A simple biological method for detecting streptococcal nicotinamide adenine dinucleotide glycohydrolase

N. E. GREEN

From the Department of Microbiology, Worthing Hospital, Worthing, Sussex, UK

SUMMARY A biological method for detecting streptococcal nicotinamide adenine dinucleotide glycohydrolase (NADG) is presented, based on its ability to inhibit the growth of *Haemophilus parainfluenzae*. Three hundred clinical isolates of β -haemolytic streptococci were tested. All isolates producing NADG belonged to Lancefield's groups A, C, or G.

Streptococcal NADG (NAD'ase, DPN'ase) has been extensively characterised (Carlson *et al.*, 1957) and shown to be an extracellular product of growth with classical enzymic properties. It has been purified and shown to catalyse specifically the cleavage of nicotinamide adenine dinucleotide at the nicotinamide-ribose linkage. NADG does not appear to be inducible since it is formed in a defined minimal medium free of substrate and the addition of substrate does not influence its production (Bernheimer *et al.*, 1957).

Streptococcal NADG has been described as cardiotoxic (Van Heyningen, 1962) and postulated as a leucotoxin distinct from the streptolysins (Bernheimer *et al.*, 1957; Wilson, 1957). Although there is a high correlation between NADG activity and leucotoxicity, subsequent evidence points towards streptolysin S as the substance responsible for leucocyte destruction (Ofek *et al.*, 1970). It is nevertheless possible that more than one streptococcal product is involved. NADG inhibits the oxidation of citrate, fumarate, and α -keto-glutarate by mitochondria (Carlson *et al.*, 1956).

To date, the only microbes found to produce specific NADG are *Neurospora crassa*, certain strains of streptococci of Lancefield's groups A, C, and G (Bernheimer *et al.*, 1957), and *Yersinia pestis* (Ajl *et al.*, 1956). A very similar but less specific enzyme, NAD pyrophosphatase, is found in *Mycobacterium butyricum*, *Bacillus subtilis*, *Proteus morgani*, *Proteus vulgaris*, and *Proteus rettgeri* but is very unusual in that it requires 'heat activation' (Swartz *et al.*, 1956).

Correlation between production or lack of produc-

tion of NADG by group A streptococci and their M type has been reported (Lazarides and Bernheimer, 1957). M-types 3, 4, 6, and 12 were found to produce NADG with striking regularity, whereas M-types 1, 5, 14, 19, and 24 tended not to produce NADG. Association between NADG production and serum opacity factor (SOF) has also been studied in an attempt to relate them to the organisms M- and T-type (Ofek *et al.*, 1971). This provided a close association between serotypes, as tested by T-agglutination, and the ability to produce NADG and/or SOF, which provided a simple means of dividing given T-patterns into four enzymic combinations.

Originally, NADG was detected by a spectrophotometric method (Bernheimer *et al.*, 1957), which measured residual NAD after incubation of a known amount of substrate with dialysed culture supernatant. These methods required large quantities of culture supernatant (in one method 15 litres), dilutions in buffer, and the use of a water bath, ice bath, and spectrophotometer. Recently, a slightly simpler method has been described (Lütticken *et al.*, 1976) which utilises the fluorescence of NAD under ultraviolet light that develops on the addition of strong alkali. However, this method still requires buffer and culture supernatants in addition to the use of an ice bath, strong alkali, and an ultraviolet light source.

Principle

The method presented here is much simpler than those previously described. The basis of the test is the fact that the substrate for NADG is also the specific growth-requiring factor for *Haemophilus parainfluenzae*. Growth of *H. parainfluenzae* on a

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minimal medium with the addition of NAD (V factor) was found to be inhibited by NADG-producing streptococci. Non-producers of NADG had no effect on the growth of *H. parainfluenzae* under the same conditions.

That this inhibition is not due to some non-specific substance produced by certain streptococci which kills *H. parainfluenzae* was indicated by the fact that the zone size was inversely proportional to the substrate concentration.

Three hundred clinical isolates of β -haemolytic streptococci were examined for NADG production using this biological method.

Material and method

IDENTIFICATION OF ISOLATES TESTED

All of the isolates included in this study conformed with the definition of β -haemolytic streptococci (Cowan and Steel, 1974) in that they were Gram-positive, catalase-negative cocci possessing a tendency to form chains and producing zones of β -haemolysis on Columbia blood agar.¹ An attempt was made to identify the Lancefield group of each isolate by the Rantz and Randall method (Rantz and Randall, 1955) using anti-sera A-G.²

Indicator strain of *H. parainfluenzae*

Although clinical isolates of *H. parainfluenzae* were used, two reference strains (NCTC 7857 and NCTC 10665) gave equally satisfactory results. There was no discernible difference between the performance of the capsulated reference strain (NCTC 10665) and the non-capsulated reference strain (NCTC 7857). The indicator strain was maintained on chocolate Columbia blood agar at 37°C in an atmosphere containing 5-7% CO₂³ and was subcultured twice weekly.

Performance of the test

A tryptone soya agar (TSA) plate¹ was seeded with a suspension of the indicator organism using a sterile cottonwool swab to cover the entire plate. The inoculum size was chosen by trial and error, and optimum results were obtained by emulsifying five to six well isolated colonies of *H. parainfluenzae* in 1-2 ml peptone water. Too light an inoculum caused difficulty in reading, whereas an extremely heavy inoculum still gave readable results, although the zone sizes were usually much reduced. A stab inoculum of a single colony (from an overnight blood

agar purity plate incubated at 37°C in a Gas Pak⁴ anaerobic jar) of each test strain was then made into the seeded plate. V factor discs⁵ were placed aseptically on the plate, so arranged that each test inoculum was approximately 8-12 mm from a disc. Plates were incubated at 37°C in an atmosphere containing 5-7% CO₂,³ and examined the next day for the presence of zones of inhibition of the indicator organism produced by the test strains. Part of a typical test plate is illustrated in the Figure. Provided that the test organism produced a zone of inhibition, it was classified as an NADG producer (zone sizes, under identical conditions, varied from 1 to 15 mm, most being about 5 mm diameter). If the test organism had no effect on the indicator organism it was classified as a non-producer of NADG.

Figure Part of a typical test plate (see text for details) seeded with *H. parainfluenzae* and the following streptococcal isolates, stab inoculated: (top left) group A (+ve), (bottom left) group G (+ve), (right) group D (-ve).

Since it is known that streptococci produce NAD, the possibility of omitting the substrate source was investigated. Although a few test organisms produced a zone in this way, the results were variable, some test organisms failing to produce any growth of the indicator organism. Even when a zone was evident, it was only so after prolonged incubation (two or

¹Oxoid Limited, Wade Road, Basingstoke, Hants.

²Wellcome Reagents Limited, Beckenham, Kent.

³Hotpack CO₂ incubator, Hotpack, Philadelphia, USA.

⁴Gas Pak BBL, B-D Cockeysville, Md 21030, USA.

⁵Mast Laboratories, Liverpool.

three days). Consequently, V factor discs were used throughout this study.

Once established, a positive and negative control were included on each test plate.

Results

The results for the production of NADG by the 300 isolates of β -haemolytic streptococci are presented in the Table; 84% of the group A isolates, 58% of the group C isolates, and 67% of the group G isolates tested produced NADG. There was no NADG producer which did not belong to groups A, C, or G.

Table Results of NADG production by 300 clinical isolates of β -haemolytic streptococci

Lancefield group	No. strains tested (% of total)	No. producing NADG (%)
A	93 (31)	78 (84)
B	24 (8)	0 (0)
C	38 (13)	22 (58)
D	9 (3)	0 (0)
E	—	—
F	8 (3)	0 (0)
G	69 (23)	46 (67)
No precipitation with antisera A–G	59 (20)	0 (0)
Totals	300 (100)	146 (47)

It should be noted that only one method of Lancefield grouping was used, and variation of success rates between various methods has been demonstrated (Broomfield, 1978). By using other methods of Lancefield grouping, the relatively high number of isolates (20%) that were not identified as belonging to groups A–G may have been reduced.

Using the described technique, approximately 8–12 tests can be incorporated on one TSA plate. However, if the substrate was included in the medium, up to 20 tests could be accommodated on a single plate. This compares very favourably with the economics of the chemical methods.

The enhancing effect of 1% horse serum on streptococcal NADG production (Bernheimer *et al.*, 1957) was investigated using 10 positive and 10 negative isolates. A demonstrable effect was not found.

Conclusion and discussion

When compared with previous results (Bernheimer *et al.*, 1957), an unexpectedly high percentage of the group A isolates tested produced NADG (84% in this study, 55% previously). However, this could be explained by a predominance of particular M-types among the group A streptococci tested.

Compared with the chemical methods for detecting NADG, this biological method is simpler, faster, less laborious, and far less expensive. No dangerous chemicals are involved in this method, but they are in one of the spectrophotometric methods involving cyanide and in the ultraviolet method which utilises strong alkali.

This is believed to be the first time that a biological method for screening streptococcal NADG has been reported. Although this method has not yet been compared with the previously used chemical methods (for example, for sensitivity), it was a success in that the results agreed in essence with previous findings. All the NADG producers belonged to Lancefield groups A, C, or G, and all the non-NADG producers did not. This biological method may prove useful as a supplement to the M- and T-typing of group A streptococci.

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- Requests for reprints to: N. E. Green, Microbiology Department, Worthing Hospital, Lyndhurst Road, Worthing, Sussex, UK.