Application of Enzyme Production Properties in Subtyping of Group A Streptococci According to T Type

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The production of extracellular nicotinamide adenine dinucleotide glycohydrolase (NADG) and the cell-bound lipoproteinase (serum opacity reaction, SOR) by strains of different serological types of group A streptococci, in relation to the T typing, was studied. The production of both NADG and SOR, or only one of them, was found to be characteristic of serotypes, as determined by M and T antigen. No difference in the production of these enzymes was found in relation to M-positive and M-negative variants. Investigation into NADG and SOR production as related to the T type enabled the division of a single agglutination pattern into four main groups, each of which corresponds to one specific M type or more. Of the 370 strains belonging to 12 different T-agglutination patterns, 21% produced both enzymes and 42.5% failed to produce any of them, whereas the remaining 36.5% produced only one out of the two enzymes. Five streptococcal types which did not produce NADG and SOR also failed to synthesize streptolysin S at the early logarithmic phase of growth, indicating that streptolysin S production by young cultures may be also related to serotype. No correlation was found between the production of NADG-SOR as related to serotype and the production of streptolysin O, acid phosphatase, esterase, N-acetylglucosaminidase, hyaluronidase, streptokinase, and the cell-sensitizing factor. The practical and potential usefulness of NADG and SOR production in epidemiological studies is discussed.

Members of group A streptococci produce an extracellular enzyme, nicotinamide adenine dinucleotide glycohydrolase (NADG; reference 10), which splits the nicotinamide-ribose linkage of NAD, and a cell-bound lipoproteinase (16), which acts upon serum lipoproteinase and is responsible for the serum opacity reaction (SOR).

Extensive investigation into the relationship between the production of streptococcal lipoproteinase by strains of different serological types and production of M antigen has been made by Top et al. (16) and Widdowson et al. (17). It was found that in large numbers of streptococcal types lipoproteinase production was related to certain serotypes as determined by M antigen. It was also found that there was no variability between M-positive strains and their M-negative variants with regard to the production of this enzyme (16, 17). On the other hand, only a few M types were previously tested for NADG production (10), where it was found that enzyme production was also related to certain M types. No data were, however, given on the relationship of M-negative variants of those types and the production of NADG.

In the present investigation, a large number of group A streptococcal strains were tested for their simultaneous production of SOR and NADG. This series of strains included 35 different M types, 14 of which were not previously tested for NADG and 4 of which were not tested for the simultaneous production of both enzymes. Three hundred seventy T strains submitted for routine serotyping were likewise tested.

MATERIALS AND METHODS

Streptococcal strains. The strains used in this study were obtained either from a large number of cultures submitted for routine serotyping or from stock M vaccine strains obtained from W. R. Maxted of Cross-Infec tions, Colindale, and M. D. Moody of Communicable Disease Center, Atlanta, Ga. (CDC). In addition, M-negative (glossy) variants were selected...
from a stock of M types grown on horse serum or blood-agar plates, or both.

All strains were classified by T agglutination (6) and M precipitation in agar-gel by using absorbed M antisera prepared in our laboratory (9a).

**Determination of streptococcal products.** Streptococcal products were determined on either the supernatant fluid or on washed cells derived from 18-hr cultures grown in Todd Hewitt broth.

**Other determinations.** (i) NADase was determined by the method of Bernheimer et al. (1), by using oxidized NAD (Nutritional Biochemical Corp., Cleveland, Ohio). One per cent horse serum was incorporated into the Todd Hewitt broth. Results are expressed as units of NADG per ml of supernatant fluid. Production of NADG was considered positive when the supernatant fluid contained more than 50 units per ml of the enzyme. (ii) SOR was performed by the method of Top et al. (16). (iii) Streptolysin S (SLS) was determined by a method described previously (5). (iv) Cell-sensitizing factor (SF) was determined by passive hemagglutination (4). The cell-sensitizing antigen was obtained either from the supernatant fluid of streptococcal cultures (4) or after treatment of washed cell suspensions by phenol as described elsewhere (13). Rabbit serum to heat-killed group A streptococci was used as a source of anti-SF antibodies. (v) Determinations of phosphatase, esterase, and N-acetylglucosaminidase were performed on supernatant fluids or on washed streptococcal suspensions as described in another report (5a). (vi) Hyaluronidase and streptokinase were determined by methods described elsewhere (9).

**RESULTS**

In a preliminary examination of stock strains obtained from the CDC in Atlanta, Ga., and from the Cross-Infection Center in Colindale, London, as well as from strains received for routine typing, a distinct association between M type and NADG or SOR production was found. All of the strains of a given type (except type 49) which we examined consistently produced either NADG or SOR, or both, or consistently failed to produce either. Examination of M types 2, 3, 6, and 17 and their M-negative variants (glossy) showed no variations in their ability to produce NADG and SOR. The results concerning SOR were in agreement with those reported previously (16, 17).

With respect to the formation of NADG and SOR, it is possible to divide the M stock strains received from Colindale, CDC, and from our own stock cultures into four main groups. Table 1 shows that whereas the majority of M types tested either produced both enzymes or failed to produce either, types 3, 6, 12, and 31 produced NADG only and type 60 produced SOR only. Although the two latter groups are in minority (only 5 out of 35 M types studied in this investigation), they represent, however, the most common types found in a community.

**Table 1. Production of nicotinamide adenine dinucleotide glycohydrolase (NADG) and serum opacity reaction (SOR) by group A streptococci**

| Group | Enzyme produced | No. of types tested | M types a |
|-------|-----------------|---------------------|-----------|
|       | NADG | SOR | 20 | 1, 5, 14, 15, 17, 18, 19, 23  |
|       |      |      | 10 | 3, 6, 12, 28, 48 |
|       |      |      | 4  | 3, 6, 12, 31b |
|       |      |      | 1  | 56b |

a Seventeen M types (without footnotes) were also tested for SOR production by Top et al. (16) and for NADG production by Lazairides et al. (10) and Bernheimer et al. (1). Their results are in full agreement with those described above. At least three strains of each of the following M types: 8, 13, 15, 22, 23, 25, 26, 27, 29, 33, 36, 41, 46, 48, 52, 53, 54, 57 and at least 10 representative strains of each of the other M types were tested.

These M types were tested for NADG by our laboratory only, whereas the SOR was also tested by Top et al. (2). The latter obtained similar results to those described above.

These M types were tested for NADG and SOR production in our laboratory only.

**Screening of streptococcal strains for SOR and NADase.** Further screening of streptococcal M types belonging to the agglutination patterns 8/25/Imp. 19 and 4/28 were studied.

The T-agglutination pattern 8/25/Imp. 19 included five established M types which could be divided into three enzymatic groups: (i) nonproducers of either enzyme which included types 55 and 57; (ii) producers of both enzymes which included types 8 and 25; and (iii) producers of NADG only; these included cultures isolated in Israel which could not be typed with the available antisera present in our laboratory. A representative of this group was designated as strain number 1101. Later on, antiserum prepared against this strain reacted specifically with the homologous strains as well as with type 31 obtained from Colindale and CDC. These findings were recently confirmed by these laboratories (personal communication).

The T pattern 4/28 included eight established M types which could also be divided into three enzymatic groups: (i) nonproducers, included
the M types 24, 26, 29 and 46; (ii) producers, included the M types 4, 28, and 48; and (iii) producers of SOR only, which included strains found in the fall of 1968. These strains were probably associated with an epidemic of scarlet fever and reappeared again recently. Antiserum produced against one of these strains proved to be provisional type 60 (Bergner-Rabinowitz et al., Israel J. Med. Sci. 7: 1105). On retesting the strains isolated, this type was found to be present in Israel in the summer of 1967 in association with an outbreak of severe pharyngitis.

Table 2 shows the production of NADG and SOR by routine group A streptococcal strains as typed by the T-agglutination method. The variation in enzyme production in the various T patterns indicates that they include distinct strains, as characterized by their capacity to produce the enzymes. The results on the 370 routine strains (Table 2) show that more than one-third of the strains could be differentiated only when assays for the enzymes were made.

The possibility that the formation of several other group A streptococcal products was also associated with certain serotypes was investigated. It was found that all of the streptococcal strains listed in Table 1 consistently produced phosphatase, esterase, N-acetylglucosaminidase, streptolysin O, hyaluronidase, and streptokinase. The production of the cell-sensitizing factor was demonstrated by all except one type 19 strain (out of five).

**Possible relationship of SLS production to serotype.** It has been previously shown that

| T-agglutination pattern | No. of strain | Group 1 N− S− | Group 1 N+ S+ | Group 2 N+ S− | Group 3 N− S+ | Group 4 N+ S+ |
|------------------------|--------------|---------------|---------------|---------------|---------------|---------------|
| 1                      | 15           | 11            | 2             |               |               |               |
| 2                      | 15           | 2             | 1             | 2             |               |               |
| 3/13/B3264             | 69           | 45            | 8             | 10            | 6             |               |
| 4/28                   | 25           | 2             | 2             | 4             | 9             |               |
| 5/27/44                | 28           | 14            | 5             | 1             | 8             |               |
| 6                      | 11           | 2             | 2             | 7             | 2             |               |
| 11                     | 9            | 4             | 5             |               |               |               |
| 12                     | 45           | 3             | 14            | 25            |               |               |
| 14/49                  | 27           | 11            | 7             | 3             | 5             |               |
| 15/17/19/23            | 15           | 6             | 5             |               |               |               |
| 28/56                  | 16           | 15            |               |               |               |               |
| Total                  | 370          | 157           | 78            | 67            | 68            |

* N− = no production of nicotinamide adenine dinucleotide glycohydrolase (NADG), N+ = production of NADG, S− = no production of serum opacity reaction (SOR), S+ = production of SOR.

strains of certain serotypes (types 1, 5, and 19; references 14, 18) were nonleukotoxic when harvested at the early logarithmic phase of growth. It was later shown that the leukotoxic factor is probably identical with SLS and that the nonleukotoxicity of these serotypes was due to the nonproduction of this hemolysis at this early stage of growth (14). Table 1 indicates that these serotypes also failed to produce SOR and NADG. Further studies showed that type 55, which also failed to synthesize SLS by young cultures, was a nonproducer of NADG and SOR. Thus, it seems that the production of SLS at the early logarithmic phase of growth is also associated with serotypes. It is also of interest that types 14 and 36, which were found to be nonleukotoxic (1, 18), also belong to the nonproducers of either NADG or SOR (see Table 1).

**DISCUSSION**

The present data were obtained from a large number of stock strains received from Colindale and CDC or isolated in our laboratory from clinical material submitted for serotyping. These data confirmed and extended for additional M types the findings of Lazarides and Bernheimer (10) on the association of NADG with serotype as determined by M antigen. Subsequent studies of M-positive and M-negative variants of stock strains types 2, 3, 6, and 17 revealed a consistent association between the ability to produce NADG and the serotypes as determined by M or T antigens, or both. When strain classification could be accomplished by the T-agglutination method only, particularly in the more complex T patterns such as 4/28, 8/25/Imp. 19, 3/13/B3264, etc., variations in SOR and NADG production were seen in members of these patterns. Some of these variations were due to the inclusion within these serotypes of distinct, currently unclassifiable M types (such as provisional M60 of the T pattern 4/28) or classifiable but unrelated M types (such as M type 22 of the T pattern 12, 12). The only exception found was among strains of type 49 which produced these enzymes irregularly. No explanation for this phenomenon can be given at present.

The close association between serotype as tested by the T-agglutination method and the ability to produce NADG and SOR provides a simple means of dividing a given T pattern into four enzymatic combinations. Each combination can thus be differentiated by one or more different M types as classified by the precipitin method. Since the majority of streptococcal strains isolated from clinical material are M-non-typable (15), this enzymatic subclassification of
these T strains could be of special importance in epidemiological studies of streptococcal disease. This is well documented in the study of our T-strain series. For example, the majority of the strains of T pattern 3/13/B3264 (Table 2) are not M-negative variants of the M type 3 or 13, but rather belong to M-negative variant of M types 33, 41, 52, and 53 or possibly to a new M type.

Further investigation into the search for other streptococcal products as related to serotype was done. Thus far, esterase, acid phosphatase, hyaluronidase, streptokinase, cell-sensitizing factor, and streptolysin O were found to be regularly produced by all members of group A streptococci tested.

On the other hand, at least four serotypes, 1, 5, 19, and 55, which are nonproducers of either SOR and NADG, also failed to synthesize SLS and to kill leukocytes (leukotoxicity) when harvested from the early logarithmic phase of growth. It is also of interest that two additional serotypes, 14 and 36, which are both nonproducers of SOR or NADG were also found to be nonleukotoxic (1, 18). It is thus probable that assays for SLS production by young cultures may also be useful in the classification of streptococci according to serotype.

Since the biological role of NADG and SOR in the economy of the streptococcus is not fully understood, it would be worthwhile to investigate the association between the nonproduction of NADG and SOR and some biological properties of these types. In this respect, it is interesting that only strains of types 5 and 19 (which are nonproducers of NADG and SOR) were found to share common antigen with mammalian heart tissue (8). Lyampert et al. (11) and Danilova (3) showed that antisera to streptococci of types 5 and 29 (both nonproducers of NADG and SOR), cross-reacted with cardiac myofibrils of mammalian heart tissue. Furthermore, type 1 (a nonproducer) was found to be the only streptococcal type to possess histocompatibility antigens (7). On the other hand, all of the group A strains tested which belong to the producer group (types 4, 6, 11, and 49) and to the nonproducer group (types 5 and 14) were found to possess the property of induction of homograft sensitivity (2). Further studies along these lines would shed more light on the possible relationship of serotype and the production of NADG, SOR, and SLS to certain biological properties of the streptococcus in relation to poststreptococcal diseases.

LITERATURE CITED

1. Bernheimer, A. W., P. D. Lazarides, and A. T. Wilson. 1957. Diphasophorpyridine nucleotidase as an extracellular product of streptococcal growth and its possible relationship to leukotoxicity. J. Exp. Med. 106:27–37.
2. Chase, R. M., and F. T. Rappaport. 1965. The bacterial induction of homograft sensitivity. I. Effect of sensitization with group A streptococci. J. Exp. Med. 122:721–732.
3. Danilova, T. A. 1966. An immunofluorescent study of the antigens of group A streptococcus and heart tissue from human and experimental animals. Fed. Proc. 25:1099–1102.
4. Dahan, T., R. Finkel, Z. Marcus, and I. Ginsburg. 1967. Cell-sensitizing products of streptococci. Immunology 13:555–564.
5. Ginsburg, I., and T. N. Harris. 1965. Oxygen-stabile hemolysins of group A streptococci. IV. Studies on the mechanism of lysis by cell-bound hemolysin of red blood cells and Ehrlich ascites tumor cells. J. Exp. Med. 121:647–656.
6. Griffith, F. 1934. The serological classification of Streptococcus pyogenes. J. Hyg. 34:542–584.
7. Hirata, A. A., and P. I. Terasaki. 1970. Cross-reaction between streptococcal M-proteins and human transplantation antigens. Science 168:1093–1096.
8. Kaplan, N. H., and J. O. Frengley. 1969. Autoimmunity to the heart in cardiac disease. Current concept of the relation of autoimmunity to rheumatic fever postcardiomyopathy and postinfarction syndromes and cardiomyopathies. Amer. J. Cardiol. 24:459–473.
9. Kwapiński, J. B., and M. Snyder. 1962. The immunology of rheumatism. Appleton-Century-Crofts, New York.
10.LASch., E., V. FrankeI, P. Vardy, S. Bergner-Rabinowitz, I. Ofek, and K. Rabinowitz. 1971. Epidemic glomerulonephritis in Israel. J. Infec. Dis. 124:141–147.
11. Lazarides, P. D., and A. W. Bernheimer. 1957. Association of production of diphosphopyridine nucleotidase with serological type of group A streptococcus. J. Bacteriol. 74:412–413.
12. Lyampert, I. M., O. I. Vvedenskaya, and T. A. Danilova. 1966. Study on streptococcus group A antigens common with heart tissue elements. Immunology 11:313–320.
13. Maxted, W. R., and H. A. Valkenburg. 1969. Variation in the M-antigen of group A streptococci. Med. Microbiol. 2:199–210.
14. Moskowitz, M. 1966. Separation and properties of red cell sensitizing substance from streptococcus. J. Bacteriol. 91:2200–2204.
15. Ofek, I., S. Bergner-Rabinowitz, and I. Ginsburg. 1970. Oxygen-stabile hemolysins of group A streptococcus. VII. The relation of the leukotoxic factor to streptolysin S. J. Infec. Dis. 122:517–522.
16. Parker, M. T. 1967. International survey of the distribution of serotypes of Streptococcus pyogenes (group A streptococci). Bull. W. H. O. 37:513–527.
17. Top, F. H., Jr., and L. W. Wannamaker. 1968. The serum opacity reaction of Streptococcus pyogenes: frequency of production of streptococcal lipoproteins by strains of different serological types and the relationship to M-protein production. J. Hyg. 66:49–58.
18. Widdowson, J. P., W. R. Maxted, and D. L. Grant. 1970. The production of opacity in serum by group A streptococci and its relationship with the presence of M antigen. J. Gen. Microbiol. 61:343–353.
19. Wilson, A. T. 1957. The leukotoxic action of streptococcal. J. Exp. Med. 105:463–484.