 0.15 M saline. Washed cells were suspended in 50 ml of saline and killed by heating for 30 min in a water bath at 56 C. The killed cells were sedimented by centrifugation, suspended in 50 ml of 0.05 M sodium phosphate buffer (*p*H 8) containing 50 mg of crystalline trypsin (Calbiochem), and incubated for 2 hr at 37 C. The trypsinized cells were collected by centrifugation and washed three times with saline, and their acid extracts were examined by capillary precipitation (7) for the absence of type 12 M protein. Streptococcal antisera were twice adsorbed at 37 C for 1 hr by suspending 2 volumes of serum in 1 volume of packed trypsinized cells. The absence of group A specific antibody in the adsorbed serum was verified by capillary precipitation.

Adsorbed homologous blocking antiserum (AHB) was prepared by adsorbing with homologous, heat-killed cells which had not been subjected to trypsin treatment.

FA reagents. Immunoglobulin was precipitated three times in 40% saturated ammonium sulfate at 4 C. The precipitate globulin was dissolved in saline and freed from ammonium sulfate by gel filtration through Sephadex G-25 gel (fine grade, Pharmacia). The globulin was further fractionated overnight by

low-ionic-strength precipitation by dialysis against 0.01 M sodium phosphate buffer (*p*H 7.6) at 4 C and by centrifugating the precipitated protein at 2,000 × *g*. The soluble globulin supernatant was dialyzed against 0.01 M sodium carbonate-bicarbonate buffer (*p*H 9.2) and conjugated with 20 μg of fluorescein isothiocyanate (FITC; BBL) per mg of protein. Conjugation was carried out at room temperature for 6 hr with constant stirring (4). Free FITC was removed from the conjugate by filtration through Sephadex G-25 gel.

FA staining. Dilution of FA conjugate was prepared in 0.01 M sodium phosphate-buffered saline, (*p*H 7.6; PBS). Air-dried, heat-fixed smears from 18-hr cultures were stained with conjugate diluted 1:80 for 30 min at room temperature in a moist chamber and then were rinsed for 30 min in PBS. Stained smears were gently blotted dry and mounted under carbonate-buffered glycerine, (*p*H 9). Visualization of fluorescent staining was accomplished by using a Bausch & Lomb PB-252 microscope equipped with an American Optical Fluorolume ultraviolet illuminator. Intensities of cell wall staining were visually recorded as 4+ for intense staining, 3+ for bright staining, 2+ for moderate staining, 1+ for dull staining, and 0 for negligible or no staining.

Typing by capillary precipitation. All isolates exhibiting any degree of FA staining were examined for the presence of type 12 M protein by capillary precipitation with acidic extracts (7). A random 10% sampling of isolates exhibiting no immunofluorescence was also examined for type 12 M protein.

RESULTS

Streptococcal M type 12 FA conjugates prepared in these studies exhibited 4+ staining intensities with the immunizing strain and most other type 12 strains at dilutions as high as 1:160. These conjugates were tested against 37 M serotypes and were observed to exhibit considerable staining with several serotypes other than type 12 (Table 1). Eleven type 4 and three type 41 strains were examined and, with the exception of one type 4 strain, all exhibited significant FA staining. In the case of the other serotypes examined, more than one strain was tested in all cases, except for types 32, 39, and 43, and the results were usually very similar to those presented in Table 1. The addition of preimmune serum had no significant effect on altering the staining of heterologous types (Table 2); however, addition of AHB eliminated or greatly reduced heterologous staining with the exception of type 4 (Table 2). Whenever staining of heterologous cells was not completely eliminated by the presence of AHB, the degree of staining was sufficiently reduced to allow them to be distinguished from type 12 strains.

To evaluate the diagnostic application of this FA conjugate, 2,000 group A streptococcal isolates were examined from throat cultures (Table 3). These were grouped by the staining with unblocked conjugate as 164 isolates giving 3+ or 4+ intensities, 294 giving 2+ intensities, and 1,542

TABLE 1. *Immunofluorescent staining of streptococcal M serotypes with an M serotype 12 FA conjugate*

M serotype	FA staining intensity[a]
12	4+
4, 41	4+
33, 14	3+
1, 2, 18, 32	2+
5, 15, 31, 36, 39, 43, 44	1+
3, 6, 8, 9, 11, 17, 19, 23, 24, 25, 26, 29, 30, 34, 35, 40, 41, 42, 43, 46, 47	0

[a] Intense staining, 4+; bright staining, 3+; moderate staining, 2+; dull staining, 1+; negligible or no staining, 0.

TABLE 2. *Effect of the addition of preimmune serum and AHB on heterologous staining with a streptococcal M type 12 FA conjugate*[a]

M serotype	Unblocked conjugate	1:10 Preimmune serum	1:10 AHB
12	4+	4+	4+
4	4+	4+	2+
41	4+	2+	0
14	3+	3+	0
33	3+	2+	0
1	2+	2+	0
2	2+	2+	0
18	2+	1+	1+

[a] Intense staining, 4+; bright staining, 3+; moderate staining, 2+; dull staining, 1+; negligible or no staining, 0.

TABLE 3. *Unblocked and AHB-blocked FA staining of group A streptococcal isolates with an M type 12 FA conjugate*

No. of isolates	FA staining reaction[a]		M type 12 precipitation
	Unblocked	AHB-blocked	
104	4+	3+ or 4+	+
22	3+	2+ or 3+	+
8	4+	2+	0
30	3+ or 4+	0 or 1+	0
294	2+	0 to 2+	0
1,542[b]	0+ or 1+	0 or 1+	0[b]

[a] Intense staining, 4+; bright staining, 3+; moderate staining, 2+; dull staining, 1+; negligible or no staining, 0.

[b] We examined 150 isolates for the presence of type 12 M protein by capillary precipitation.

giving 0 or 1+ intensities. When unblocked staining and blocked staining of the first group were compared, 126 isolates showed no significant reduction in staining intensity and gave complete agreement with the results of the capillary precipitin technique for type 12 M antigen. From these data, it can be concluded that type 12 strains can be identified accurately by a comparison of unblocked and blocked staining.

DISCUSSION

The immunofluorescent techniques described here, in most cases, enabled identification of type 12 streptococcal isolates without resorting to conventional precipitin assays. If particular attention is given to the degree of cell wall staining, most questionable isolates can be presumptively eliminated, since all type 12 strains encountered during these studies displayed very marked peripheral staining of the cell wall.

The problem of heterologous staining of strains of other M serotypes was largely overcome by using AHB-blocked FA conjugate (Table 3). In this regard, it is noteworthy that only 4 of the 38 M serotypes tested cross-reacted strongly with type 12 FA conjugate (Table 1), and only the staining of type 4 cells could not be completely masked by the AHB (Table 2). It would therefore seem that the most likely error would arise from misidentification of type 4 strains. We have, however, encountered a number of strains which could not be typed by the precipitin reaction that gave significant staining reactions with unblocked conjugate.

The nature of the serum constituents responsible for blocking is not known but is assumed to be antibody to certain common antigens shared by the streptococci. Blocked staining with preimmune serum did not appreciably reduce nonspecific staining, indicating that the active constituents in AHB are the products of a specific immune response.

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LITERATURE CITED

1. Karakawa, W. W., E. K. Borman, and C. R. McFarland. 1964. Typing of group A streptococci by immunofluorescence. I. Preparation and properties of type 1 fluorescein-labeled antibody. J. Bacteriol. 87:1377–1382.
2. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrate of variant strains. J. Exp. Med. 162:11–28.
3. Moody, M. D., E. C. Ellis, and E. L. Updyke. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. 75:553–560.
4. Olson, W. P. 1968. Maximal brightness and yield of fluorescent antibody. J. Bacteriol. 95:1176–1177.
5. Redys, J. J., A. B. Parzick, and E. K. Borman. 1963. Detection of group A streptococci in throat cultures by immunofluorescence. Pub. Health Rep. 78:222–226.
6. Rotta, J., M. Hejnova, R. Bocova, B. Curik, S. Mickova, and J. Salacova. 1968. Surveillance of haemolytic streptococcus group and type (*Streptococcus pyogenes*) distribution over the territory of Czechoslovakia. J. Hyg. Epidemiol. Microbiol. Immunol. 12:176–186.
7. Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing group A streptocccci by M precipitin reactions in capillary tubes. J. Exp. Med. 78:127–133.
8. Vincent, W. F., and K. J. Lisiewski. 1969. Improved growth medium for group A streptococci. Appl. Microbiol. 18:954–955.