

Neuraminidase Activity in *Streptococcus sanguis* and in the Viridans Group, and Occurrence of Acylneuraminase Lyase in Viridans Organisms Isolated from Patients with Septicemia

H. E. MÜLLER

Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, D 53 Bonn-Venusberg, West Germany

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The enzyme neuraminidase (EC 3.2.1.18) was found to be strongly active in different types of *Streptococcus sanguis* and *S. viridans*, and, in addition, the occurrence of the enzyme acylneuraminase pyruvate lyase (EC 4.1.3.3) was described in *S. viridans*. The enzyme-active bacteria strains were isolated from blood cultures of patients with septicemia. Whereas *S. sanguis* lost its strong neuraminidase activity after some weeks, *S. viridans* retained its enzyme activity for a long time in culture. Immunoelectrophoretic studies of the blood cultures of patients with streptococcal infections showed the loss of neuraminic acid in most glycoproteins of the serum, proving the in vivo action of neuraminidase. The pathogenic role of neuraminidase is discussed in streptococcal septicemia from the viewpoint of present knowledge.

Some years ago, Hayano and co-workers (3-6) and also Pinter and co-workers (20, 21) described the occurrence of neuraminidase in many streptococci species such as streptococci groups A, B, C, D, E, F, G, H, K, L, M, N, and O, and also *Streptococcus salivarius*, *S. sanguis*, *S. thermophilus*, and *S. viridans*. They used mucine from cattle and bovine *N*-acetylneuraminylactose as substrates, which were split by the enzymes of these bacteria. Neuraminidase was found in small amounts in these strains, which were obtained from culture collections.

Recent studies from our laboratory described the occurrence of strongly active neuraminidase and acylneuraminase pyruvate lyase in an *S. sanguis* strain isolated from the blood culture of a patient with sepsis (17). This study also revealed the presence of both enzymes in large amounts of series of blood cultures of patients with septicemia and endocarditis caused by *S. sanguis* and *S. viridans*. These findings support the possible pathogenic role of neuraminidase in such infections by streptococci.

MATERIALS AND METHODS

Blood cultures. A 25-ml amount of brain heart infusion broth (Difco) was inoculated with 5 ml of patients' blood and was incubated at 37 C. The time needed for detection of growth in the broth ranged from 24 to 96 h at which time the broth was subcultured to sheep blood agar and the serum samples were investigated.

Serum samples. The positive blood cultures were centrifuged and concentrated by vacuum evaporation in finger-shaped collodium membranes at room temperature (Sartorius-Werke, Göttingen, W. Germany).

Immunoelectrophoresis. The immunoelectrophoresis of the concentrated serum samples, using normal human serum as controls, were performed in the micromodification of Scheidegger (22). The agar was prepared by dissolving 2 g of pure agar (Behringwerke, Marburg, W. Germany) in 100 ml of diethylbarbiturate acetate buffer, pH 8.2 ($\mu = 0.05$). Quantities (3 ml each) of the hot agar solution were pipetted onto precleaned glass slides (76 by 26 mm). The agar wells were cut by means of a template. The buffer used was a Michaelis diethylbarbiturate acetate buffer, pH 8.2 ($\mu = 0.1$). The electrophoretic separation of the serum was achieved within 180 min. The voltage was 1 V/cm. The specific rabbit antisera directed against the different human glycoproteins which were used in this study were obtained from Behringwerke, Marburg, W. Germany, and are listed in Table 2. After the application of antiserum, precipitin lines were formed within 24 h (Fig. 1-8) (13).

Streptococci. The streptococci specimens isolated from blood cultures used in this study and their biotypes (I, II, and IV) are summarized in Table 1. The serological and biochemical typing of the streptococcus strains was done by W. Heeschen, Streptokokzentrale, Kiel, W. Germany, according to the cited procedures (1, 2, 23). All strains were propagated on sheep blood agar, stored at room temperature, and subcultured weekly.

To prove the neuraminidase activity of the subcultured *S. viridans* strains against human plasma proteins, the bacteria were inoculated heavily into plates of tryptose agar medium (Difco) containing

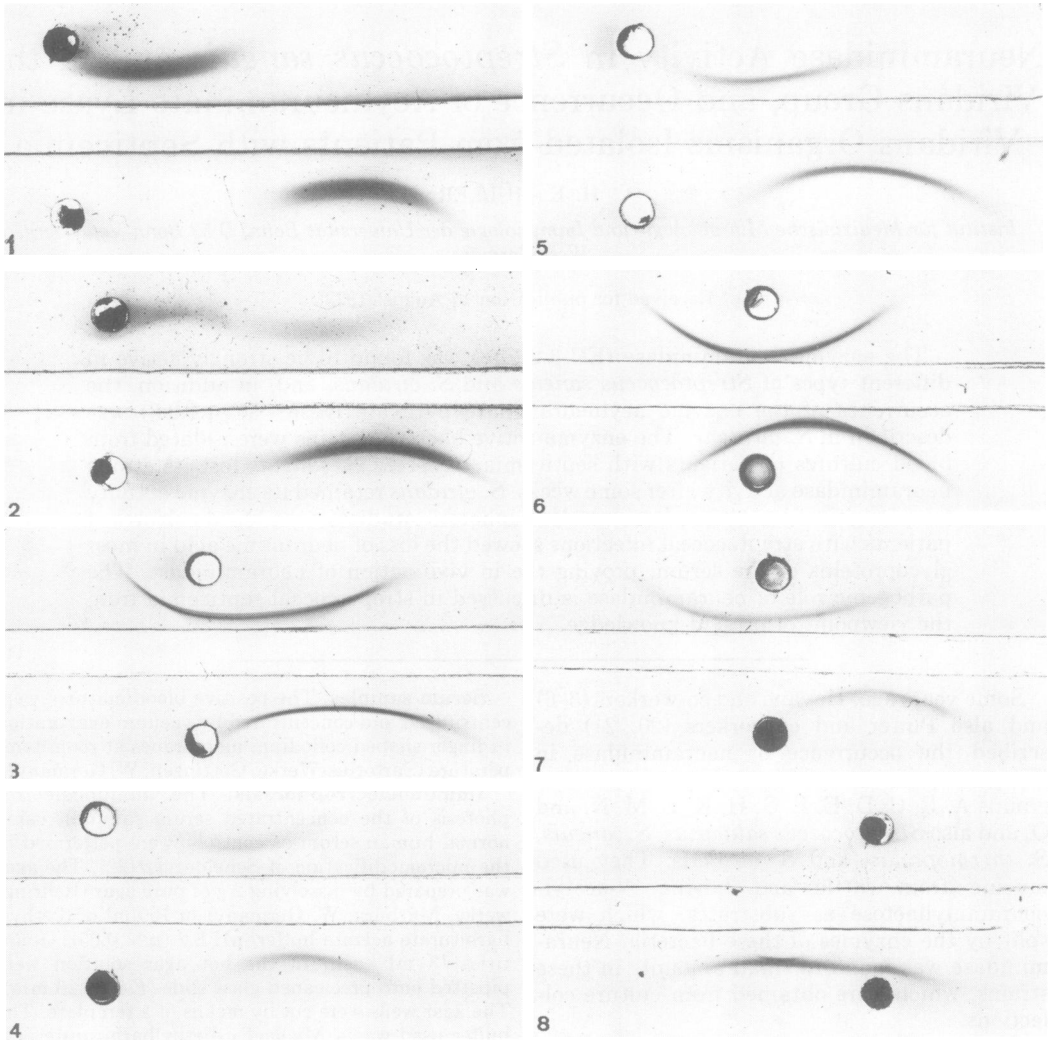


FIG. 1-8. Agar microimmunoelectrophoresis of human proteins from serum samples. The upper bands in each of the figures represent the samples with split-off *N*-acetylneuraminic acid; the lower bands represent normal controls. The anode is towards the right of the figures. The used antisera are monospecifically directed against the investigated glycoproteins degraded by *S. viridans* *in vitro* and *in vivo*. Fig. 1, acid α_1 -glycoprotein; Fig. 2, α_1 -antitrypsin; Fig. 3, haptoglobin; Fig. 4, ceruloplasmin; Fig. 5, α_2 HS-glycoprotein; Fig. 6, transferrin; Fig. 7, hemopexin; Fig. 8, immunoglobulin A.

50% human plasma (blood group AB). After 48 h of incubation at 37 C, small blocks of plasma agar immediately beneath the colonies were removed and implanted on agar gel for the performance of the microimmunoelectrophoresis. Uninoculated human plasma agar incubated under identical conditions as the test material was used as the control. The procedure has been described in detail elsewhere (13, 14).

Paper chromatography. *S. viridans* colonies, grown on sheep blood agar, were collected with a loop and suspended in a solution 1:1 (vol/vol) of 2% *N*-acetylneuraminylactose and 2% *N*-acetylneurami-

nate in an acetate buffer (0.15 M, pH 5.5). The suspensions were incubated for 3 h in a 37 C water bath. Thereafter the mixture was applied to the paper. The ascending chromatography was performed with an ethanol-water-ammonia solution (79.5:19.5:1) as described previously (15, 16).

RESULTS

Alpha-hemolytic streptococci not belonging to the Lancefield groups are found frequently in blood samples of patients with sepsis or endocarditis, or both. Table 1 summarizes the

findings of the bacteriological investigations regarding this problem.

There is some evidence that *S. sanguis* and *S. viridans* develop two different pathogenic mechanisms having an effect on human plasma proteins at least in vitro but probably also in vivo. Not only the plasma proteins but also endothelia and other cell structures are affected in a similar manner. The immunoelectrophoretic studies of the blood samples containing

streptococci show, after incubation, either neuraminidase action or a proteolytic action on the proteins of the human blood.

This study reflects only upon the neuraminidase production of streptococci. Indeed, proteases of streptococci have long been known, but their character and mode of action are not yet known. It was not possible, in our cases, to reproduce the proteolytic action observed in the original blood samples by immunoelectrophoresis (Table 1) in the first subcultures of the streptococci to plasma agar plates as described in the case of neuraminidase-producing strains.

The neuraminidase action results in typical cathodic shifts of neuraminic-containing glycoproteins as listed in Table 2. The characteristic patterns of the neuraminidase-altered glycoproteins, demonstrated by the immunoelectrophoresis, are due to the loss of negative charges. The same neuraminidase effect on human glycoproteins by streptococci is seen in plasma agar plates inoculated with streptococci from the blood culture (Fig. 1-8).

Whereas *S. sanguis* (types I and II) lose their neuraminidase activity in the course of a few weeks so that no or very weak activity is demonstrable by immunoelectrophoresis, as was described previously (17), *S. viridans* (types II and IV were investigated) retain their enzyme activity for some months. For more than 1 year, *S. viridans* strain 014984/71 showed approximately the same enzyme activity (Table 2).

TABLE 1. Enzyme actions of streptococci in blood cultures of patients

Expt no.	Blood culture no.	Species	Bio-type	Enzyme
1 ^a	11035/71	<i>Streptococcus sanguis</i>	II	N ^b
2	014984/71	<i>S. viridans</i>	II	N
3 ^a		<i>S. viridans</i>	IV	N
4	0291/72	<i>S. sanguis</i>	I	N
5	0876/72	<i>S. viridans</i>	II	P
6	4761/72	<i>S. sanguis</i>	II	P
7	5663/72	<i>S. viridans</i>	II	P
8 ^a	7909/72	<i>S. sanguis</i>	I	N
9	8253/72	<i>S. viridans</i>	II	P
10	08903/72	<i>S. viridans</i>	II	P
11	011376/72	<i>S. viridans</i>	IV	N
12 ^a	011785/72	<i>S. viridans</i>	II	N
13		<i>S. viridans</i>	IV	Not tested

^a Described in Table 2.

^b N, Neuraminidase action on human serum proteins; P, protease action on human serum proteins.

TABLE 2. Neuraminidase activity of *S. viridans* and *S. sanguis* strains in blood samples in the first and consequent subcultures

Neuraminidase action on human glycoproteins	Strains											
	Expt 12			Expt 13			Expt 8			Expt 1		
	Blood sample	1st SC ^a	10th SC	Blood sample	1st SC	63rd SC	Blood sample	1st SC	25th SC	Blood sample	1st SC	25th SC
Acid α_1 glycoprotein	+ ^b	+	+	+	+	+	+	+	- ^c	+	+	+
α_1 -Antitrypsin	• ^d	•	+	+	+	+	+	+	-	+	-	-
α_1 -Antichymotrypsin	+	-	•	•	•	•	•	•	-	+	•	-
Haptoglobin	+	+	+	+	+	+	+	+	-	+	-	-
Ceruloplasmin	+	+	+	+	+	(+) ^e	+	+	-	+	-	-
α_2 -Macroglobulin	+	(+)	-	-	-	-	(+)	(+)	-	-	-	-
α_2 HS-glycoprotein	+	+	+	+	+	(+)	+	+	-	+	+	-
Transferrin	+	+	+	+	+	+	+	+	-	+	+	-
Hemopexin	+	+	•	+	+	+	+	+	-	+	+	(+)
β_2 -Glycoprotein-I	-	-	-	(+)	-	-	•	+	-	-	-	-
IgA	•	•	+	-	-	-	-	-	-	-	-	-

^a SC, Subculture.

^b +, Positive neuraminidase action.

^c -, Negative neuraminidase action.

^d•, Enzyme reaction not tested.

^e(+), Weak positive neuraminidase action.

The existence of the enzyme neuraminidase in *S. viridans* was also proved by paper chromatography by using the neuraminidase-specific substrate *N*-acetylneuraminylactose (left side of Fig. 9). This substrate is split by neuraminidase of *S. viridans* into lactose and *N*-acetylneuraminate (NAN). NAN, in its turn, is split by another enzyme into *N*-acetyl-D-mannosamine and pyruvate. This enzyme is therefore the acylneuraminate pyruvate lyase subsequent

to neuraminidase in the series of reaction steps which degrade molecules. The effect of splitting off NAN is shown on the right side of Fig. 9. Only the *N*-acetyl-D-mannosamine is detectable by the applied color reaction in Fig. 9; the pyruvate, however, is no longer visible.

There is also proof, by the paper chromatogram of Fig. 9, of the existence of the enzyme neuraminidase (sialidase = mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18)

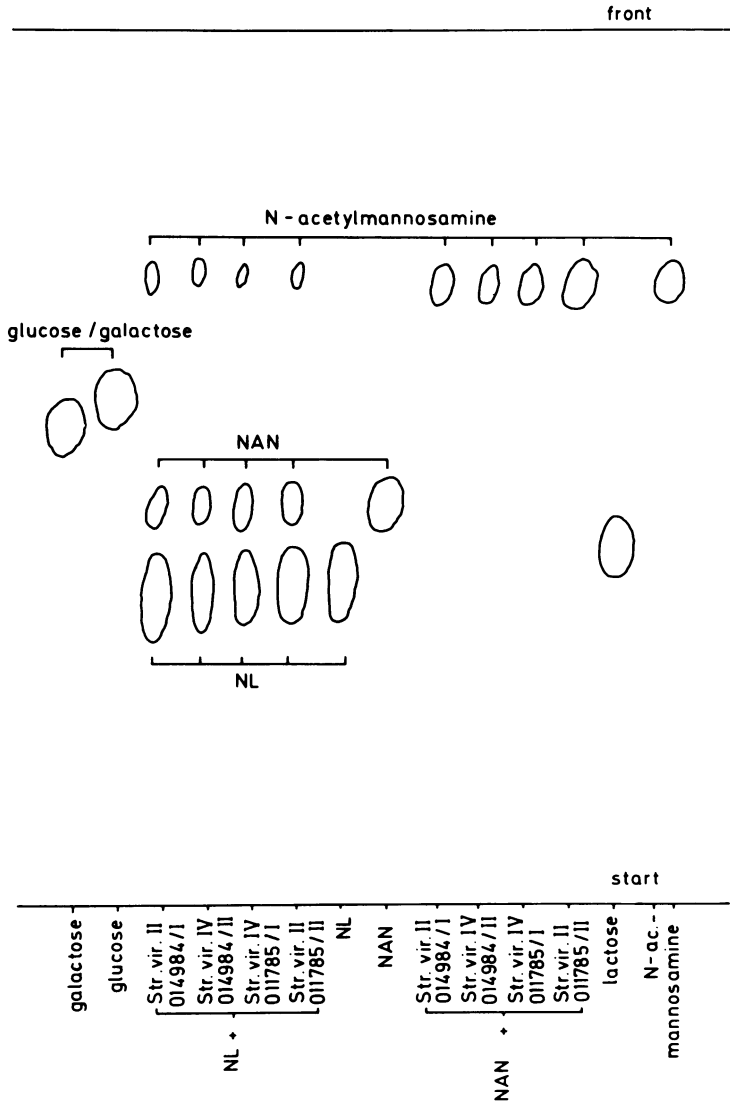


FIG. 9. Paper chromatogram showing the action of neuraminidase and acylneuraminate lyase of *S. viridans* (*S. viridans* II, strains 014984/I and 011785/II; *S. viridans* IV, strains 014984/II and 011785/I) on the substrates *N*-acetylneuraminylactose (NL) and NAN. Neuraminidase splits *N*-acetylneuraminylactose into *N*-acetylneuraminate and lactose. Acylneuraminate lyase splits *N*-acetylneuraminate into *N*-acetylmannosamine and pyruvate. A galactosidase-like enzyme splits lactose into glucose and galactose.

and acylneuraminate pyruvate lyase (*N*-acetylneuraminate lyase, EC 4.1.3.3) in *S. viridans*.

DISCUSSION

The demonstration of neuraminidase in *S. sanguis* and *S. viridans* by Hayano et al. (6) was performed by using mucine as the substrate. They found only very weak neuraminidase activity and could not detect the activity of acylneuraminate pyruvate lyase. However, they investigated collection strains, whereas in our laboratory freshly isolated strains from pathological material (i.e., blood samples of patients with sepsis) were studied. Under these conditions, streptococci seem to develop strong neuraminidase activity.

Some years ago, Kelly and co-workers studied the neuraminidase production of pneumococci (8). They also found strong enzyme activity in bacteria in the macroorganism, or in material freshly isolated from it, and a decrease of neuraminidase activity in the course of subcultures. The same effect is observed in *S. sanguis* (17).

S. viridans, however, differs from *S. sanguis* in that its neuraminidase seems to be a noninducible enzyme. However, neuraminidase activity of *S. viridans* varies greatly, and most strains isolated from human pharynges show only very weak enzyme activity. Whereas *S. sanguis* and also *Diplococcus pneumoniae* may possess inducible neuraminidases produced in great amounts under pathological conditions such as pneumonia (7), meningitis (10), or endocarditis (17), *S. viridans* may be selected under these conditions in the animal or human host as a consequence of the enzyme production, i.e., neuraminidase or proteolytic enzymes.

The same effect of inducible neuraminidase was described in a previous study of *Fusobacterium polymorphum* (18). Also, in the case of actinomycosis as a mixed infection with *Fusobacterium*, we found strong activity of neuraminidase in the bacteria of the first and second subculture. However, after some weeks the neuraminidase activity decreased to a very low level.

The pathogenicity of pneumococcal neuraminidase, at least, is well established by different investigations. Kelly and Greiff described the toxicity of pneumococcal neuraminidase for the brain of mice (9), verifying an old observation in bacteriological laboratories that mice surviving pneumococcal infection frequently show cerebral damage. Another path of pathogenic action of neuraminidase during pneumococcal infection, especially pneumonia, was demonstrated

by Fischer and co-workers (7). They found severe thrombosis and a Sanarelli-Schwartzman-like syndrome caused by a Thomsen-Friedenreich phenomenon, i.e., by the panagglutinability of the red cells as the effect of pneumococcal neuraminidase. In our laboratory, the effect of neuraminidase on the glycoproteins of the cerebrospinal fluid in the meningitis caused by pneumococci was shown also as an indication of the pathogenic action of this enzyme (10). In a similar manner, neuraminidase of clostridia of the gas gangrene group alters the glycoproteins in infected wounds (11, 12), and the neuraminidase of *Bacteroides fragilis* shows protein alterations in the abscesses caused by this bacterium (19).

There is, therefore, some suggestion of the pathogenic action of the neuraminidase of *S. sanguis* and *S. viridans*, respectively, especially under the conditions of endocarditis. The strong activity of this enzyme may alter not only the plasma glycoproteins but also the surfaces of red cells as is shown for pneumococcal pneumonia and, in addition, the endothelia cells. But this last point must be proved.

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