INFECTION AND IMMUNITY, Nov. 1979, p. 563-572
0019-9567/79/11-0563/10$02.00/0

Vol. 26, No. 2

Neuraminidase-Dependent Hemagglutination of Human Erythrocytes by Human Strains of *Actinomyces viscosus* and *Actinomyces naeslundii*

A. H. COSTELLO,[1]* J. O. CISAR,[2] P. E. KOLENBRANDER,[2] AND O. GABRIEL[1]

Georgetown University, School of Dentistry, Washington, D.C. 20007,[1] and Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20205[2]

Received for publication 28 June 1979

Human A, B, and O erythrocytes (RBC) were agglutinated by many human strains of *Actinomyces viscosus* and *A. naeslundii*. At 37°C, these bacterium-mediated hemagglutination reactions required the action of bacterial neuraminidase upon the RBC; however, at 4°C, the requirement for neuraminidase was not as striking. Bacterial cell suspensions which caused hemagglutination at 37°C contained both soluble extracellular and cell-associated neuraminidase activities as shown by enzyme assays using a soluble substrate (i.e., α_1-acid glycoprotein). Bacterium-mediated hemagglutination occurred only in the presence of soluble neuraminidase activity, and the rate of hemagglutination could be inhibited by 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, a competitive inhibitor of purified soluble neuraminidase from *A. viscosus* T14V. Suspensions of bacteria which contained only cell-associated neuraminidase activity were unable to initiate hemagglutination, but they caused immediate hemagglutination when mixed with neuraminidase-treated RBC. All hemagglutination reactions were reversible in the presence of 0.02 M lactose and were abolished by heating (85°C for 30 min) the actinomycete cells but not the RBC. The proposed mechanism of hemagglutination involves two sequential steps: (i) the action of neuraminidase to unmask galactose-containing receptors on the RBC and (ii) the multivalent binding of these receptors by many low-affinity lectin sites on the bacterial surface.

The adherence of certain bacteria to mucosal surfaces may involve lectin-like sites on the bacterial surface which bind to specific sugar residues on mammalian cells. This concept was illustrated by the mannose-inhibited binding of *Escherichia coli* to erythrocytes (RBC) and to certain other mammalian cells (9, 12, 24-26, 33, 34) and by the fucose-inhibited adherence of *Vibrio cholerae* to human RBC and rabbit intestinal brush border membranes (15). Lectin-like binding also has been described for another group of bacteria, the oral actinomycetes, as shown by the ability of lactose to inhibit cell-cell interactions between many human strains of *Actinomyces viscosus* or *A. naeslundii* and certain isolates of *Streptococcus sanguis* and *S. mitis* (6, 20). These interactions, which may contribute to the formation of dental plaque, are thought to result from specific sites on the actinomycetes which bind galactose-like residues on the streptococcal surface.

The mechanisms of adherence which allow actinomycetes to reside on oral surfaces are of interest because these bacteria have been associated with various disease states (4). Though commonly found in plaque, the isolation of actinomycetes from oral mucosal surfaces (10) implies that mechanisms exist for the direct attachment of these bacteria to mammalian cells. Indeed, adherence of *A. naeslundii* to human buccal epithelial cells (11) and hemagglutination of human RBC by *A. viscosus* (14, 31) have been demonstrated.

The present investigation focuses on the mechanism by which *A. viscosus* and *A. naeslundii* cells adhere to human RBC, resulting in hemagglutination. These hemagglutination reactions, like cell-cell interactions between certain actinomycetes and plaque streptococci (6, 20), were reversed by lactose and appeared to involve lectin sites on the actinomycetes. The production of neuraminidase by many strains of actinomycetes and its essential role in hemagglutination are described.

(Preliminary reports of this work have appeared elsewhere [O. Gabriel, J. Dent. Res. 57: 202, Abstr. 510, 1978; A. H. Costello, J. O. Cisar, P. E. Kolenbrander, and O. Gabriel, J. Dent. Res. 58:342, Abstr. 1000, 1979]. This work was done in partial fulfillment of the requirements

for the Ph.D. from the Department of Biochemistry, Georgetown University.)

MATERIALS AND METHODS

Bacterial strains. Listed in Table 1 are the host origin and the strain designation of the actinomycetes studied. The source of most strains was given in a previous report (6), and the culture source of additional strains was as follows: *A. viscosus* strain A32B1 from S. Socransky; *A. viscosus* strain W1628 from the Center for Disease Control (S. L. Bragg); *A. viscosus* strain 626 from a National Institute of Dental Research stock culture originally obtained from J. M. Slack; *A. naeslundii* strain N16 from M. A. Gerencser; *A. naeslundii* strain CROB 2039 from B. Williams; and *A. bovis* ATCC 13683 from the American Type Culture Collection.

Bacterial growth conditions. Each strain was grown aerobically in static culture at 37°C in a complex growth medium (100-ml volume) containing 0.5% yeast extract, 0.5% Tryptone (Difco Laboratories), 0.5% K_2HPO_4, and 0.05% Tween 80, with glucose (0.2%) as the source of energy (6). Cultures were harvested during the midexponential phase of growth (about 100 to 150 Klett units) as determined by use of a Klett Summerson colorimeter fitted with a red filter (660 nm). The pH of cultures at the time of harvest varied from 6 to 7. Each culture was divided into two portions, and the cells were prepared for hemagglutination or neuraminidase assays by the procedures outlined below.

Bacterium-mediated hemagglutination. Bacterial cells were harvested from culture, washed three times by centrifugation (10,000 × *g* for 10 min) in 0.02 M phosphate-0.85% NaCl, pH 7.2 (PBS), containing 0.02% sodium azide, and suspended in buffer to an optical density of 2.0 at 650 nm (about 10^{10} cells/ml). Human RBC from blood drawn in anticoagulant citrate phosphate dextrose solution (Fenwal Laboratories, Deerfield, Ill.) were washed three times by centrifugation (300 × *g* for 10 min) in PBS and were used within 1 week of collection. Enzyme treatments of washed RBC were performed with 15% (vol/vol) packed-cell suspensions and incubation periods of 2 h at 37°C. The enzymes used were type V neuraminidase from *Clostridium perfringens* (N2876; Sigma Chemical Co., St. Louis, Mo.) at 30 μg/ml, protease from *Streptomyces griseus* (P5130; Sigma) at 2 mg/ml, and trypsin (T8253; Sigma) at 2 mg/ml. Enzyme-treated RBC along with control RBC were washed three times by centrifugation in PBS containing 0.1% gelatin before use.

Visual assays for bacterium-mediated hemagglutination were performed in round-bottom microtiter plates or in small tubes. Serial dilutions of bacterial cell suspensions prepared in PBS containing 0.1% gelatin were mixed with equal volumes of either 1 or 4% (vol/vol) RBC suspensions at 4°C, room temperature, and 37°C. Assays were examined immediately for hemagglutination and also were examined after various incubation periods. Reversal of hemagglutination was accomplished by the addition of lactose to a final concentration of 0.02 M. In assays for the inhibition of hemagglutination by sugars, 25 μl of the sugar solution and 25 μl of a bacterial suspension (about 2 × 10^7 bacteria) were mixed before the RBC suspension was added (20 μl containing about 2 × 10^6 RBC).

Bacterium-mediated hemagglutination also was followed by a turbidimetric method (3), using a model 300 Chrono-Log platelet aggregometer (Chrono-Log Corp., Broomall, Pa.) in conjunction with a model 481

TABLE 1. *Neuraminidase activity displayed by various Actinomyces strains and the ability of these strains to cause lactose-reversible hemagglutination of neuraminidase-treated human A, B and O RBC*

Strain	Host origin	Lactose-reversible hemagglutination	Enzyme activity[a]
A. viscosus			
T14V	Human	+	284
MG1	Human	+	187
A1B1	Human	+	137
Keyes	Human	+	101
A32B1	Human	+	90
W1628	Human	+	76
626	Human	−	113
T14AV	Human	−	6 (100)[b]
A828	Hamster	−	84
X-602	Hamster	−	42
T6-1600	Hamster	−	32
R-28	Rat	−	8
A. naeslundi			
W1544	Human	+	108
N16	Human	+	96
W752	Human	+	89
CROB2039	Human	+	76
WVU820	Human	+	68
WVU45	Human	+	59
I	Human	+	33
W1096	Human	+	28
WVU398A	Human	+	17
ATCC 12104	Human	+	14
W826	Human	+	0
A. odontolyticus			
ATCC 17982	Human	−	198
A. parabifidus (*Bifidobacterium bifidum*)	Human	−	35
A. bovis W827			
ATCC 13683	Bovine	−	2

[a] Measured as nanomoles of *N*-acetylneuraminic acid released per hour at 37°C by 150 μl of a washed-cell suspension which contained cell-associated neuraminidase.

[b] Activity after mechanical shearing of culture; see text.

recorder (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Cell mixtures for aggregometry contained about 2×10^7 RBC and 10^8 bacteria in 0.5 ml of PBS containing 0.1% gelatin. These mixtures were stirred at 1,200 rpm in glass cuvettes (7 by 50 mm) maintained at 37°C. The aggregometer was adjusted so that 10% transmission corresponded to the RBC alone in 0.475 ml, and 80% transmission was achieved for maximum hemagglutination with neuraminidase-treated RBC and the bacteria in 0.5 ml. In hemagglutination experiments, a stable base line was established for the RBC in 0.475 ml before the addition (25 μl) of bacterial suspensions. Solutions of 1 M lactose and 10^{-3} M 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid were added to cell mixtures to obtain the desired final concentrations of these substances. The latter compound was provided by Roland Schauer, Christian-Albrechts-Universitat, Kiel, Germany.

Assays for neuraminidase activity. Enzyme assays were carried out either in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 6.4) containing 0.005 M $CaCl_2$ or in PBS.

Bacteria were harvested, washed twice with either buffer, and suspended in buffer to approximately 10^{10} cells/ml. Reaction mixtures, containing 150 μl of the bacterial cell suspension and 50 μl of a solution of α_1-acid glycoprotein (10 mg/ml), were incubated at 37°C for either 2 or 6 h. Bacteria were removed from these mixtures by centrifugation, and supernatants were assayed for the amount of free sialic acid released, using the colorimetric procedure described by Warren (37).

A total of 906 nmol of bound *N*-acetylneuraminic acid was present in each incubation mixture. All values for neuraminidase activity shown in Tables 1, 2, and 3 were derived from data that were obtained within the linear range of the assay.

As a second independent assay, the formation of asialo α_1-acid glycoprotein was demonstrated by its electrophoretic mobility, which was slower than that of the native glycoprotein. In these determinations, polyacrylamide disc gel electrophoresis was performed according to Davis (8) with 7.5% polyacrylamide gel at pH 8.9, and gels were stained for protein with Coomassie blue as described by Chrambach et al. (5). Soluble neuraminidase in culture medium and in buffer was measured by the above procedures, except that the incubation mixture contained 150 μl of cell-free enzyme solution and 50 μl of α_1-acid glycoprotein (10 mg/ml).

TABLE 2. *Effect of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid on the enzymatic activity of soluble neuraminidase from A. viscosus T14V*

Inhibitor concn (μM)	Relative enzyme activity (%)
None	100[a]
10	50
25	28
250	14

[a] 100% relative activity equals 292 U (nanomoles of *N*-acetylneuraminic acid released per hour).

TABLE 3. *Requirement of soluble neuraminidase activity for hemagglutination by several strains of Actinomyces*

Strain	Sample[a]	Enzyme activity[b]	Hemagglutination[c]
A. viscosus T14V	Supernatant	31	+
	Cells	185	−
A. viscosus MG1	Supernatant	21	+
	Cells	74	−
A. naeslundii W1544	Supernatant	16	+
	Cells	63	−
A. naeslundii W826	Supernatant	(5)[d]	−
	Cells	(9)	−

[a] Bacterial cells were incubated overnight in buffer at 37°C to obtain a cell suspension with soluble and cell-associated neuraminidase activity. Centrifugation of this suspension yielded a cell-free supernatant fraction and the bacterial cells, which were washed and adjusted to their original density in fresh buffer.

[b] Nanomoles of *N*-acetylneuraminic acid released per hour.

[c] With human O RBC. Bacterial cells were included in assays of the supernatant fractions.

[d] Values in parentheses are not significant.

Release of galactose from asialo α_1-acid glycoprotein. The preparation of asialo α_1-acid glycoprotein labeled with tritium on terminal D-galactose residues was carried out as described for ceruloplasmin (22). Incubation of this specifically labeled substrate with *A. viscosus* cells in the presence or absence of unlabeled α_1-acid glycoprotein was carried out under conditions identical to those in the assay for neuraminidase described above (Tris-maleate buffer, pH 6.4). After incubation for 2 h, the release of terminal galactose residues was determined by two procedures. In the first, the sample was centrifuged to remove bacterial cells and the supernatant was subjected to polyacrylamide gel electrophoresis (conditions described above). The amount of radioactivity located in the area corresponding to the mobility of asialo α_1-acid glycoprotein was determined and compared with the total radioactivity initially present in the sample. Alternatively, the sample was applied to a Sephadex G-15 column (1-cm diameter × 30 cm), and the materials eluting in the void volume and in the salt volume were collected separately and assayed for radioactivity.

RESULTS

Hemagglutination of human RBC by actinomycete cells. Several human strains of *A. viscosus* and *A. naeslundii* caused agglutination of human A, B, and O RBC. These reactions occurred slowly and required incubation periods ranging from minutes to hours at room temperature or 37°C. When the RBC were pretreated with neuraminidase from *C. perfringens*, immediate hemagglutinations were observed with 17 human strains of *A. viscosus* and *A. naeslundii*,

but reactions did not occur with two human strains of *A. viscosus* and with various other actinomycetes (Table 1). The immediate hemagglutinations with neuraminidase-treated RBC and the slower reactions with untreated RBC were inhibited or reversed by the addition of lactose to a final concentration of 0.02 M. Significantly, about half of the bacterial strains gave immediate hemagglutination with untreated RBC when the assays were performed at 4°C. These cold hemagglutinations, like the slower reactions, at higher temperatures, were lactose reversible. The inhibitory activity of lactose appeared to involve the terminal galactose moiety of the disaccharide because β-methyl-galactoside inhibited hemagglutination at concentrations up to 0.1 M. Hemagglutinations that were inhibited by lactose were prevented by heating (85°C for 30 min) the actinomycete cells but not by similar heating of the neuraminidase-treated RBC. These findings suggested a mechanism of hemagglutination which involved the interaction of lectins on the actinomycetes with carbohydrate receptors on the RBC surface.

Production of neuraminidase by bacterial strains. Washed-cell suspensions of the actinomycetes listed in Table 1 were assayed for neuraminidase. Most of the strains displayed considerable activity as measured by the release of sialic acid from α_1-acid glycoprotein (Table 1) and by the corresponding formation of asialo α_1-acid glycoprotein which was detected by disc gel electrophoresis (electrophoretic patterns not shown). In a few instances, very low or questionable neuraminidase activities were present as judged by values for the release of sialic acid (*A. viscosus* R-28, *A. naeslundii* W826, *A. bovis* W827, and *A. viscosus* T14AV); however, with *A. bovis* W827 and *A. viscosus* T14AV, significant activity was present as shown by the appearance of asialo glycoproteins in disc gel electrophoresis. Although *A. viscosus* T14AV displayed low activity, mechanical shearing or freezing and thawing of the culture resulted in an amount of neuraminidase activity which was comparable to that found with *A. viscosus* T14V.

Neuraminidase-dependent hemagglutination. The immediate hemagglutination of neuraminidase-treated RBC by several human strains of *A. viscosus* and *A. naeslundii* suggested that hemagglutination of normal RBC at room temperature or 37°C required the action of neuraminidase. This suggestion was supported by studies with three actinomycetes (*A. naeslundii* strains W1544 and W826 and *A. viscosus* MG1) in which hemagglutination at 37°C was followed by aggregometry. RBC pretreated with neuraminidase from *C. perfringens* (Fig. 1A) or from *A. viscosus* T14V (data not shown) agglutinated immediately when actinomycete cells were added. By contrast, untreated RBC and washed bacterial cells did not react until a small amount of purified neuraminidase from *A. viscosus* T14V was added (Fig. 1B). RBC pretreated with protease from *S. griseus* also were not agglutinated by the actinomycete cells until neuraminidase was added (Fig. 1B), and similar results were obtained with trypsin-treated RBC (results not shown). Significantly, the extent of neuraminidase-dependent hemagglutination was less with protease or trypsin-treated RBC than with untreated RBC (Fig. 1B). With all hemagglutination reactions, the addition of lactose to a final concentration of 0.02 M caused complete reversal. Thus, lactose-reversible hemagglutination of normal RBC at 37°C was initiated by neuraminidase and not by proteolytic enzymes.

Additional strong evidence for neuraminidase-dependent hemagglutination at 37°C was provided by studies with a competitive inhibitor for neuraminidase (21), 2-deoxy-2,3-dehydro-*N*-acetyl-neuraminic acid. This compound at a concentration of about 10^{-5} M gave 50% inhibition of the purified extracellular neuraminidase from *A. viscosus* T14V (Table 2) and also inhibited the rate of hemagglutination resulting from the addition of neuraminidase to a mixture of RBC and actinomycete cells (Fig. 2). With the amount of neuraminidase used in the experiments presented in Fig. 2, the rates of hemagglutination at inhibitor concentrations between 1.4×10^{-5} and 3.4×10^{-6} M displayed a proportional, inverse relationship to the inhibitor concentration. Thus, under these conditions the progress of hemagglutination depended directly on the action of neuraminidase.

Role in hemagglutination of soluble versus cell-bound neuraminidase. In view of the finding that hemagglutination at 37°C was neuraminidase dependent, a puzzling aspect of preliminary studies was that the time required for hemagglutination of normal RBC by suspensions of bacteria was not related clearly to the total amount of neuraminidase activity (i.e., soluble plus cell associated) detected in these suspensions by enzyme assays using a soluble glycoprotein substrate. This was explained when bacterium-mediated hemagglutination of normal RBC was found to depend on neuraminidase activity which was soluble rather than cell associated. To demonstrate this point, suspensions of bacteria containing both soluble and cell-associated neuraminidase activity were prepared. This was done conveniently by overnight incubation of washed bacterial cells in buffer at 37°C.

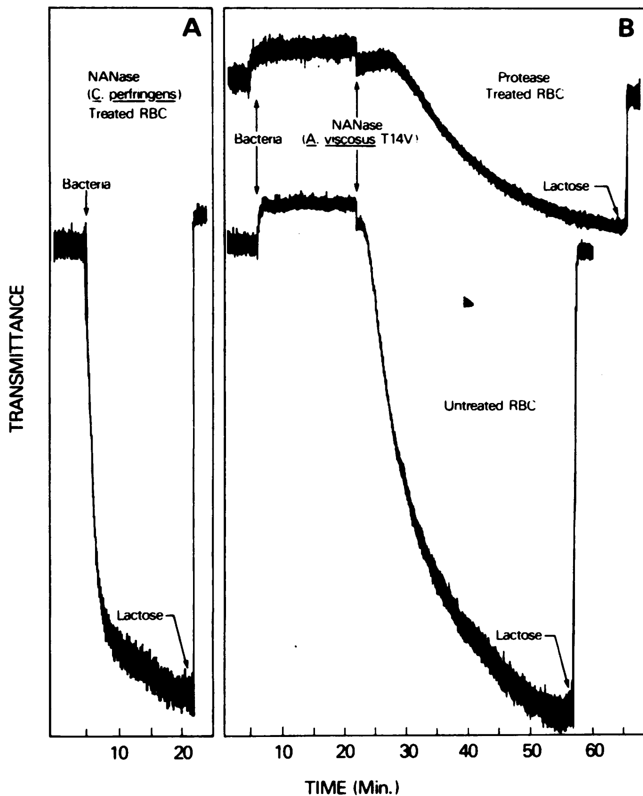

FIG. 1. *Ability of neuraminidase to promote hemagglutination of human RBC by A. naeslundii W1544 cells. The progress of hemagglutination was followed by the continuous recording of turbidity measurements, using an aggregometer. Bacterial cells were added to: (A) RBC treated with neuraminidase from C. perfringens; immediate hemagglutination occurred. (B) Protease-treated (upper curve) or untreated RBC (lower curve); hemagglutination did not occur until purified neuraminidase from A. viscosus T14V was added to these cell mixtures. All hemagglutination reactions were reversed by the addition of lactose to a final concentration of 0.02 M.*

The soluble neuraminidase activity in these suspensions was measured in assays of cell-free supernatants, and cell-associated enzyme activity was determined with the washed bacteria freshly suspended in buffer (Table 3). Visible hemagglutination of normal RBC did not occur in the presence of freshly washed bacteria but proceeded when the supernatant was added to the mixture of RBC and bacteria (Table 3). This observation was unexpected at first glance because the levels of cell-associated neuraminidase activity displayed by freshly washed bacteria were two to four times greater than the amounts of soluble neuraminidase activity in the cell-free supernatants.

The absolute nature of the requirement for soluble versus cell-bound neuraminidase in the hemagglutination reaction was shown by aggregometry. A suspension of *A. naeslundii* W1544 cells containing both soluble and cell-associated neuraminidase activity caused slow hemagglutination of normal RBC at 37°C (Fig. 3A). This reaction was shown to be neuraminidase dependent by the ability of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid to inhibit the rate of hemagglutination (Fig. 4). When bacterial cells from the suspension were collected, washed by centrifugation in fresh buffer, and added to normal RBC, hemagglutination did not occur over a period of 40 min (Fig. 3B) even though 72% of the original neuraminidase activity remained associated with the bacterial cells and was detected by enzyme assays with soluble α_1-acid glycoprotein as the substrate. The cell-free supernatant from the original suspension of bacteria contained soluble neuraminidase activity (28% of the original) and initiated slow hemagglutination when added to the mixture of RBC and washed bacterial cells. In the converse experiment (Fig. 3C), incubation with the cell-free

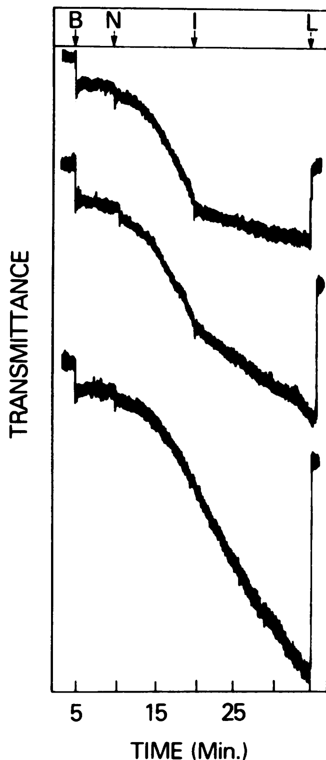

FIG. 2. *Hemagglutination reactions with human RBC and A. naeslundii W1544 cells in the presence of purified neuraminidase from A. viscosus T14V, and the inhibition of these reactions by a competitive neuraminidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. The rate of hemagglutination was followed by the continuous recording of turbidity measurements, using an aggregometer. Bacterial cells (B) and purified neuraminidase (N) were added separately to the RBC suspension. After the initiation of hemagglutination by neuraminidase, the neuraminidase inhibitor (I) was added to final concentrations of: 1.4×10^{-5} M (upper curve); 3.4×10^{-6} M (middle curve); no inhibitor (lower curve). All hemagglutination reactions were reversed by the addition of lactose (L) to a final concentration of 0.02 M.*

supernatant for 40 min yielded RBC which were agglutinated immediately by the washed bacterial cells. Thus, both soluble and cell-associated neuraminidase were detected with a soluble substrate (i.e., α_1-acid glycoprotein), but only soluble neuraminidase acted on the RBC to initiate hemagglutination by the actinomycete cells.

The complete reversal of all hemagglutination reactions by 0.02 M lactose suggested that the actinomycetes interacted with galactose residues which would be exposed on the RBC surface by the action of soluble neuraminidase. This suggestion was consistent with results from an experiment in which *A. viscosus* cells were incubated with asialo α_1-acid glycoprotein which contained terminal D-galactose residues specifically labeled with tritium. During a 2-h incubation period, more than 80% of the radioactivity remained associated with the asialo glycoprotein. Thus, terminal galactose residues exposed by the action of neuraminidase remained linked to the glycoprotein.

DISCUSSION

In the present investigation, hemagglutination of human RBC by human strains of *A. viscosus* and *A. naeslundii* was studied to help define mechanisms which may contribute to the adherence of these bacteria to oral mucosal surfaces (11) and possibly to the glycoprotein pellicle of teeth (31). The results show clearly that at 37°C, bacterium-mediated hemagglutination requires neuraminidase which acts on the RBC glycoproteins (Fig. 5) and glycolipids. In addition, these hemagglutination reactions were inhibited or reversed specifically by relatively high concentrations (0.02 M) of lactose and α-methylgalactoside. Finally, hemagglutination was abolished by heating (85°C for 30 min) the actinomycete cells but not the RBC. These findings are consistent with a mechanism of hemagglutination which involves two sequential steps: (i) the action of neuraminidase to expose galactosyl groups on the RBC surface and (ii) the binding of these groups by lectin-like molecules on the actinomycete cells.

An important additional characteristic of several bacterium-mediated hemagglutinations was their similiarity to cold agglutination reactions. At 37°C in the absence of soluble neuraminidase, freshly washed bacteria did not cause agglutination of normal RBC, but at 4°C, these cell mixtures gave immediate hemagglutination reactions which were lactose reversible. Studies from Ginsburg's laboratory (35, 36) have shown that cross-linking of erythrocytes by immunoglobulin M cold agglutinins occurs at 4°C but not 37°C when the density of receptors on the RBC surface is relatively low. These findings, when applied to the present results, suggest that adherence of actinomycete cells to RBC requires the combined effect of many low-affinity lectin sites on the bacterial surface. Thus, bacterium-mediated hemagglutination may not occur at 37°C in the absence of neuraminidase because the low density of galactosyl receptors on the RBC surface does not permit enough multivalent binding for strong cross-linking of the RBC. By contrast, multivalent binding and hemagglutination may be favored at 4°C by a small increase in affinity of individual binding sites

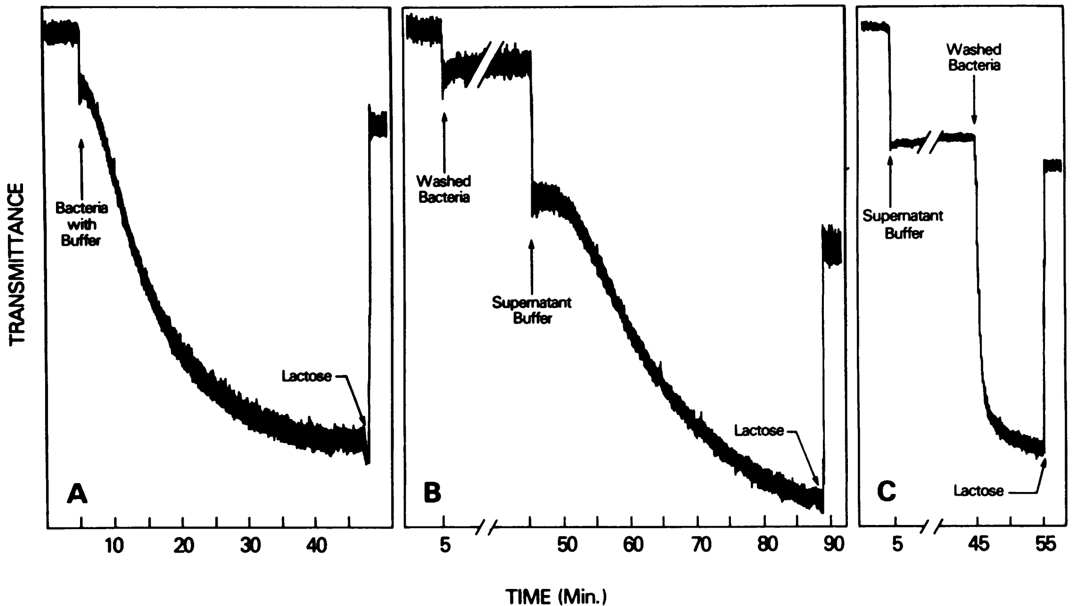

FIG. 3. *Ability of soluble but not cell-associated neuraminidase activity to promote hemagglutination of human RBC by A. naeslundii W1544 cells. The progress of hemagglutination was followed by the continuous recording of turbidity measurements using an aggregometer. (A) A bacterial cell suspension, which contained both soluble and cell-associated neuraminidase activity, caused slow hemagglutination of normal RBC. The bacterial cell suspension was centrifuged to obtain the cell-free supernatant buffer which contained soluble neuraminidase activity (84 U/ml), and the pelleted bacteria were used to prepare a suspension of freshly washed bacteria which contained only cell-associated neuraminidase activity (211 U/ml). Experiments with normal RBC revealed: (B) no hemagglutination after addition of the washed bacteria and slow hemagglutination after a subsequent addition of the supernatant buffer; (C) no hemagglutination after addition of the supernatant buffer and immediate hemagglutination after a subsequent addition of the washed bacteria. Addition of the supernatant buffer in panels B and C caused a drop in transmittance due to dilution. All hemagglutination reactions were reversed by the addition of lactose to a final concentration of 0.02 M.*

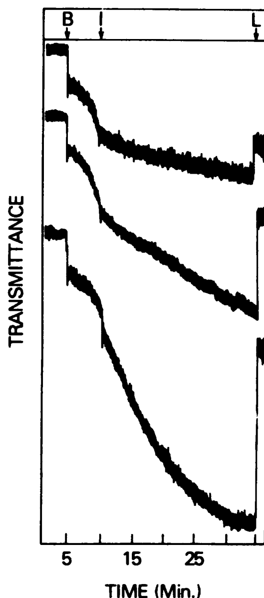

FIG. 4. *Hemagglutination of human RBC by a suspension of A. naeslundii W1544 cells containing neuraminidase activity (28% soluble, 72% cell associated), and the inhibition of hemagglutination by a competitive neuraminidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. The rate of hemagglutination was followed by the continuous recording of turbidity measurements, using an aggregometer. The bacterial cell suspension (B) was added to the RBC suspension. After the initiation of hemagglutination, the neuraminidase inhibitor (I) was added to final concentrations of: 4.2×10^{-5} M (upper curve); 1.4×10^{-5} M (middle curve); no inhibitor (lower curve). All hemagglutination reactions were reversed by the addition of lactose (L) to a final concentration of 0.02 M.*

which would be expected at this temperature. The observation that relatively high concentrations of lactose (0.02 M) were required to inhibit these reactions is consistent with the notion that bacterial attachment involves multivalent binding by many low-affinity sites. Such a mechanism may enable the bacterial surface to adhere

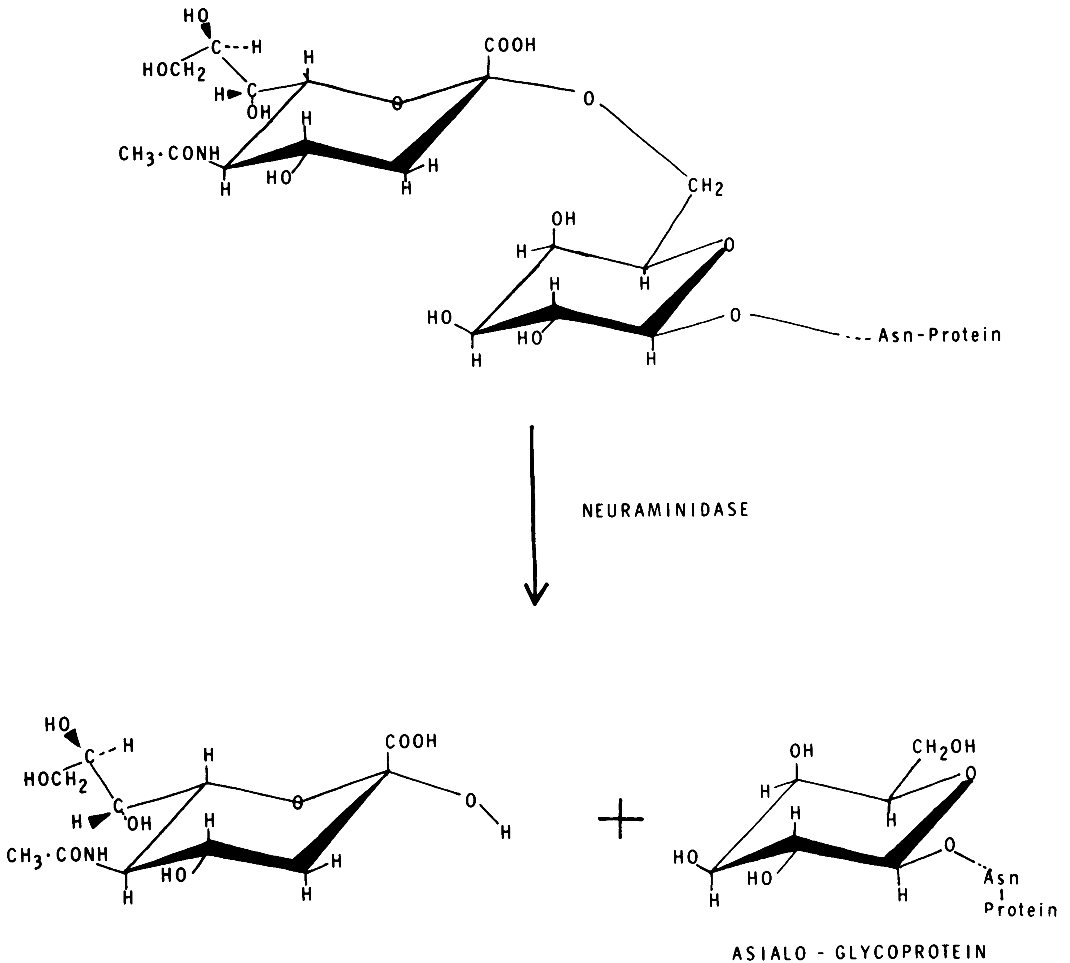

FIG. 5. *Action of neuraminidase upon glycoprotein.*

to host cells in the presence of salivary asialo glycoproteins containing galactose termini.

The production of neuraminidase by many pathogenic or saprophytic microorganisms suggests an important function for this enzyme in the colonization and penetration of mucosal surfaces (23, 27). This idea is consistent with the present results, which show that at 37°C, hemagglutination caused by many actinomycete strains proceeds only after the removal of neuraminic acid from the RBC surface. The absolute requirement for neuraminidase in these reactions was demonstrated clearly by the ability of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid to act as a potent competitive inhibitor of bacterium-mediated hemagglutination (Fig. 2 and 4). This compound appears to be a transition-state analog for neuraminidase (Fig. 6; 21), and as such, it is bound more tightly in the catalytic site than is the substrate. The essential role for neuraminidase in the interaction of actinomycete cells with human RBC raises the possibility that this enzyme also functions in the adherence of these bacteria to oral surfaces. Indeed, it is possible that the actinomycetes contribute significantly to the neuraminidase activity of human saliva (17, 29).

An important feature of the neuraminidase activity in actinomycete cultures was that a fraction of the total activity appeared as a soluble extracellular enzyme, whereas the remainder was associated with the bacterial surface. Significantly, only soluble neuraminidase acted on the RBC to initiate hemagglutination by the actinomycete cells. By contrast, both the soluble and cell-associated neuraminidase acted on soluble α_1-acid glycoprotein as shown by two independent assays: (i) the colorimetric measure-

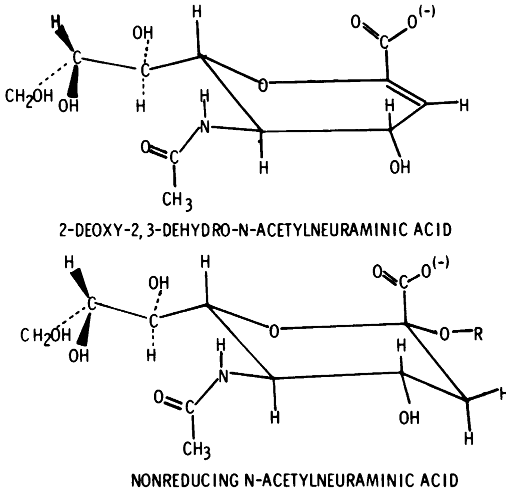

FIG. 6. *Structural similarity between 2-deoxy-2,3-dehydro-N-acetylneuraminic acid and terminal nonreducing N-acetylneuraminic acid.*

ment (37) of released neuraminic acid and (ii) the electrophoretic demonstration of asialo α_1-acid glycoprotein. The latter method was used to ensure the detection of neuraminidase activity in cases where released *N*-acetylneuraminic acid was converted to products which no longer reacted in the colorimetric assay. It is interesting that the neuraminidase from *V. cholerae*, when immobilized on Sepharose, retained some ability to release neuraminic acid from human RBC (28). Thus, the complete inability of cell-associated neuraminidase to initiate hemagglutination (Fig. 3B) suggests that the enzyme exists on the bacterial surface in a location which is inaccessible to the RBC. Studies are underway to identify this location and also to examine both the biosynthesis and substrate specificity of the soluble and cell-associated neuraminidase.

It is well established that the removal of neuraminic acid from mammalian glycoproteins exposes the penultimate galactosyl residues which then can serve as receptors for lectins and antibodies of appropriate specificity. Perhaps the best-studied example of this recognition mechanism has come from the work of Ashwell and colleagues (1, 2) on binding of asialo glycoproteins by a galactose-specific, hepatic lectin. Similarly, the action of neuraminidase on human RBC exposes the T antigen, which is recognized by naturally occurring anti-T antibodies (13) and by the galactose-specific peanut lectin (18). Both the MN antigens and the T antigen are carried by glycophorin, the major glycoprotein of the RBC membrane (19); the removal of neuraminic acid to expose galactosyl residues destroys MN reactivity and creates T determinants which include Gal $\beta(1 \rightarrow 3)$GalNAc sequences (16, 18, 32). Other sugar sequences which contain terminal or penultimate galactosyl residues have been identified in glycoproteins, and similar sequences also occur in glycolipids (30). Thus, many possibilities exist for the RBC receptors in neuraminidase-dependent hemagglutination, and studies are underway to define the structures which are involved. In this regard, the reduced extent of hemagglutination with protease-treated RBC as compared with untreated RBC (Fig. 1B) is consistent with structures which occur on membrane glycoproteins rather than glycolipids.

Previous results (6, 20) have shown that most human strains of *A. viscosus* and *A. naeslundii* display a surface-associated, lectin-like activity which mediates lactose-inhibitable interactions of these bacteria with certain oral streptococci. The present findings show that these actinomycete strains also cause lactose-reversible hemagglutination reactions with neuraminidase-treated RBC. Recent results (J. Cisar, unpublished data) indicate that these interactions with streptococci and RBC are mediated by fimbriae (7) on the actinomycetes, and studies are in progress to characterize the lactose-inhibitable binding sites on these structures. The possibility that lectin-like sites contribute to adherence of the actinomycetes provides additional support for the idea that interactions of a lectin-carbohydrate type represent a significant mechanism for the attachment of microorganisms to mammalian cell surfaces (25). A better understanding of these lectin-receptor interactions may suggest new and useful approaches for the control of certain bacterial infections.

ACKNOWLEDGMENTS

We thank Ann Sandberg (National Institute of Dental Research) for her helpful suggestions and Shelley Berg for her excellent assistance.

This work was supported in part by Public Health Service grants DE-04201 and AI-07241 from the National Insititutes of Health. Human α_1-acid glycoprotein was provided by the American Red Cross Fractionation Center with the partial support of Public Health Service grant HL 13881 from the National Institutes of Health.

LITERATURE CITED

1. **Ashwell, G., and A. G. Morell.** 1974. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Adv. Enzymol. **41**:99–128.
2. **Ashwell, G., and A. G. Morell.** 1977. Membrane glycoproteins and recognition phenomena. Trends Biochem. Sci. **2**:76–78.
3. **Baumgartner, H. R., and G. V. R. Born.** 1968. Effects of 5-hydroxytryptamine on platelet aggregation. Nature (London) **218**:137–141.
4. **Bowden, G. H., and J. M. Hardie.** 1973. Commensal and pathogenic *Actinomyces* species in man, p. 277–299.

In G. Sykes and F. A. Skinner (ed.), Actinomycetales: characteristics and practical importance. Society for Applied Bacteriology Symposium Series no. 2. Academic Press Inc., London.
5. **Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari.** 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. **20:**150–154.
6. **Cisar, J. O., P. E. Kolenbrander, and F. C. McIntire.** 1979. The specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. Infect. Immun. **24:** 742–752.
7. **Cisar, J. O., and A. E. Vatter.** 1979. Surface fibrils (fimbriae) of *Actinomyces viscosus* T14V. Infect. Immun. **24:**523–531.
8. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121:**404–427.
9. **Duguid, J. P., and R. R. Gillies.** 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. **74:**397–411.
10. **Ellen, R. P.** 1976. Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect. Immun. **14:**1119–1124.
11. **Ellen, R. P., D. L. Walker, and K. H. Chan.** 1978. Association of long surface appendages with adherence-related functions of the gram-positive species *Actinomyces naeslundii*. J. Bacteriol. **134:**1171–1175.
12. **Eshdat, Y., I. Ofck, Y. Yashauv-Gan, N. Sharon, and D. Mirelman.** 1978. Isolation of a mannose-specific lectin from *Escherichia coli* and its role in the adherence of the bacteria to epithelial cells. Biochem. Biophys. Res. Commun. **85:**1551–1559.
13. **Friedenreich, V.** 1930. The Thomsen hemagglutination phenomenon. Production of a specific receptor quality in red cell corpuscles by bacterial activity. Levin Munksgaard, Copenhagen.
14. **Gibbons, R. J., and J. V. Qureshi.** 1976. Interactions of *Streptococcus mutans* and other oral bacteria with blood group reactive substances, p. 163–181. *In* H. Stiles, W. Loesche, and T. O'Brien (ed.), Microbial aspects of dental caries, vol. 1. Information Retrieval, Inc., Washington, D.C.
15. **Jones, G. W., and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. **14:**240–245.
16. **Elenk, E., and G. Uhlenbruck.** 1960. Uber neuraminsaurehaltige Mucoide aus Menschenerythrocytenstroma, ein Beitrag zur Chemie der Agglutinogene. Hoppe-Seyler's Z. Physiol. Chem. **319:**151–160.
17. **Leach, S. A., and T. H. Melville.** 1970. Investigation of some human oral organisms capable of releasing the carbohydrates from salivary glycoproteins. Arch. Oral Biol. **15:**87–88.
18. **Lotan, R., E. Skuteisky, D. Danon, and N. Sharon.** 1975. The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). J. Biol. Chem. **250:**8518–8523.
19. **Marchesi, V. T., T. W. Tillack, R. L. Jackson, J. P. Segrest, and R. E. Scott.** 1972. Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane. Proc. Natl. Acad. Sci. U.S.A. **69:**1445–1449.
20. **McIntire, F. C., A. E. Vatter, J. Baros, and J. Arnold.** 1978. Mechanism of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34. Infect. Immun. **21:**978–988.
21. **Miller, C. A., P. Wang, and M. Flashner.** 1978. Mechanism of *Arthrobacter sialophilus* neuraminidase: the binding of substrates and transition-state analogs. Biochem. Biophys. Res. Commun. **83:**1479–1487.
22. **Morrell, A. G., C. J. A. VanDen Hamer, I. H. Scheinberg, and G. Ashwell.** 1966. Physical and chemical studies on ceruloplasmin. IV. Preparation of radioactive, sialic acid-free ceruloplasmin labeled with tritium on terminal D-galactose residues. J. Biol. Chem. **241:** 3745–3749.
23. **Muller, H. E.** 1974. Neuraminidases of bacteria and protozoa and their pathogenetic role. Behring Inst. Mitt. **55:**34–56.
24. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. Infect. Immun. **22:**247–254.
25. **Ofek, I., E. H. Beachey, and N. Sharon.** 1978. Surface sugars of animal cells as determinants of recognition in bacterial adherence. Trends Biochem. Sci. **3:**159–160.
26. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature (London) **265:**623–625.
27. **Pardoe, G. I.** 1974. The inducible neuraminidases of pathogenic microorganisms. Behring Inst. Mitt. **55:**103–122.
28. **Parker, T. L., A. P. Corfield, R. W. Veh, and R. Schauer.** 1977. Immobilized *Clostridium perfringens* neuraminidase substrate cleavage and enzyme release during incubation. Hoppe-Seyler's Z. Physiol. Chem. **358:**789–795.
29. **Perlitsh, M. J., and I. Glickman.** 1966. Salivary neuraminidase: I. The presence of neuraminidase in human saliva. J. Periodontol. **37:**368–373.
30. **Rauvala, H., and J. Finne.** 1979. Structural similarity of the terminal carbohydrate sequences of glycoproteins and glycolipids. FEBS Lett. **97:**7–8.
31. **Rolla, G., and M. Kilian.** 1977. Haemagglutination activity of plaque-forming bacteria. Caries Res. **11:**85–89.
32. **Sadler, J. E., J. C. Paulson, and R. L. Hill.** 1979. The role of sialic acid in the expression of human MN blood group antigens. J. Biol. Chem. **254:**2112–2119.
33. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type I *Escherichia coli* pili. J. Exp. Med. **146:**1169–1181.
34. **Salit, I. E., and E. C. Gotschlich.** 1977. Type I *Escherichia coli* pili: characterization of binding to monkey kidney cells. J. Exp. Med. **146:**1182–1194.
35. **Tsai, C.-M., D. A. Zopf, and V. Ginsburg.** 1978. The molecular basis for cold agglutination: effect of receptor density upon thermal amplitude of a cold agglutinin. Biochem. Biophys. Res. Commun. **80:**905–910.
36. **Tsai, C.-M., D. A. Zopf, R. K. Yu, R. Wistar, Jr., and V. Ginsburg.** 1977. A Waldenstrom macroglobulin that is both a cold agglutinin and a cryoglobulin because it binds N-acetylneuraminosyl residues. Proc. Natl. Acad. Sci. U.S.A. **74:**4591–4594.
37. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234:**1971–1975.