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Neuraminidase Activity in *Diplococcus pneumoniae*

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ABSTRACT

KELLY, R. T. (Marquette University School of Medicine, Milwaukee, Wis.), D. GREIFF, AND S. FARMER. Neuraminidase activity in *Diplococcus pneumoniae*. J. Bacteriol. **91**:601–603. 1966.—A method for the quantitation of neuraminidase in the presence of *N*-acetylneuraminic acid aldolase is described. The neuraminidase content of *Diplococcus pneumoniae* was found to be dependent on the media employed for growth; the highest enzyme activity per milligram of bacterial protein was obtained with Todd-Hewitt broth. Neuraminidase production was stimulated in *D. pneumoniae* by the addition of *N*-acetylneuraminlactose, *N*-acetylneuraminic acid, or *N*-acetylmannosamine to the growth medium. Three rough strains of *D. pneumoniae*, which were nonpathogenic for mice, lacked neuraminidase activity. Seven of 12 smooth strains contained neuraminidase; enzyme activity was not detected in the remaining 5 smooth strains. There was no correlation between the presence of neuraminidase activity and the capsular type or between neuraminidase production and animal virulence.

Neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, E.C.3.2.1.18) has been reported in a variety of bacteria and in the myxoviruses (6). The partial purification of this enzyme, with *Diplococcus pneumoniae* (type I) as source material, has been described (3). Because of our finding that *N*-acetylneuraminic acid aldolase (*N*-acetylneuraminate pyruvate-lyase, E.C. 4.1.3.3) is present in pneumococci, it was necessary to develop a method for determining the level of neuraminidase activity in the presence of this enzyme. This assay eliminated the erroneously low values for neuraminidase activity that otherwise would result if the split product was measured directly. Using this procedure, we have investigated the effects of growth media and enzyme inducers on the production of neuraminidase by *D. pneumoniae*. We have studied also the neuraminidase activities of various types and strains of pneumococci and the relationship of neuraminidase to animal pathogenicity.

MATERIALS AND METHODS

Pneumococci, grown in Todd-Hewitt, tryptose phosphate, or Brain Heart Infusion broths at 37 C, were harvested by centrifugation in the log phase of growth, washed in saline, and resuspended in distilled water. Samples (2 ml) were disrupted by sonic treatment at 0 C in the small-volume attachment of a Branson Sonifier. After 3 min of sonic treatment, preparations were centrifuged at 27,000 × *g* for 15 min. Protein concentrations of the supernatant fluids were determined by the method of Lowry et al. (5). Crystalline bovine albumin was used as the standard.

Neuraminidase activities of the supernatant fluids were determined by depletion of substrate, a modification of the method used for determining the inhibition of influenza virus neuraminidase by *N*-acetylneuraminic acid (8). Sodium borohydride was added after incubation of the enzyme with substrate to destroy the liberated *N*-acetylneuraminic acid; the uncleaved *N*-acetylneuraminic acid was determined with the Ehrlich reagent (10).

The reacting mixture (0.75 ml) contained: suitably diluted pneumococcal supernatant fluid, tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (*p*H 6.5) at a final concentration of 0.1 M, and neuraminlactose at a final concentration of 1.0 μM. After incubation for 15 min at 37 C, the reaction was stopped by the addition of 0.1 ml of 0.1 N NaOH containing 8 mg of $NaBH_4$. After 30 min, the excess borohydride was decomposed by the addition of 0.1 ml of 1 N HCl. The remaining *N*-acetylneuraminic acid was determined (10) and subtracted from that amount present originally, the difference being the quantity split by the action of the enzyme. A unit of neuraminidase activity was defined as that amount of enzyme which split 1 μmole of *N*-acetylneuraminic acid from an excess of neuraminlactose in 1 min at 37 C (6).

Neuraminlactose was either purchased (Sigma Chemical Co., St. Louis, Mo.) or prepared from bovine colostrum (7). *N*-acetylneuraminic acid and *N*-acetylmannosamine were obtained from Sigma Chemical Co. *D. pneumoniae* typing sera were obtained from the Communicable Disease Center, Atlanta, Ga.

RESULTS

The effects of different growth media on the levels of neuraminidase of pneumococci (type II, ATCC 6302) were investigated. Although variations in levels of neuraminidase were observed for different lots of a given medium, the levels of activities in organisms grown in Todd-Hewitt medium were consistently higher than those of organisms grown in other media, averaging 0.389 units per mg of bacterial protein. The levels of activities in organisms grown in tryptose phosphate broth or in Brain Heart Infusion broth were 0.319 and 0.075 units per mg of bacterial protein, respectively.

The effect of reduced oxygen tension on enzyme production by pneumococci (type II) was investigated by adding sodium thioglycolate (0.05%, w/v) to Todd-Hewitt medium. A 10% increase in neuraminidase activity per milligram of bacterial protein was found in organisms grown in broth containing thioglycolate.

The association between pathogenicity and neuraminidase activity of pneumococci was investigated by quantitative enzyme analyses of virulent and avirulent organisms grown in Todd-Hewitt medium of the same lot number (Table 1). The three rough strains uniformly lacked neuraminidase activity. The highest neuraminidase activity was found in type XIV, a strain avirulent for mice. The type I strain obtained from the American Type Culture Collection (6301) had a high level of enzyme activity, whereas neuraminidase was not detected either in the SV-1 strain or the transformed R36-NC-T-1 strain. To rule out the possibility that the latter strains had changed to an avirulent form by passage in artificial media, mice were inoculated intraperitoneally with the SV-1 and R36-NC-T-1 strains. The animals died within 24 hr, and type I pneumococci were recovered from heart blood and were grown in Todd-Hewitt medium. Neuraminidase was not detected in either strain.

TABLE 1. *Neuraminidase activities of various types and strains of Diplococcus pneumoniae grown in Todd-Hewitt medium*

Type	Strain	Neuraminidase (units/mg of bacterial protein)
I	ATCC 6301*	0.4133
I	SV-1†	Not detected
I	SV-1 (mouse passaged)	Not detected
I	R36-NC-T-1 (transformed)†	Not detected
I	R36-NC-T-1 (transformed—mouse passaged)	Not detected
II	ATCC 6302*	0.4390
III	ATCC 6303*	0.1294
IV	Parke, Davis‡	Not detected
V	Parke, Davis‡	Not detected
VIa	Parke, Davis‡	0.0973
VII	Parke, Davis‡	Not detected
VIII	Parke, Davis‡	0.0659
IX	Parke, Davis‡	0.4037
XIV	ATCC 6314*	0.5102
Rough	Derived from type I, ATCC 12213*	Not detected
Rough	Derived from type II ATCC 11733*	Not detected
Rough	Derived from type II R36-NC†	Not detected

* Obtained from American Type Culture Collection (ATCC).

† Obtained from R. Austrian, University of Pennsylvania School of Medicine.

‡ Obtained from M. Fisher, Parke, Davis & Co., Detroit, Mich.

TABLE 2. *Effect of additives on the production of neuraminidase by Diplococcus pneumoniae, type II, ATCC 6302, grown in Todd-Hewitt broth*

Additive	Concn (μmoles/ml of medium)	Activity (units/mg of bacterial protein)	Increase
			%
Control	—	0.3681	—
N-acetylneuraminlactose	0.1	0.4152	12.8
N-acetylneuraminic acid	1.0	1.0457	184.1
N-acetylmannosamine	1.0	0.5429	47.5

Increased neuraminidase synthesis has been reported for cultures of *Vibrio cholerae* grown in the presence of *N*-acetylneuraminlactose, *N*-acetylneuraminic acid, or *N*-acetylmannosamine (1, 2). These compounds stimulated also neuraminidase synthesis in pneumococci (type II) when added to Todd-Hewitt medium (Table 2). Because of a limited supply of neuraminlactose, this compound was used at one-tenth the concentration of *N*-acetylneuraminic acid or *N*-acetylmannosamine.

DISCUSSION

Neuraminidases are capable of cleaving terminal sialic acids from a wide variety of glycolipids, glycoproteins, and oligo-saccharides (6). Many of these substrates are essential components of tissues and body fluids, and could be degraded by neuraminidases from an infecting organism with injury to the host. Experimental evidence of the above is quite sparse, and the role of bacterial or viral neuraminidases in infectious disease has not been clearly defined. The dis-

appearance of sialic acid containing mucoproteins from the chorioallantoic membranes, and an elevation in the levels of free sialic acid in the chorioallantoic fluids of embryonate eggs infected with influenza virus has been reported (4). These alterations, corresponding to the time at which maximal amounts of virus were present in the chorioallantoic fluids, suggested that the viral enzyme functioned in the release of newly synthesized virus. The data of a recent study indicate that viral neuraminidase may be responsible for the convulsions and death which occur in mice inoculated intracerebrally with influenza virus (Kelly and Greiff, Biochim. Biophys. Acta, *in press*).

Toxigenic *Corynebacterium diphtheriae* strains produce neuraminidase; nontoxigenic forms may or may not contain the enzyme (9). In our study of *D. pneumoniae*, all three rough strains, which were nonpathogenic for mice, uniformly lacked neuraminidase activity. Seven of 12 smooth strains were found to contain neuraminidase; in the remaining 5 smooth strains, neuraminidase activity was not detectable. The presence or absence of neuraminidase was not type-specific; the enzyme was present in one strain of type I (6301), but absent in others, SV-1 and R36-NC-T-1. These strains were equally virulent for mice when injected by the intraperitoneal route. The highest level of neuraminidase activity was found in pneumococcus type XIV, a type nonpathogenic for mice. These findings indicate that the presence or absence of neuraminidase in pneumococci is not related to mouse pathogenicity via the intraperitoneal route.

Neuraminidase synthesis was stimulated in type II pneumococci by the addition of substrate (neuraminlactose) to the culture medium. A product of the enzymatic reaction, *N*-acetylneuraminic acid, also stimulated enzyme production. We observed that pneumococcus, type II, contains *N*-acetylneuraminic acid aldolase, which further splits *N*-acetylneuraminic acid to *N*-acetylmannosamine and pyruvic acid. The former, *N*-acetylmannosamine, was found to be an enzyme inducer. Pneumococcal neuraminidase should be added, therefore, to the list of inducible enzymes of bacterial origin.

The increased level of neuraminidase in bacteria grown in culture medium containing thioglycolate may have been related to the final *p*H of the medium. At the end of the growth period, the *p*H of medium containing thioglycolate was 6.48; that of medium without thioglycolate, 6.38. The autolysis of pneumococci is known to be related inversely to the hydrogen ion concentration, i.e., the lower the *p*H, the greater the autolysis. The increased leakage of enzyme from organisms grown in medium without thioglycolate would result, therefore, in lower levels of enzyme.

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