Application of a New Method for Detecting Streptococcal Nicotinamide Adenine Dinucleotide Glycohydrolase to Various M Types of *Streptococcus pyogenes*

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The production of extracellular nicotinamide adenine dinucleotide glycohydrolase (NADase) by *Streptococcus pyogenes* can easily be demonstrated using the fluorescence of nicotinamide adenine dinucleotide in ultraviolet light occurring on addition of strong alkali. The new method described here uses microtiter plates and can be read with the naked eye. It permits the screening of large numbers of strains in a short time and with minimal amounts of reagents. The sensitivity of the new method proved to be good in comparison with the bisulfite spectrophotometric method. Culture supernatants of 177 group A streptococci were tested for NADase production by the microtiter fluorescence method. We could confirm former findings that strains within a certain M type of *S. pyogenes* are usually either producers or nonproducers of the enzyme. The usefulness of the test for screening of streptococcal NADase production is discussed.

Streptococcal nicotinamide adenine dinucleotide glycohydrolase (NADase) has been reported to be consistently present or absent within most M types of *Streptococcus pyogenes* (3, 7, 9). On this basis, Ofek et al. (9) have recommended examining group A streptococci (*S. pyogenes*) for production of NADase and serum opacity factor (8, 12) as a method of subclassifying these strains.

Unfortunately, the photometric methods used for NADase determinations (1, 4) are not very suitable for screening large numbers of strains or in the routine laboratory. We took advantage of the fluorescence of nicotinamide adenine dinucleotide (NAD), which develops on treatment with strong alkali (5), to detect production of streptococcal NADase. This fluorescence of NAD is excited by ultraviolet light of 366-nm wavelength. If the NAD has been split by NADase at its nicotinamide-ribose linkage, no fluorescence occurs after the addition of alkali (5, 13).

MATERIALS AND METHODS

Streptococcal strains. One hundred and seventy-seven group A streptococci from the collection of the University of Minnesota laboratory were used in this study. These strains included prototype strains used as reference strains for M typing and strains from cultures submitted for serotyping between the years 1960 and 1975. Additional strains were obtained through the courtesy of R. Facklam from the

Center for Disease Control, Atlanta, Ga. All strains have been kept in the frozen (-20 C) or lyophilized state until used for further tests. M typing of the streptococcal strains (and, on some isolates, also T typing) had been performed by standard procedures (11, 14).

Determinations of NADase. For determinations of NADase, a few colonies of the strains were inoculated from the growth on blood agar plates into tubes with 10 ml of Todd-Hewitt broth (Difco). After overnight incubation at 35 C, 0.1 ml of these cultures was transferred to tubes with Todd-Hewitt broth containing 1% horse serum (3). The cells of the second culture were spun down after approximately 20 h of incubation, and the supernatants were taken for the NADase test. The supernatants and the reagent solutions to be used in the test were kept cool in an ice bath.

For this new method of detecting NADase production, we use microtiter plates (Cook or Linbro; one batch of plates of the latter brand appeared to be less transparent for ultraviolet light and did not give satisfactory results) and 0.1 M potassium phosphate buffer, pH 7.35, containing 0.1% bovine serum albumin. The procedure consists of the following steps. (i) Drop 1 drop (0.025 ml) of buffer into each well of the plates. (ii) Add 1 drop (0.025 ml) of culture supernatant to be tested. (iii) Add 1 drop (0.025 ml) of beta-NAD solution (in buffer, 1.8 mg/ml; oxidized form; Sigma 7004) to each well and mix by slightly agitating the plates. (iv) Incubate the plates (covered with an empty microtiter plate) for 20 min at 35 C. (v) Add 2 drops (0.025 ml) of 5 N sodium hydroxide, seal plates with plastic tape, and keep them for 1 h at room temperature in the dark. After

that they are read in a dark room using an ultraviolet lamp of 366-nm wavelength and a microtiter reading mirror. The ultraviolet light is either directed onto the mirror or from below directly onto the plate to be read. By this means the alkaline fluorescence of NAD can easily be seen. Culture supernatant (without NAD) controls and NAD (with broth instead of culture supernatant) controls are always included; the fluorescence of the latter controls is graded as 4+. Supernatants giving no or definitely diminished fluorescence (2+ or less) are read as NADase positive.

To obtain information about the sensitivity of the new method, experiments were performed in which twofold serial dilutions of culture supernatants of three streptococcal strains were tested for NADase by both the plate method and the photometric method of Ayoub and Ferretti (1) using bisulfite, which has a sensitivity of about 50 U per ml of culture supernatant (3). For comparison, NADase from *Neurospora crassa* (Sigma N 9629) was also used in both test systems.

RESULTS

The new microtiter fluorescence method proved to be rather sensitive; the lower limit of detection was approximately 50 U (6) of streptococcal NADase per ml of culture supernatant

(47 to 50 U/ml in several experiments; highest amount not being detected, 37 U/ml). With NADase from *N. crassa* the lower limit of detection was similar (42 U/ml). The reactions in the plates were stable at room temperature in the dark for several days. Figure 1 shows serial twofold dilutions of 12 different streptococcal supernatants (diluted in the wells of a microtiter plate beginning from the bottom of the figure, columns 1-12, rows H-C) demonstrating NADase activity (columns 1-3, 5, 6, 10, 12) and lack of detectable enzyme activity (columns 4, 7-9, 11). Wells A9 through 12 (at top of the figure) contain NAD controls showing bright fluorescence; wells B1 through 12 are supernatant controls; wells A1 through 8 are empty.

For screening of the 177 streptococcal strains for NADase production, undiluted culture supernatants were examined. Some of the strains were repeatedly tested and gave the same results. In each instance the test showed clearly either NADase production (i.e., about 50 U of the enzyme per ml of culture fluid or more) or no detectable production.

The relationship of NADase production to M type of the test strains is shown in Tables 1

FIG. 1. Microtiter fluorescence test for detection of streptococcal NADase performed with twofold serial dilutions (starting from the bottom wells) of 12 different culture supernatants. Positive: columns 1, 2, 3, 5, 6, 10, 12; negative: columns 4, 7, 8, 9, 11. NAD controls: wells A9-12; supernatant controls: wells B1-12.

TABLE 1. *M* types in which all strains examined were found to be producers of streptococcal NADase

<i>M</i> type	No. of strains tested
3	6
4	5
6	11
8	3
9	3
11	4
12	6
22	4
25	4
27	3
28	3
48	1
49	6
50	1
58	3
59	3
60	3
61	3
62	2
63	1
64	1

through 3. For most *M* types at least three strains were examined for NADase production. We found *M* types 2, 13, and 31 to be the only types in which both producers and nonproducers occurred.

DISCUSSION

This new method for screening group A streptococci for NADase production presents a reliable and simple test. It permits testing of large numbers of strains in a short time with minimal amounts of reagents. The sensitivity of the new test system seems to be sufficient for screening purposes. It detects about 50 U of the enzyme per ml; however, 49 of 51 NADase-positive strains were found to yield more than 100 U/ml of culture fluid by Bernheimer et al. (3).

The usefulness of screening strains of *S. pyogenes* for NADase production was recently discussed by Ofek et al. (9). These authors were able to subdivide strains of *S. pyogenes* (group A streptococci) into four subgroups according to their NADase and serum opacity factor production. We could not confirm *M* type 60 as a nonproducer of NADase, thus possibly leaving only three of their subgroups.

On the whole our data support earlier reports (3, 7, 9), which indicated that within a certain *M* type of group A streptococcal strains are either NADase producers or nonproducers. However, there were a few *M* types in which both NADase producer and nonproducer strains were encountered, as was previously

shown by Bernheimer et al. and Lazarides and Bernheimer (3, 7).

The demonstration of NADase production by this new method may be an additional tool useful in subtyping group A streptococci for epidemiological and other purposes, especially in those situations in which one of the established serological methods (e.g., *M* typing) cannot be performed. NADase production of *S. pyogenes* seems to be a quite stable property of the strains, but no detailed studies of its genetics have been made. One report suggests that there may be two different enzymes with NADase activity associated with group A strep-

TABLE 2. *M* types in which no strains examined were found to be producers of streptococcal NADase

<i>M</i> type	No. of strains tested
1	7
5	4
14	4
15	3
17	2
18	3
19	4
23	3
24	3
26	2
29	3
30	3
32	2
33	4
34	1
36	3
37	2
38	2
39	3
40	1
41	3
42	2
43	3
46	3
47	2
51	3
52	3
53	2
54	2
55	3
56	3
57	3

TABLE 3. Streptococcal *M* types in which both producer and nonproducer strains of NADase were found

<i>M</i> type	No. of strains tested	Producer	Nonproducer
2	3	2	1
13	3	2	1
31	4	3	1

tococci (10). It has been suggested by Bernheimer et al. (3) that streptococcal NADase may be involved in the pathogenesis of acute glomerulonephritis, but this has not been established.

We have performed some preliminary experiments suggesting that the microtiter fluorescence method can also be adapted for the determination of the anti-NADase antibody titer, but the titers tend to be lower than those obtained with the standard methods (1, 2).

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