Inhibitory substances produced by *Streptococcus salivarius* and colonization of the upper respiratory tract with group A streptococci

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SUMMARY

It has been proposed that inhibitory substances produced by viridans streptococci colonizing the upper respiratory tract aid in eradication of established group A streptococcal colonization of that site. We studied the prevalence of inhibitory-substance producing strains of *Streptococcus salivarius* in throat cultures from three groups of children: 16 children with persistently positive throat cultures for group A streptococci despite receiving recommended therapeutic courses of antibiotics (group I). 26 children from whom group A streptococci were eradicated from the upper respiratory tract by antibiotic therapy (group II). and 18 children who never harboured group A streptococci in their upper respiratory tract during the study period (group III). An *in vitro* deferred antagonism method was employed to detect inhibitory substances: 5233 strains of *S. salivarius* were examined. Strains of *S. salivarius* producing inhibitory substances were isolated from 76–88% of the children in each group on at least one occasion. However. only a small percentage of subjects in each group harboured strains producing these substances in every throat culture. The mean total percentage of *S. salivarius* strains producing inhibitory substances was 21.8% in children in group I. 22.4% in children in group II. and 16.4% in children in group III: these percentages were not statistically different ($P > 0.1$). In this study. we could not confirm a significant role for inhibitory substances produced by *S. salivarius* in the eradication of group A streptococci from the upper respiratory tract of colonized individuals.

INTRODUCTION

Several studies have reported that strains of viridans streptococci producing substances that inhibit the growth of group A streptococci *in vitro* are more commonly found to colonize the upper respiratory tract of children who seem to

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be 'resistant' to upper respiratory tract colonization with group A streptococci (Crowe, Sanders & Longley, 1973; Gerasimov, 1968; Grahn & Holm, 1983; Sanders, 1969).

In two separate studies, the percentage of viridans streptococci producing inhibitory substances was found to increase in individuals following group A streptococcal colonization of the upper respiratory tract (Crowe, Sanders & Longley, 1973; Grahn & Holm, 1983). Crowe, Sanders & Longley (1973) hypothesized that increased production of inhibitory substances may result from 'selection pressure' exerted by group A streptococcal colonization. They proposed that these substances may inhibit colonization of the upper repiratory tract by group A streptococci as well as aid in the eradication of established group A streptococcal colonization of that site.

We tested this hypothesis during the study of an outbreak of streptococcal pharyngitis in school children caused by a predominant serotype (M-1, T-1) (Smith *et al.* 1988). We compared the prevalence of strains of *Streptococcus salivarius*, a particular species of viridans streptococci, producing inhibitory substances recovered from several groups of children: a group persistently harbouring group A streptococci in the upper respiratory tract despite antibiotic treatment, a group in which group A streptococci colonizing the upper respiratory tract were successfully eradicated by antibiotics, and in a third group of children from whom group A streptococci were never recovered during the study period.

MATERIALS AND METHODS

The study was conducted in a semi-closed community of 75 families where we have conducted previous studies of streptococcal epidemiology (Kaplan, Gastanaduy & Huwe, 1981). One hundred and ninety-two children attended the parochial school on the community campus. During the months of October to December of 1984, an outbreak of group A streptococcal pharyngitis caused by a predominant serotype (M type-1, T agglutination-1) occurred in this community (Smith *et al.* 1988). Symptomatic individuals were examined by a registered nurse living in the community and throat cultures were obtained. In addition, during the month of November, screening throat cultures were obtained on two occasions from the school population because of the increasing incidence of symptomatic pharyngitis associated with positive throat cultures for group A streptococci. Over 60 % of the school-children from nursery school through the ninth grade had positive throat cultures for group A streptococci during the course of this outbreak. Over 90 % of the group A streptococcal isolates were a single serotype (M-1, T-1) (Smith *et al.* 1988). All individuals with an initial positive culture for group A streptococci received a 10-day course of oral penicillin V as recommended by the Committee of the American Heart Association (1977). Informed written consent was obtained from the parents of all children participating in this study.

Using the results of throat cultures obtained during the previously mentioned epidemiologic studies, three groups of children from this population were selected in early January of 1985 for further study: 16 children colonized with group A streptococci who continued to have positive throat cultures of the same serotype 3–5 days after they completed oral penicillin treatment (group I), 26 children

colonized with group A streptococci who had negative throat cultures for group A streptococci 3–5 days after they completed oral penicillin therapy (group II), and 18 healthy control children whose throat cultures were consistently negative for group A streptococci (group III). Children were selected using criteria of age (in order to achieve an equivalent age distribution among the three groups) and family (in order to enroll several groups of siblings).

Twenty-three percent (3 of 16) of the chldren in group I and 42% (11 of 26) of the children in group II were identified when they presented with a sore throat, clinical signs of pharyngitis, and a throat culture positive for group A streptococci. The remaining subjects in these two groups were asymptomatic and were found to be colonized with group A streptococci when a throat culture survey was carried out in the entire school population earlier in the course of the outbreak (November). The children in group III (age-matched controls) were selected from the same school population.

In group I, 6 children had negative throat cultures after a second course of antibiotic, 6 children had negative throat cultures after a third course of antibiotic, and 4 children continued to have positive throat cultures even after completing three full courses of antibiotic therapy.

Culturing

The prevalence of *S. salivarius* strains producing inhibitory substances was determined during the months of January through March of 1985 following the acute phase of the outbreak. Throat cultures were obtained from the three groups each week although each child was not cultured on every occasion. Cultures were not obtained during antibiotic treatment or for a period of 2 weeks after treatment was completed.

Each throat culture was obtained by the same individual using two calcium alginate Type II swabs. One swab was immediately inoculated on a plate containing Tryptose Blood Agar Base and 6% sheep blood. This plate was incubated overnight at 35 °C, and examined at 24 and 48 h for beta-haemolytic streptococci. streptococci were serologically grouped and typed as previously described (Kaplan, Couser & Huwe, 1979).

The second swab was inoculated on a plate containing Mitis-Salivarius Agar. This plate was incubated anaerobically using Gas Pak Anaerobic Systems (BBL, Cockeyville, MD) for 48 h at 35 °C, and examined for the presence of *S. salivarius* colonies. These colonies could be identified by their characteristic large mucoid colony morphology (Facklam, 1977).

Deferred antagonism testing of S. salivarius *strains*
Screening test for inhibitory substances

Colonies of *S. salivarius* were first tested for the production of inhibitory substances using a modification of a screening test described by Tagg *et al.* (1983). Approximately 20 individual colonies (range of 16–24) of *S. salivarius* were selected from each culture plate, then each colony was stabbed in succession in a grid pattern into a growth control plate (Tryptose Blood Agar with 6% sheep blood), and finally into two duplicate plates containing Columbia Blood Agar Base with 5% human blood and 0·5% $CaCO_3$. Calcium carbonate was incorporated

into the media as a buffer since acid production by *S. salivarius* under anaerobic conditions could cause a localized decrease in pH inhibiting the growth of the indicator strains and resulting in a false positive interpretation. Anaerobic incubation was also used to rule out the possibility of non-specific inhibition of the indicator strains because of production of hydrogen peroxide. A positive control, *S. salivarius* strain no. 5 [supplied by Tagg (Dempster & Tagg, 1982)] was also stabbed into each plate. All of the plates were incubated anaerobically for 18 h at 35 °C. The two indicator strains used were the *S. salivarius* strain T-18 used by Tagg *et al.* (1983) and a representative group A streptococcal strain (M-1, T-1) recovered at this time from the study population. These indicator strains were grown overnight in Todd–Hewitt Broth with 2% Neopeptone at 35 °C. A 10^1 dilution of the *Staphylococcus epidermidis* broth culture was made and one ml of this dilution was applied to one of the two duplicates plates. A 10^4 dilution of the group A streptococcal broth culture was made and one ml of this dilution was applied to the other duplicate plate. These plates were incubated aerobically overnight at 35 °C.

Strains of *S. salivarius* producing inhibitory substances produced zones of inhibited growth of the indicator strains (Fig. 1). A positive result was defined as a zone of 3 mm or more in diameter of inhibited growth of either of the indicator strains. Preliminary studies in our laboratory showed that essentially all strains of *S. salivarius* that produced zones of inhibition smaller than 3 mm gave negative results when further tested using the individual test described below (unpublished observations). Strains identified as producing inhibitory substances were isolated from the growth control plate and frozen in skim milk at −20 °C for further testing (maximum of five strains per culture plate).

Individual test for inhibitory substances

Selected strains of *S. salivarius* identified as producing inhibitory substances by the screening test were then tested further using a modification of the technique described by Tagg *et al.* (1983). An overnight Todd–Hewitt Broth culture of the strain was swabbed onto a plate containing Columbia Blood Agar Base with 5% human blood and 0·5% $CaCO_3$ in 1 cm wide streak (producer streak). The plate was incubated anaerobically for 18 h at 35 °C. Five indicator strains, the two strains described previously (*S. salivarius* strains T-18 and the group A streptococcal strain, M-1 T-1) plus three additional group A streptococcal strains isolated from this population during this outbreak (M-5, T-5/27/44; M-non-typable, T-3/13/B; and M-non-typable, T-12, Serum Opacity Reaction type 22) were grown overnight in Todd–Hewitt Broth with 2% neopeptone at 35 °C. Cotton swabs charged with growth from these broth cultures were inoculated on the plate perpendicular to the producer streak. The plates were incubated aerobically overnight at 35 °C.

The following day, the plates were examined for inhibition of the growth of the indicator strains. A positive result was defined as complete or partial (few scattered colonies) inhibition of the growth of the indicator strains and was interpreted as confirming production of inhibitory substances by the *S. salivarius* strain tested (Fig. 2).
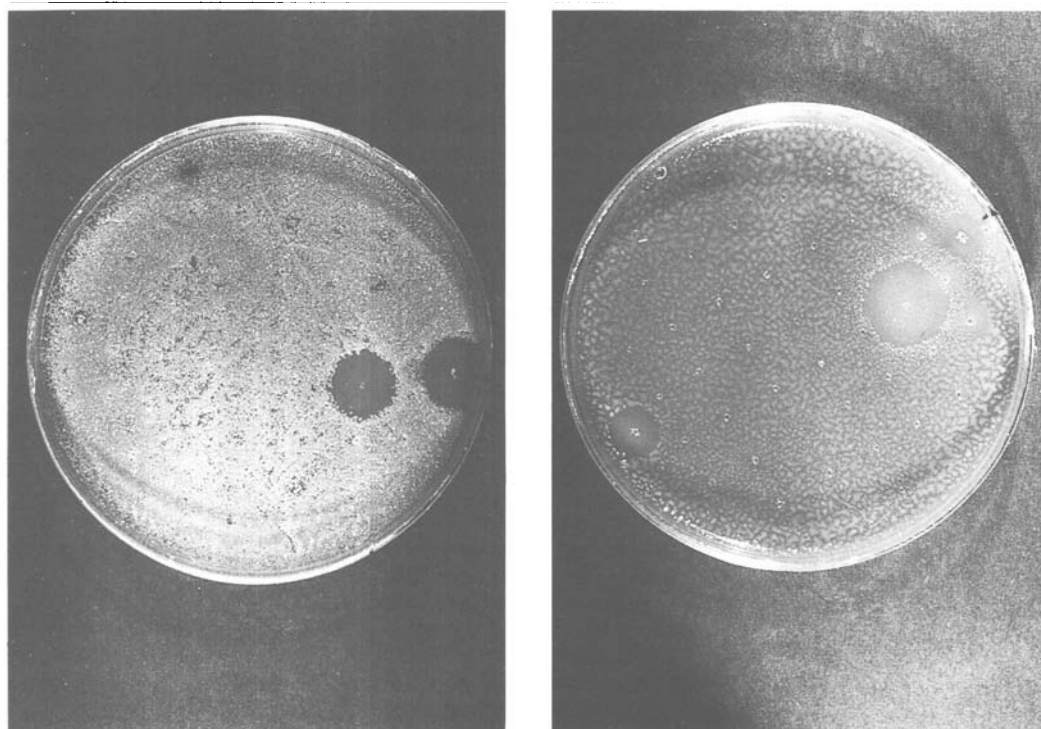
Fig. 1. The screening test used for *S. salivarius* straining producing inhibitory substances. The agar plate demonstrates zones of inhibition of the indicator strains (*S. epidermidis* on the left panel and group A streptococci on the right panel) produced by *S. salivarius* strains producing inhibitory substances.

Statistical analysis

The methods of statistical analysis used were the χ^2 test, the Mann–Whitney U test, and the Kruskal Wallis test. The Mann–Whitney U test is a non-parametric test for two independent samples. The Kruskal–Wallis test is an extension of the Mann–Whitney U test for multiple independent samples. Non-parametric tests were used because the data analysed were not distributed normally: these tests take this fact into consideration.

RESULTS

The demographics and culture data for the three clinical groups of subjects are shown in Table 1. The mean ages and the range of ages are similar among groups I, II, and III. The mean number of days of antibiotic treatment was different among the groups for obvious reasons; the duration of treatment in group I was almost three times that or group II. Since all subjects were not cultured each week, the mean number of cultures obtained per child and consequently the mean number of *S. salivarius* colonies tested per child varied among the three groups.

A total of 5233 colonies of *S. salivarius* from throat cultures obtained from all three groups of subjects were examined by the screening test for the production
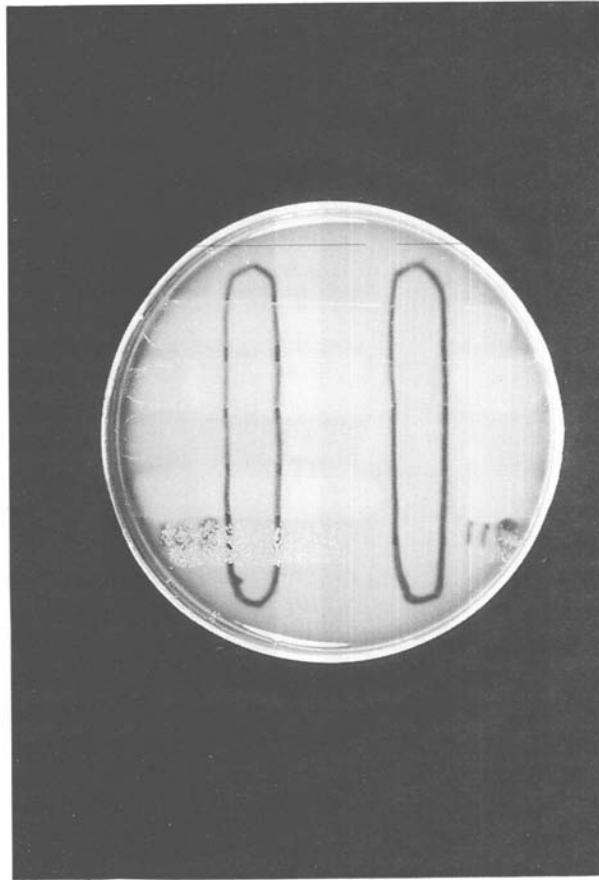
Fig. 2. The individual test for *S. salivarius* strains producing inhibitory substances. The plate demonstrates inhibition of the growth of the indicator strains over the producer streak on the right (positive result) and no inhibition of the growth of the indicator strain over the producer streak on the left (negative result).

Table 1. *Demographic and culture data*

	Group 1	Group II	Group III
Number of children	16·0	26·0	18·0
Mean age in years	9·4	9·0	9·6
(range)	(6 12)	(6–13)	(7–13)
Mean number of days of antibiotic treatment	27·5	10·0	0·0
Mean number of cultures per child	3·1	4·2	5·6
(range)	(3–5)	(2–7)	(3–6)
Meana number of *S. salivarius* colonies tested	62	110	86
per child (range)	(56 99)	(58 151)	(96-132)

of inhibitory substances. Twenty-one percent (1073/5233) of the *S. salivarius* strains tested were interpreted as producers (positive) using the screening test. Five hundred and forty-eight of the 1073 strains (57 %) producing positive results by the screening test were subsequently retested using the individual test. These 548 strains represented a cross-section of the strains screened from each group of
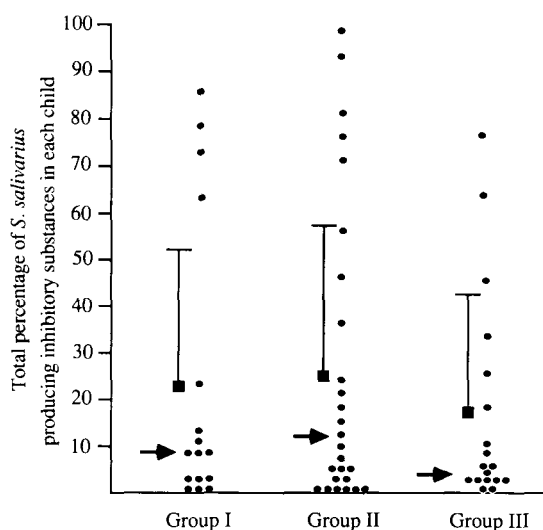
Fig. 3. The total percentage of *S. salivarius* producing inhibitory substances in each child in groups I, II. and III. Each point represents the total percentage of *S. salivarius* producing inhibitory substances from all the cultures from each child. The mean percentage (■) standard deviation. and the median percentage (➤) are shown (see text).

subjects. Five hundred and twelve of these 548 *S. salivarius* strains showing positive results on the screening test also gave positive results on the individual test. Consequently, the percentage of false positive results from the screening test was 7% (36/548).

An additional 85 *S. salivarius* strains which produced negative results on the screening test were also retested using the individual test. These 85 strains represented 2% (85/4160) of the 4160 (5233 strains tested minus 1073 positive) *S. salivarius* strains that produced negative results on the screening test. These strains were also selected from a cross-section of the strains screened from each group. Seventy-nine of these 85 strains producing negative results on the screening test also produced negative results on the individual test. The percentage of false negative results from the screening test identified by this testing was 7% (6/85). Because of the demonstrated reliability of the screening test, the results reported in the following sections represent results of the screening test only.

A comparison of the prevalence of *S. salivarius* strains producing inhibitory substances in each of the three groups of subjects is shown in Fig. 3. The results of all throat cultures taken from each child during the entire period of observation (3 months) were added to determine the total percentage of *S. salivarius* strains producing inhibitory substances inhibited from each child. Each point in the figure represents the total percentage (from all cultures obtained from each subject) of *S. salivarius* strains producing inhibitory substances.

At least one strain of *S. salivarius* producing inhibitory substances was isolated from a majority of children in each group (81% in group I, 76% in group II, and 88% in group III). There was wide variation in the percentage of *S. salivarius* strains producing inhibitory substances among children in each of the three

groups. However, as shown in Fig. 3, most of the subjects in each group harboured a low percentage of *S. salivarius* strains isolates that produced inhibitory substances. The mean total percentage of *S. salivarius* producing inhibitory substances among children in each group was 21·8 % (±30·8) for group I, 22·4 % (±32·9) for group II, and 16·4 % (±25·8) for group III. A non-parametric analysis of variance (Krusal–Wallis test) showed no significant difference ($P = 0.66$) when the three groups were compared.

Fluctuations in the percentages of *S. salivarius* strains producing inhibitory substances between cultures from the same individual but taken at different times were noted in some instances. It was for this reason that the cumulative results from all cultures taken from each child were determined. These fluctuations were random in nature and did not reflect any general changes with time in the percentage of *S. salivarius* strain producing inhibitory substances when the data from individual subjects were compared. There was no association between the percentage of *S. salivarius* strains producing inhibitory substances and the age of the children or their clinical presentation (symptomatic *vs.* asymptomatic). In addition, no obvious correlation was noted in percentages of *S. salivarius* strains producing inhibitory substances recovered from cultures obtained from siblings in the 16 sibling groups which were included in the study population. The distribution of the percentages of *S. salivarius* producing inhibitory substances among the children in group I is somewhat suggestive of a bimodal distribution. However, a careful review of the clinical history of the children in group I did not reveal any distinguishing feature(s) that may have characterized those children at the upper end of the distribution from those children at the lower end of the distribution.

Finally, we compared the percentage of *S. salivarius* strains producing inhibitory substances recovered from single throat cultures from subjects in group I (from which group A streptococci were simultaneously isolated) with the percentage of *S. salivarius* strains producing inhibitory substances isolated from single cultures from subjects in group III (from which no group A streptococci were recovered). The purpose of this analysis was to determine if the continued presence of group A streptococci in the upper respiratory tract induced any changes in the percentage of *S. salivarius* producing inhibitory substances. These was no significant difference ($P = 0.67$, Mann–Whitney U test) in the distribution of percentages of *S. salivarius* producing inhibitory substances in single cultures positive for group A streptococci (from group I) compared to single cultures negative for group A streptococci (from group III).

DISCUSSION

Previous studies have documented that viridans streptococci isolated from the upper respiratory tract may produce substances that inhibit the growth of group A streptococci *in vitro* (Crowe, Sanders & Longley, 1973; Dajani, Tom & Law, 1976; Dempster & Tagg, 1982; Gerasimov, 1968; Grahn & Holm, 1983; Sanders, 1969; Sanders & Sanders, 1982; Tagg, Dajani & Wannamaker, 1976; Tagg *et al.* 1983). Biochemical analysis of some of these substances has indicated that they may be low-molecular weight antibiotics (Sanders & Sanders, 1982) or that they may be similar to bacteriocins (Dajani, Tom & Law, 1976; Dempster & Tagg,

1982). Several epidemiologic studies have postulated that inhibitory substances produced by viridans streptococci might reduce the susceptibility of individuals to colonization of the upper respiratory tract by group A streptococci (Crowe, Sanders & Longley, 1973; Grahn & Holm, 1983; Sanders, 1969; Sanders, Nelson & Sanders, 1977).

Two of these reports also suggest that the percentage of viridans streptococci producing inhibitory substances may even increase following group A streptococcal colonization (Crowe, Sanders & Longley, 1973; Grahn & Holm, 1983). Crowe, Sanders & Longley (1973) speculated that under the 'selective pressure' of group A streptococcal colonization, the number of viridans streptococci producing inhibitory substances increased. No mechanism for this selective pressure was proposed. The authors further suggested that this increase in the production of inhibitory substances by the viridans streptococci may have influenced the eradication of group A streptococci in the asymptomatic colonized subjects, since these children were colonized with group A streptococci for a relatively short time (mean 3 weeks, range 1–11 weeks) and did not receive any antibiotics.

We examined this possibility by studying the prevalence of *S. salivarius* strains producing inhibitory substances in a semi-closed community of children. Our original protocol called for a prospective examination of the prevalence of *S. salivarius* strains producing inhibitory substances before colonization with group A streptococci and before antibiotic therapy to determine if inhibitory substances produced by *S. salivarius* prevented colonization of the upper respiratory tract with group A streptococci. However, the speed with which the predominant group A streptococcal serotype spread through this population and the high prevalence of culture positive individuals made this an impossibility (Smith *et al.* 1988). Nonetheless, by completing the study we sought to determine if the presence of strains of *S. salivarius* producing inhibitory substances played a role in the eradication of group A streptococci from the upper respiratory tract. We compared the prevalence of strains of *S. salivarius* producing inhibitory substances in three groups of children: a group persistently harbouring group A streptococci despite antibiotic treatment, a group in which group A streptococci were successfully eradicated with antibiotics, and a third group from whom group A streptococci were never recovered.

No difference in the prevalence of *S. salivarius* strains producing inhibitory substances was found among these three groups of children. In addition, the persistence of group A streptococci in the upper respiratory tract did not appear to influence the prevalence of *S. salivarius* strains producing inhibitory substances. Although the administration of antibiotics represents an uncontrolled variable (which will be discussed below) in this study, the percentage of *S. salivarius* isolates producing inhibitory substances did not appear to be a significant factor associated with eradication of group A streptococci from the upper respiratory tract. Other explanations for the persistent carriage of group A streptococci in the supper respiratory tract, such as the inactivation of penicillin by beta-lactamases produced by normal oral flora (Brook, 1984), might have been present in this population during this outbreak (Smith *et al.* 1988).

This study, as well as others previously cited (Crowe, Sanders & Longley, 1973; Grahn & Holm, 1983; Sanders, 1969), utilized an *in vitro* assay to detect the

production of inhibitory substances by normal flora of the upper respiratory tract. As in any investigation which attempts to correlate observed clinical phenomena with the results of *in vitro* assays, the results must be interpreted with caution.

Potential technical problems in such an *in vitro* study include: first, the method(s) used may not be sufficiently sensitive to detect *in vitro* production of those inhibitory substances causing *in vivo* effects on the microenvironment of the upper respiratory tract. We attempted to minimize this possibility by using culture media and conditions which have been reported to be optimal for production of inhibitory substances by *S. salivarius* (Dempster & Tagg, 1982). Preliminary evaluations in our laboratory confirmed this. In addition, when comparing the results of the screening test with the individual test we showed that the smaller inoculum used in the screening test does not significantly affect the sensitivity of the assay.

A second possible technical problem with an *in vitro* assay of this kind is non-specific inhibition related to culture conditions which may be falsely interpreted as specific inhibition of group A streptococcal growth. The methods used in this study eliminated two important causes of non-specific inhibition (acidic pH and hydrogen peroxide) as possible sources of false positive results. Studies of other streptococcal species have demonstrated that both acidic pH and hydrogen peroxide may cause nonspecific growth inhibition (Malke *et al.* 1974; Tagg & Wong, 1983). While previous epidemiologic reports of inhibitory substances produced by viridans streptococci have mentioned non-specific causes of growth inhibition (Crowe, Sanders & Longley, 1973; Grahn & Holm, 1983; Sanders, 1969; Sanders, Nelson & Sanders, 1977), those studies did not incorporate precautionary measures to eliminate these factors.

This study is also unique among clinical/epidemiologic studies of inhibitory substances since it focuses only on a single species of viridans streptococci, *S. salivarius*. While other investigators have speciated the viridans streptococci studied, only cumulative data have been presented. Consequently it is unclear whether the observed inhibitory effect of normal upper respiratory tract flora was due to the production of inhibitory substances by one or by several species of viridans streptococci. *S. salivarius* has previously been described as being among the most prevalent producers of inhibitory substances among the species of viridans streptococci that normally inhabit the upper respiratory tract (Crowe, Sanders & Longley, 1973; Sanders & Sanders, 1982). Preliminary studies in our laboratory confirmed this observation (unpublished observations). By focusing our attention on *S. salivarius* alone we have been able to evaluate its role in the microbial ecology of the upper respiratory tract in more detail.

The different antibiotic treatment regimens received by children in the three groups may have affected these results since *S. salivarius*, like group A streptococci, are very sensitive to penicillin. Sanders *et al.* studied the effect of a 7-day course of oral penicillin in ten adult volunteers, all of whom were colonized with viridans streptococci producing inhibitory substances prior to penicillin administration (Sanders, Sanders & Harrowe, 1976). During the penicillin treatment, the number of viridans streptococci producing inhibitory substances was reduced. Furthermore, those investigators were unable to isolate strains of viridans streptococci producing these substances in five of the ten subjects even 3

weeks after the penicillin was discontinued. The explanation suggested was that strains of viridans streptococci producing inhibitory substances prior to antibiotic treatment were replaced during or after treatment by other strains of viridans streptococci that did not produce these substances.

To consider this possibility, we conducted a small trial to examine any effect of a 7-day course of oral penicillin on the production of inhibitory substances by *S. salivarius* in an additional eight normal children (after informed written consent was obtained). We found that the penicillin did substantially reduce the percentage of *S. salivarius* producing inhibitory substances during the 7 days of penicillin administration, but only for a limited time after penicillin was discontinued. In contrast to the report by Sanders, Sanders & Harrowe, (1976) within 2 weeks after penicillin treatment had been discontinued, *S. salivarius* colonies producing inhibitory substances were isolated from all of the six normal subjects in whom they were present prior to penicillin administration. Although the different number of courses of antibiotic therapy received by children in this study represented an uncontrolled variable, based on the results of this small trial, it is unlikely that this factor alone could account for our observations.

The eradication of group A streptococci from the upper respiratory tract is undoubtedly a complex interaction of the host immune system, the streptococcus itself, the antimicrobial effect of penicillin, and the normal flora of the upper respiratory tract. However, even considering the theoretical limitations of this study, we found no evidence to suggest that inhibitory substances produced by *S. salivarius* significantly affect the eradication of group A streptococci from the upper respiratory tract.

ACKNOWLEDGEMENTS

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