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Adherence of *Streptococcus sanguis* to Saliva-Coated Hydroxyapatite: Evidence for Two Binding Sites

E. JANE MORRIS AND BARRY C. McBRIDE*

Departments of Microbiology and Oral Biology, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada

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The characteristics of bacterial adherence to saliva-coated hydroxyapatite were examined for a salivary aggregating strain of *Streptococcus sanguis*, strain 12, and for its nonaggregating variant, strain 12na. Both strains were found to adhere in similar numbers to saliva-coated hydroxyapatite that had been preincubated at 4°C overnight. Preincubation of saliva-coated hydroxyapatite overnight at 37°C reduced subsequent adherence of *S. sanguis* 12 by approximately 10%, whereas adherence of *S. sanguis* 12na was reduced by over 80%. Preincubation at 37°C in the presence of neuraminidase reduced adherence of *S. sanguis* 12 by over 90% and caused some additional reduction in adherence of *S. sanguis* 12na. The data were analyzed with Langmuir isotherms, Scatchard plots, and Hill plots. Some evidence of cooperativity was seen. A peak in the Scatchard plot for *S. sanguis* 12 binding to saliva-coated hydroxyapatite preincubated at 4°C disappeared after preincubation at 37°C, suggesting the loss of a salivary receptor. Many more organisms were found to bind when adherence was measured by assays counting the number of organisms remaining in suspension after the beads had settled. These weakly binding organisms, which were removed by washing, demonstrated adherence characteristics similar to those of the firmly bound organisms. Both strains were strongly hydrophobic. It is proposed that the binding of *S. sanguis* 12 and 12na involves two types of receptor on the salivary pellicle. One type of receptor is stable at 37°C, but sensitive to neuraminidase; the second type is inactivated by prolonged incubation at 37°C. *S. sanguis* 12 may bind to both types of receptor, whereas *S. sanguis* 12na binds only to the second type. The neuraminidase-sensitive receptor might be involved in saliva-mediated aggregation.

Streptococcus sanguis binds to the tooth surface in the oral cavity, forming an important component of human dental plaque (8, 21). Adherence is believed to be mediated by salivary glycoproteins, which form a pellicle coating the tooth enamel (1, 9). Ionic, hydrophobic, and lectin-like interactions have been proposed as binding mechanisms holding the bacteria to the pellicle. One type of lectin-like interaction appears to involve the recognition by the bacteria of specific carbohydrate sequences carrying terminal sialic acid residues (14). Adherence is affected by treatment of saliva-coated hydroxyapatite (S-HA) with neuraminidase (4, 20; E. J. Morris, S. Crowley, and B. C. McBride, J. Dent. Res. [Special Issue] **62**:A649, 1983) and is inhibited by sialic acid-containing compounds (W. F. Liljemark, C. G. Bloomquist, and L. J. Fenner, J. Dent. Res. [Special Issue] **62**:A791, 1983). Recently, it was shown that *S. sanguis* can also interact specifically with a proline-rich salivary glycoprotein that is not affected by neuraminidase treatment (M. W. Stinson, R. P. Dittmer, M. J. Levine, L. A. Tabak, A. Prakobphol, P. A. Murray, and M. S. Reddy, J. Dent. Res. [Special Issue] **62**:A960, 1983). Gibbons et al. (5) have indicated that S-HA possesses multiple binding sites with various affinities for *S. sanguis*. Doyle and co-workers (3, 17) have demonstrated positive cooperativity between binding sites for *S. sanguis* and have suggested that binding is initiated by ionic or lectin-like bonds that are stabilized by hydrophobic forces. Gibbons et al. (R. J. Gibbons, E. C. Moreno, and I. Etherden, J. Dent. Res. [Special Issue] **62**:A790, 1983) have provided some evidence that the neuraminidase-sensitive binding sites are of high affinity and are saturated at low cell densities, whereas lower-affinity hydrophobic binding sites are occupied only at higher cell densities.

For each salivary receptor there must be a complementary bacterial adhesin, although little is known of the nature or variety of such components. Hogg and Embery (10) have proposed that lipoteichoic acid associated with bacterial surface fibrils may bind salivary glycoproteins. Liljemark and Bloomquist (12) have isolated a streptococcal cell surface protein that blocks adherence to S-HA. Certain strains of *S. sanguis* also form aggregates in the presence of saliva (7) by means of adhesins with specificity for sialic acid (11, 13), and cell surface proteins that show this specificity have been demonstrated in *S. sanguis* (14) and *Streptococcus mitior* (P. A. Murray, M. J. Levine, L. A. Tabak, and M. S. Reddy, J. Dent. Res. [Special Issue] **62**:A789, 1983). Although there are some characteristic differences between saliva-dependent aggregation and adherence (19), it is possible that the reaction responsible for aggregation may be one of a number of types of interaction that bind *S. sanguis* to S-HA.

This paper provides strong evidence for the presence of at least two types of specific interaction between *S. sanguis* and S-HA through the comparison of an aggregating strain of *S. sanguis* and a nonaggregating variant with certain altered adherence characteristics.

MATERIALS AND METHODS

Bacteria. *S. sanguis* 12, which shows strong neuraminidase-sensitive aggregation in saliva, and its variant 12na, which has lost the ability to aggregate with saliva, have been described previously (13). Cells were grown overnight in

* Corresponding author.

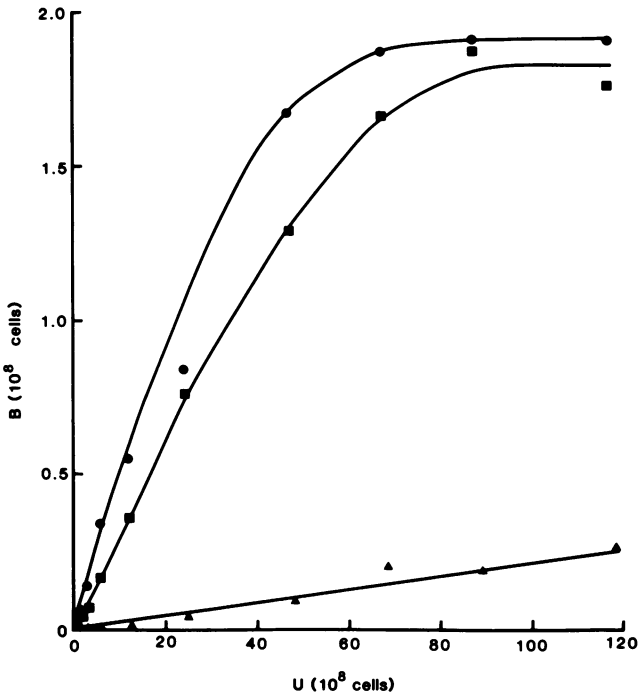

FIG. 1. Binding isotherms for adherence of *S. sanguis* 12 to S-HA. Symbols: ●, S-HA preincubated overnight at 4°C; ■, S-HA preincubated at 37°C; ▲, S-HA preincubated at 37°C with neuraminidase.

Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with yeast extract.

For adherence assays cells were radiolabeled by the addition of 1 or 2 μCi of [*methyl*-1',2',-^{3}H]thymidine (The Radiochemical Centre, Amersham, England) per ml to the culture medium. The bacteria were harvested by centrifugation and washed four times in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2). The cells were sonicated briefly (1 min) to break chains and examined microscopically to ensure that only singles and pairs were present. The bacteria were then washed twice more in HEPES buffer and suspended as required in HEPES for adherence assays. Cell densities were determined microscopically with a Petroff-Hauser counting chamber.

S-HA. Parafilm-stimulated saliva, pooled from a number of donors, was collected at 0°C and clarified by centrifugation at 20,000 × *g* for 10 min. The saliva was then heated at 60°C for 30 min to destroy endogenous enzymes; where stated below the saliva was treated at 100°C for an additional 15 min. The saliva was stored at −20°C until required. A single batch of saliva was used for all experiments described here.

Spheroidal hydroxyapatite beads (BDH Chemicals, Ltd., Poole, England) were washed extensively in water to remove fine particles and dried in a hot oven before use. Samples (40 mg) were weighed into glass scintillation vials, and saliva (0.5 ml) was added. The mixture was shaken gently at room temperature for 2 h and then placed at 4°C overnight before use.

For examination of the effects of neuraminidase on adherence, the pH of the saliva-hydroxyapatite mixture was reduced to pH 5 by the addition of 45 μl of 0.25 N HCl; 10 μl of 0.5% sodium azide was also added to prevent microbial growth during incubation. Neuraminidase (Sigma Chemical Co., St. Louis, Mo.; type VI from *Clostridium perfringens*) was dissolved in 0.05 M acetate buffer (pH 5.0) at a concentration of 50 μg/ml, and 10 μl of the solution was added to the vial. The mixture was then incubated overnight at 37°C. Controls were treated identically, except that neuraminidase was omitted.

After overnight incubation at 4 or 37°C the S-HA was washed twice with 10 ml of distilled water to remove excess saliva and once with 10 ml of HEPES buffer. Excess liquid was removed by aspiration.

In cases where it was necessary to correct for the number of bacteria adhering to the glass vial (indirect method, see below), an empty vial was coated with saliva and treated identically to vials containing HA.

Bacterial adherence to S-HA. The bacterial suspension (1 ml) was added to each sample of S-HA and shaken gently on a platform shaker for 2 h. Unattached bacteria were then removed by aspiration followed by three washes of the bacteria-S-HA complex in 10 ml of HEPES buffer. The beads were rinsed into clean plastic scintillation vials. Excess buffer was aspirated, and the beads were dried overnight at 60°C. Scintillation fluid (5 ml) was then added, and the radioactivity was monitored in a Tracor Analytic liquid scintillation counter to determine numbers of attached bacteria (*B*). Counts were corrected for quenching due to the S-HA. The percent quench (approximately 20%) was determined by counting a known quantity of radiolabeled bacteria in the presence and absence of S-HA. Cells bound to the

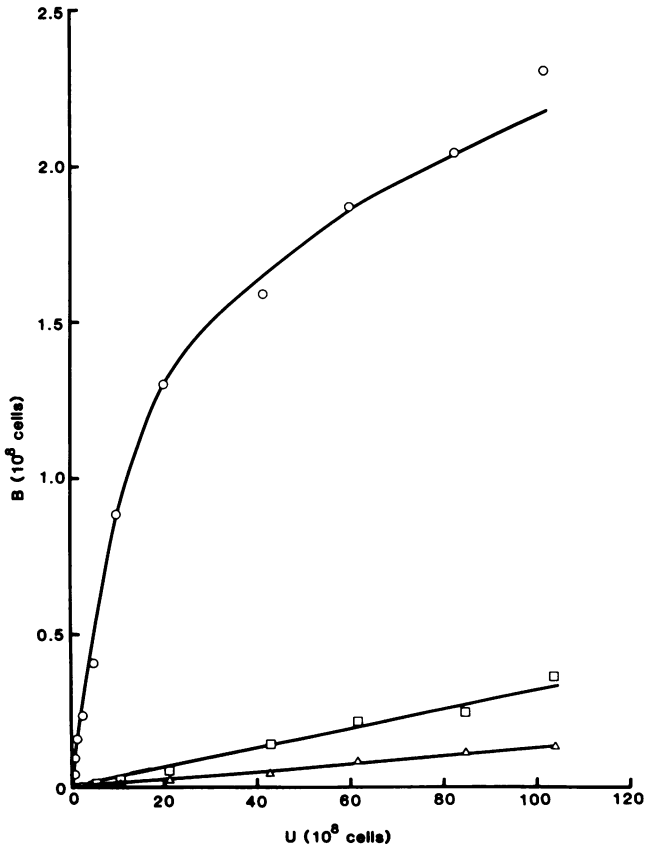

FIG. 2. Binding isotherms for adherence of *S. sanguis* 12na to S-HA. Symbols: ○, S-HA preincubated overnight at 4°C; □, S-HA preincubated at 37°C; △, S-HA preincubated at 37°C with neuraminidase.

glass vial represented only a very small percentage of the total cells added and did not alter the numbers of unbound cells sufficiently to modify the affinity constant (K) or the number of binding sites (N). Bacteria unbound at equilibrium (U) were taken as the cells added minus B.

When adherence was measured by the "indirect" method, duplicate 50-μl samples were removed from the cell suspension immediately after the addition of the bacteria and at various time intervals thereafter. In this case, bacteria adhering to S-HA (B) were determined as the loss of radioactive counts from the cell suspension after correcting for bacteria adhering to empty saliva-coated vials.

Determination of bacterial hydrophobicity. Hydrophobicity of *S. sanguis* was measured by the extent of binding to hexadecane as described by Olsson and Westergren (18), except that the cells were suspended in 0.05 M HEPES buffer (pH 7.2).

Assay for glycosidases in saliva. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide,*p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-α-D-galactoside, *o*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-α-D-mannoside, and *p*-nitrophenyl-α-L-fucoside were obtained from Sigma. Substrates were added to saliva to give a 10 mM solution, and the saliva was incubated overnight at 37°C as described above. After the addition of an equal volume of 0.4 M glycine buffer (pH 10.5), liberated *o*-nitrophenol or *p*-nitrophenol was determined by the increase in absorbance at 430 nm relative to a control containing no saliva.

RESULTS

Adherence to S-HA. The saliva-aggregating strain *S. sanguis* 12 and the nonaggregating variant 12na were tested for their ability to adhere to S-HA beads that had been incubated overnight at 4°C before adding the bacteria. Similar numbers of both strains were found adhering to the beads (Fig. 1 and 2), suggesting that adhesin-receptor complexes that govern adherence are different from those mediating aggregation.

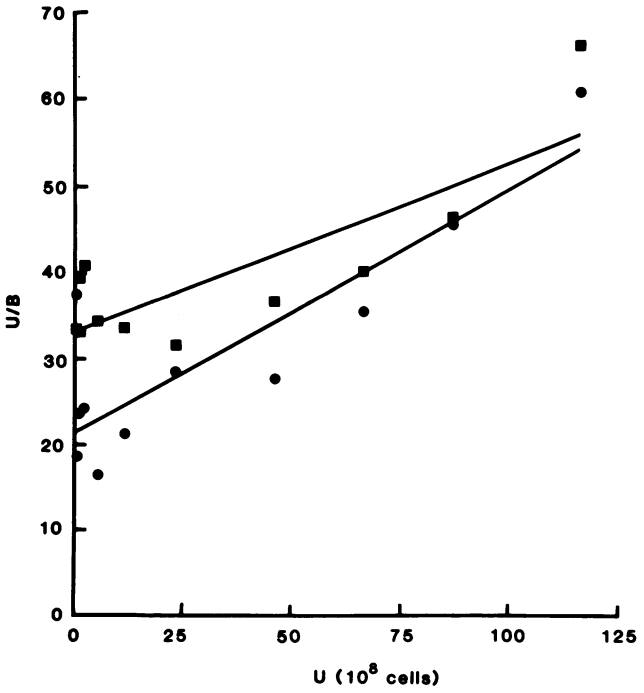

FIG. 3. Langmuir isotherms for adherence of *S. sanguis* 12 to S-HA preincubated at 4 or 37°C. Symbols as in Fig. 1.

TABLE 1. Langmuir isotherm parameters for the adsorption of *S. sanguis* to S-HA[a]

S. sanguis strain	S-HA preincubation temp (°C)	N	K	r
12	4	3.54×10^{8}	1.31×10^{-10}	0.87
12	37	5.06×10^{8}	5.98×10^{-11}	0.82
12na	4	2.72×10^{8}	4.18×10^{-10}	0.99

[a] N, Number of binding sites; K, association constant; r, correlation coefficient.

After overnight incubation of the S-HA at 37°C, *S. sanguis* 12 showed a slight reduction in adherence (Fig. 1). However, the binding of 12na to S-HA treated in the same way was reduced by over 80% (Fig. 2). It appeared that prolonged incubation of the S-HA at 37°C caused the alteration or destruction of receptor sites that were essential for the binding of strain 12na, but not for strain 12. The nature of the alteration in salivary receptors was not known, but it was thought possible that residual enzyme activity in the saliva pretreated at 60°C for 30 min might cause such changes. However, when the saliva was given a second heat treatment at 100°C for 15 min before adding it to the HA and incubating overnight at 37°C, differences in the adherence of 12 and 12na were only slightly less marked than before.

Overnight incubation of the S-HA at 37°C in the presence of neuraminidase reduced the binding of 12 to a level comparable with that found for the adherence of 12na to S-HA incubated at 37°C in the absence of neuraminidase (Fig. 1). The adherence of 12na to S-HA after neuraminidase treatment was reduced still further (Fig. 2). Unlike the findings of Gibbons et al. (J. Dent. Res. **62**:A790), neuraminidase treatment affected adherence throughout the range of cell concentrations tested.

The data for the adherence of the bacteria to S-HA were replotted in the form of the Langmuir adsorption isotherm (6): $(U/B) = [(U/N) + (1/KN)]$ where K is the affinity constant (milliliters per cell) and N represents the total number of binding sites per 40 mg of HA. Figure 3 shows Langmuir isotherms for *S. sanguis* 12 binding to S-HA incubated overnight at 4 or 37°C. Data for adherence to neuraminidase-treated S-HA are not included since the numbers of cells binding were too low to give meaningful results. In both cases there appeared to be an upward swing in the curve at the lower end, indicative of positive cooperativity (J. Benbasat, personal communication). Parameters calculated from the Langmuir isotherms are given in Table 1. The correlation coefficients for *S. sanguis* 12 were low, probably reflecting the complexity of the interactions, since the model assumes that all binding sites on the S-HA are identical. Although the data in Table 1 indicate that overnight incubation of S-HA at 37°C apparently causes some increase in the number of binding sites while reducing the association constant, the significance of these values is doubtful in view of the observed poor correlation. There was an apparent break in the Langmuir isotherm at a value of U between 25×10^{8} and 45×10^{8} cells per ml for adherence to S-HA incubated overnight at 4°C. This might reflect concentration-dependent multiple binding sites as described by

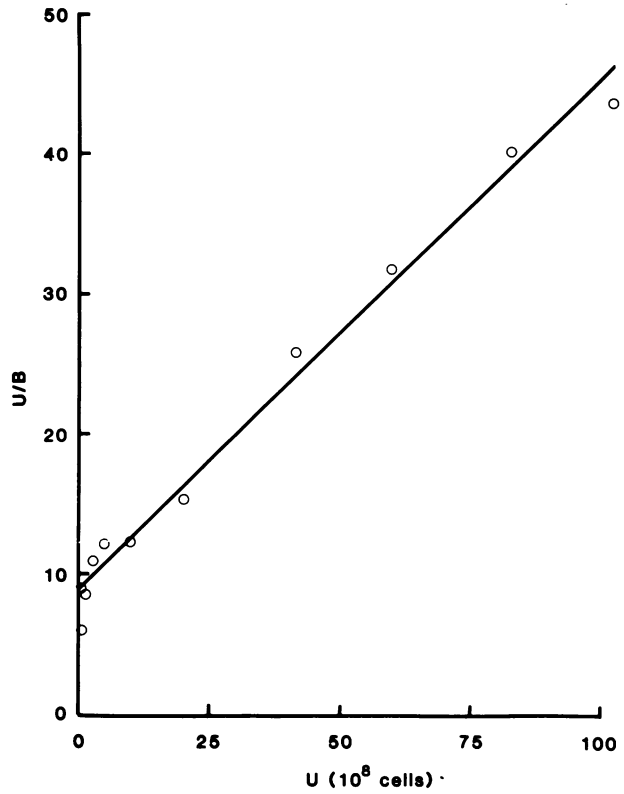

FIG. 4. Langmuir isotherm for adherence of *S. sanguis* 12na to S-HA preincubated at 4°C.

Gibbons et al. (5). However, it may be an artifact produced by the general scatter of data, particularly since the data at the lower values of *U* did not fit a straight line. No obvious break was seen in the case of adherence to S-HA incubated at 37°C.

When the data for the binding of *S. sanguis* 12na to S-HA incubated overnight at 4°C are plotted in terms of the Langmuir equation (Fig. 4), the graph shows little evidence of any upward curve at the lower end. The correlation coefficient is considerably higher than for *S. sanguis* 12, possibly indicating that fewer types of interaction are involved. The number of bacterial binding sites on the S-HA is slightly less than for *S. sanguis* 12, although the association constant is higher (Table 1).

Analysis of the data in the form of a Scatchard plot (17) reveals a graph with two peaks for the adherence of *S. sanguis* 12 to S-HA incubated overnight at 4°C (Fig. 5). A maximum in the curve indicates positive cooperativity (2, 17), the cooperativity being more pronounced as the position of this maximum shifts along the abscissa to high degrees of saturation. The significance of the first peak is unknown since errors in determination of B are greater when B is small. However, we have noticed this peak in a number of experiments with *S. sanguis* 12. A double-positive slope (Fig. 5) may be interpreted in terms of alternating positive-negative cooperativity (R. J. Doyle, personal communication). Possibly the complexity of the interactions is such that there could be more than one type of cooperativity involved. After overnight incubation of the S-HA at 37°C, the second peak disappears (Fig. 5), suggesting the loss of at least one type of salivary receptor. Possibly this is the same receptor responsible for binding 12na to S-HA preincubated at 4°C. A Scatchard plot for the binding of *S. sanguis* 12na to S-HA incubated overnight at 4°C shows a single maximum slightly to the right of the first peak seen with strain 12 (Fig. 6). However, the existence of this peak is indicated only by the results for binding at the lowest cell concentration; the remainder of the graph shows a concave curve indicative of site heterogeneity or negative cooperativity.

Finally, the data were examined in the form of the Hill plot (2) (Fig. 7 and 8). The slope of the Hill plot should provide an indication of cooperativity. The slope is unity for independent, noninteracting sites; positive cooperativity is indicated by a slope of greater than unity, whereas a slope of less than unity may indicate negative cooperativity or binding site heterogeneity. The Hill plot requires a knowledge of the total number of binding sites (*N*). When values assumed for this

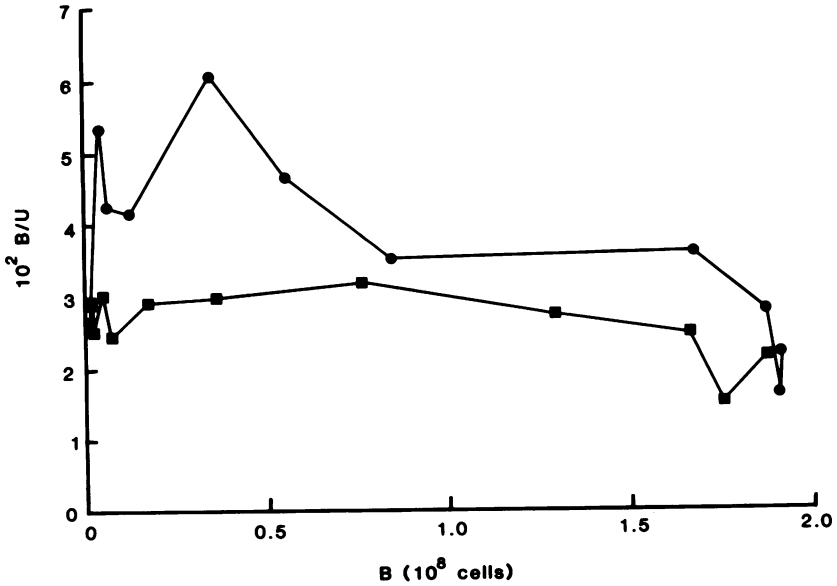

FIG. 5. Scatchard plots for adherence of *S. sanguis* 12 to S-HA preincubated at 4 or 37°C. Symbols as in Fig. 1.

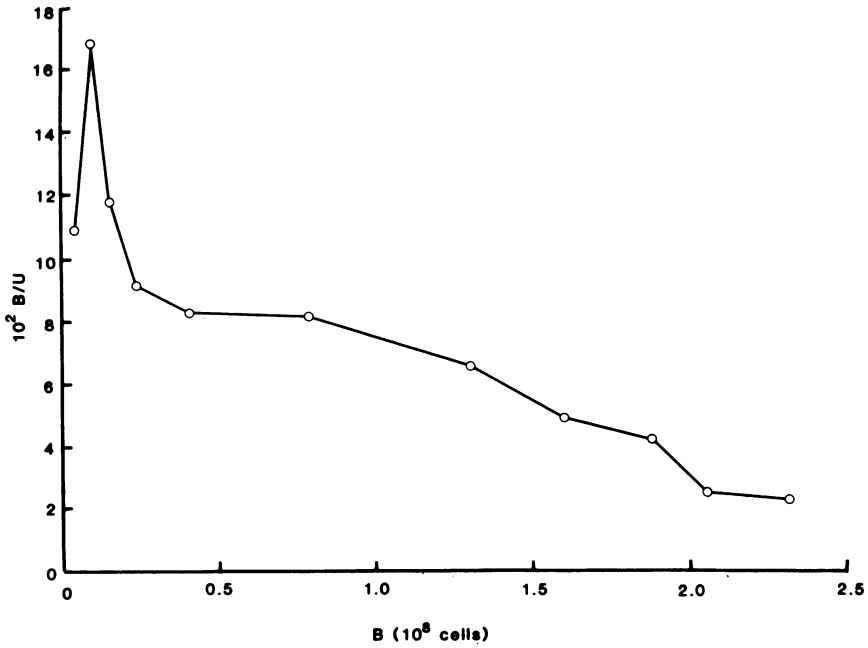

FIG. 6. Scatchard plot for adherence of *S. sanguis* 12na to S-HA preincubated at 4°C.

parameter were those calculated from the Langmuir isotherm (Table 1), Hill coefficients obtained were in all cases less than unity (Fig. 7 and 8, Table 2). However, these coefficients represent composite values of *N* that comprise both high- and low-affinity binding sites. Values for *N* applicable to the binding site(s) involved in cooperativity may be obtained by extrapolating the negative slope of the Scatchard plots to zero (Fig. 5 and 6) (17). Hill coefficients calculated by using these values of *N* and omitting higher values of *B* (those not involved in cooperativity) were substantially greater than unity (Table 2).

Determination of bacterial adherence by the indirect binding assay. Comparisons were made between the extent of adherence as measured by the direct or indirect binding assay by using *S. sanguis* 12 and 12na as well as a number of other strains of *S. sanguis* and *Streptococcus salivarius* HB

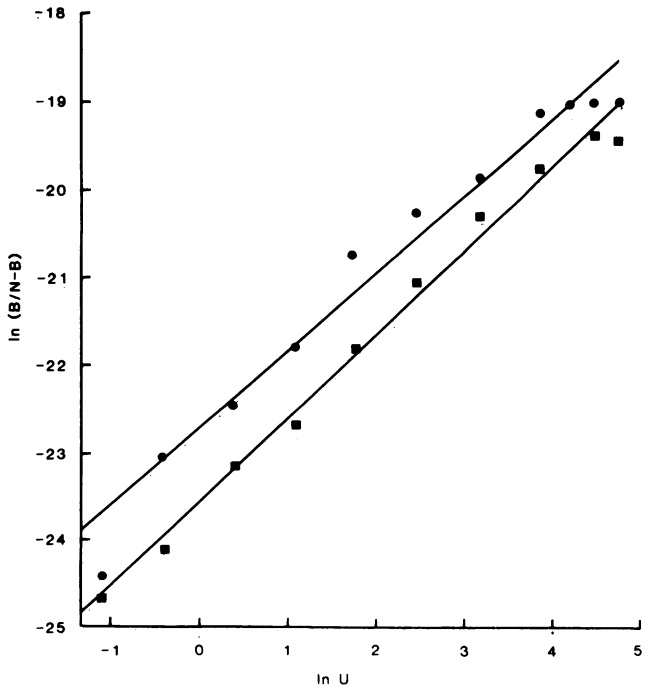

FIG. 7. Hill plots for adherence of *S. sanguis* 12 to S-HA preincubated at 4 or 37°C. Symbols as in Fig. 1.

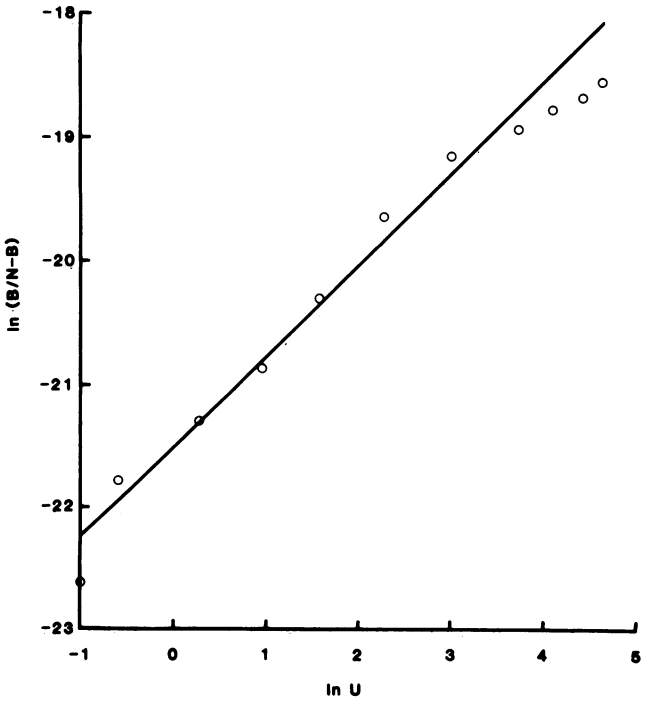

FIG. 8. Hill plot for adherence of *S. sanguis* 12na to S-HA preincubated at 4°C.

TABLE 2. Hill coefficients for the adsorption of *S. sanguis* to S-HA[a]

S. sanguis strain	S-HA preincubation temp (°C)	n_h	r
12	4	0.95[b]	0.99
		1.22[c]	0.99
12	37	0.90[b]	0.98
12na	4	0.68[b]	0.99
		1.22[c]	0.98

[a] n_h, Hill coefficient, r, correlation coefficient.
[b] N obtained from Langmuir isotherm.
[c] N obtained from Scatchard plot.

and HB-7 (Table 3). In the indirect procedure, binding is determined by measuring the number of cells remaining in suspension after the S-HA beads have settled and comparing this number with the number of cells in suspension in a control without S-HA. A cell density of approximately 5×10^9 cells per ml was used for these experiments. Most strains of *S. sanguis* consistently showed a higher number of cells bound with the indirect method than with the direct method. This suggests that cells that adhere only weakly to the S-HA might be removed during the washing of the beads and so be taken as nonadherent with the direct method. *S. salivarius* HB did not apparently show these weak interactions, since the two methods gave similar results. This confirms the findings of Weerkamp and McBride (22). As expected, the nonadhering mutant *S. salivarius* HB-7 showed no attachment with either method.

Figure 9 demonstrates the time course of adherence of *S. sanguis* 12 to S-HA as measured by the indirect method and the effects of overnight incubation of S-HA at 37°C with or without neuraminidase on this adherence. The results are similar to those obtained by the direct method, suggesting that although the extent of binding is apparently higher, the types of interactions involved are similar to those measured directly.

Bacterial hydrophobicity. Both *S. sanguis* 12 and 12na and *S. salivarius* HB and HB-7 were found to be strongly hydrophobic when measured by their ability to bind to hexadecane. The optical density of the aqueous phase was reduced by 91 and 82% for *S. sanguis* 12 and 12na, respectively, and by 99% for both strains of *S. salivarius*.

Glycosidases in saliva. α-Mannosidase activity was found in saliva treated at 60°C for 30 min, but none of the other glycosides tested was hydrolyzed. No glycosidase activity was found in saliva heated at 100°C for 15 min.

TABLE 3. Comparison of bacterial adherence to S-HA by the direct and indirect methods

Bacteria	Cells ($\times 10^8$) adhering to 40 mg of S-HA	
	Direct	Indirect
S. sanguis		
12	1.2	8.3
12na	1.4	6.7
J3	3.0	10.7
J8	4.7	12.6
J9	1.7	1.8
S. salivarius		
HB	2.3	2.7
HB-7	0.6	0

DISCUSSION

The fact that the binding of *S. sanguis* 12 and 12na to S-HA is affected in different ways by preincubation at 37°C points to the existence of at least two types of specific interaction with salivary receptors. One type of receptor is inactivated by prolonged incubation at 37°C (although the mechanism of this inactivation is unknown), whereas the other type is stable at 37°C, but inactivated by neuraminidase. The differences between strains 12 and 12na could be explained on the basis that 12na (which is also defective in salivary aggregation) lacks an adhesin to bind to the receptor that is stable to incubation at 37°C. Strain 12, on the other hand, carries adhesins specific for both types of receptor. Figure 10 explains the observed results for adherence to S-HA after incubation at 37°C and neuraminidase treatment in terms of this model.

It is likely that the salivary receptor that is stable at 37°C involves sialic acid, but the nature of the receptor that is inactivated after 37°C incubation is unknown. Murray et al. (14) found inhibition of *S. sanguis* hemagglutination with both sialic acid (*N*-acetylneuraminic acid) and galactose and suggested that the bacteria might contain separate carbohydrate-binding components, one with specificity for sialic acid and the other with specificity for galactose. Nagata et al. (15) have isolated a galactose-binding component from the surface of *S. sanguis*. However, it was thought more likely by Murray et al. (14) that a single bacterial component was involved, with specificity toward an oligosaccharide structure containing both *N*-acetylneuraminic acid and galactose. In the present instance, it is not known whether the two salivary receptor sites are totally distinct, or alternately whether both might involve a sequence resembling the *N*-acetylneuraminic acid–α2,3-galactose–β1,3-*N*-acetylgalactosamine trisaccharide recognition site as proposed by Murray

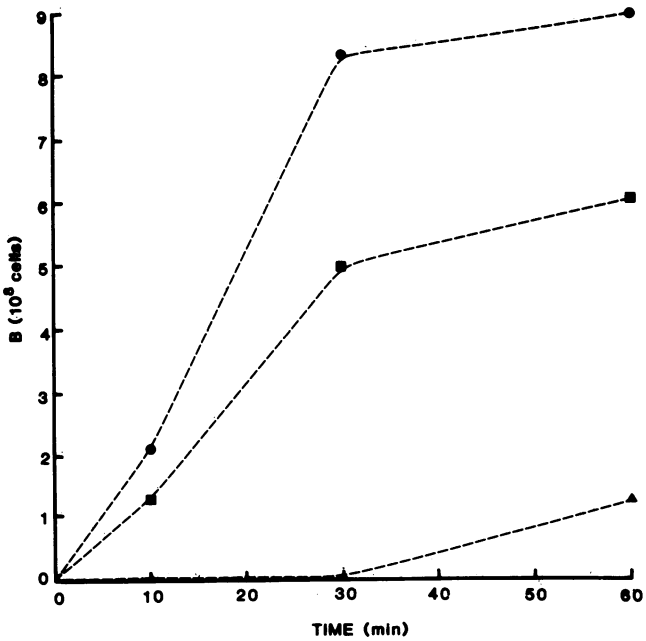

FIG. 9. Time course of adherence of *S. sanguis* 12 to S-HA (indirect method). Symbols as in Fig. 1.

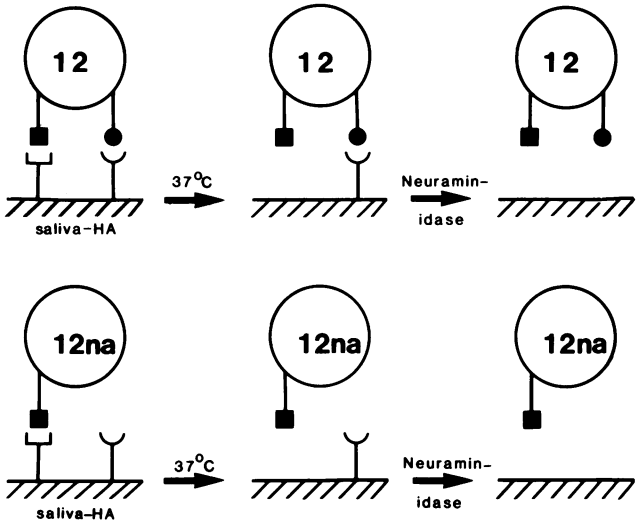

FIG. 10. Model for the adherence of *S. sanguis* 12 and 12na to S-HA.

et al. (14). In this case, an alternative model could be postulated on the assumption that proteins in the salivary pellicle would possess both this trisaccharide and the disaccharide galactose–β1,3-*N*-acetylgalactosamine from which the terminal sialic acid has been omitted. In this model *S. sanguis* 12 would have a single type of adhesin with specificity for the trisaccharide and possibly also for the disaccharide. *S. sanguis* 12na would have an altered adhesin that had lost the ability to bind to the trisaccharide, but bound strongly to the disaccharide. If the disaccharide was inactivated by incubation at 37°C (e.g., through the action of a β-galactosidase, although no such enzyme activity was found in our saliva), then binding sites for *S. sanguis* 12na would be destroyed. *S. sanguis* 12 could still bind through the trisaccharide, but cooperative interactions involving the disaccharide would be eliminated. Removal of sialic acid from the trisaccharide through neuraminidase treatment would expose the disaccharide. This would be subsequently inactivated during the 37°C incubation, eliminating binding sites for *S. sanguis* 12. This model could help to explain why some strains of *S. sanguis* show enhanced adherence after neuraminidase treatment (4). If sialic acid were removed without concomitant destruction of the uncovered disaccharide (possibly through the use of a shorter incubation time), then an increased number of binding sites for strains similar to 12na would be revealed.

Both strains of *S. sanguis* are strongly hydrophobic, and it is probable that the specific interactions described above are stabilized by hydrophobic bonds (16). Hydrophobic bonds might be particularly important in the case of the additional weak interactions measured by the indirect binding assay.

Differences in affinity of the bacteria for S-HA may be due to variations in the strength of the interaction between particular types of adhesins and receptors. However, the salivary pellicle probably presents a variety of potential bacterial binding sites, with receptors distributed at differing densities over the surface. High-affinity sites would then represent areas of the S-HA where one bacterium could bind to a number of receptors. Sites with fewer receptors would bind bacteria with a lower affinity. On the basis of this model there could be a very large number of binding sites on the S-HA, with progressively lower affinities. As proposed by Gibbons et al. (5), the highest-affinity sites would be preferentially occupied at low cell concentrations, whereas lower-affinity sites would only be occupied at high concentrations. Possibly, binding to the lowest-affinity sites can only be measured with the indirect binding assay, since weakly bound cells would easily be washed off the beads. This might explain why it is often difficult to achieve saturation of *S. sanguis* binding sites in the adherence assay. This is in contrast to the adherence of *S. salivarius* to S-HA (22), where a finite number of binding sites appears to exist. Definite saturation of the system is readily obtained, and no additional weak interactions are seen with the indirect method.

Positive cooperativity in the binding of these strains of *S. sanguis* to S-HA is apparent from the Scatchard plots and from the shape of the Langmuir isotherm for *S. sanguis* 12. In addition Hill coefficients calculated for adherence at the lower cell concentrations are greater than unity, although the binding isotherms are not obviously sigmoidal. It is difficult to understand the physical significance of cooperativity for the binding of bacteria to a surface; presumably the adherence of one cell influences the subsequent binding of others, but this could be achieved either through modification of the pellicle to produce more, or altered, binding sites, or by direct cell-cell interactions whereby bacteria could attach themselves to cells which were already adhering, rather than binding directly to the S-HA. This might be more likely to occur in strains of *S. sanguis* with the ability to self-aggregate or carry out saliva-mediated aggregation, possibly mediated by components of the pellicle. It is conceivable that the saliva is not bound to HA in a very stable fashion. Hence the binding of bacteria, along with physical abrasion during the course of the experiment, might cause the release of bacteria with attached salivary components. If these reattached either directly to the S-HA or to other adhering cells, they would provide a nucleus for the formation of aggregates on the S-HA surface. The complexity of such interactions could be partially responsible for the scatter of data on the Langmuir isotherm of *S. sanguis* 12, in comparison with that of 12na, which does not aggregate in saliva. In addition, the binding of *S. sanguis* 12 shows stronger indications of positive cooperativity than does that of strain 12na, both in the Langmuir isotherm and Scatchard plots.

It is not certain to what extent aggregation and adherence reactions involve the same bacterial adhesins. It is tempting to equate the adhesin proposed to be lacking or altered on *S. sanguis* 12na with the absence of salivary aggregation with this strain. Thus the ability to bind to a carbohydrate sequence possessing a terminal sialic acid residue would be a prerequisite for saliva-mediated aggregation to occur. However, some of the strains examined by Murray et al. (14) that were postulated to possess a lectin with specificity for sialic acid did not aggregate in saliva. Possibly there is a range in the proportions of different adhesins in different strains. A high proportion of sialic acid binding lectins might be required for the extensive cross-linking reactions involved in aggregation, or alternatively there are distinct sialic acid lectins responsible for binding and aggregation.

The role of the 37°C-sensitive salivary receptor is uncertain since it may be inactivated in the oral cavity. Nevertheless this study demonstrates clearly the complexity of the interactions between *S. sanguis* and saliva-coated surfaces. Although previous work (5) has indicated the presence of multiple binding sites, it has proved difficult to determine the nature of these sites. The use of variants of *S. sanguis* deficient in certain adherence capabilities, as described

above, provides an important tool for differentiating between individual adhesins and their salivary receptors. It is believed that the results presented here provide strong evidence for multiple interactions between *S. sanguis* and the salivary pellicle.

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