

Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media?

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The normal bacterial flora of the upper airways provides an important barrier to invading pathogens. This study investigated the production of bacteriocin-like inhibitory substances (BLIS) by streptococci isolated from the nasopharyngeal flora of children who either do or do not experience recurrent acute otitis media (AOM). Twenty children with recurrent AOM and 15 controls were tested. Swabs from the nasopharynx were evaluated for streptococci having BLIS activity against two representative strains of each of the AOM pathogens *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae* and *Moraxella catarrhalis*. Streptococci displaying strong BLIS activity were characterized further and tested for known streptococcal bacteriocin structural genes. Sixty-five per cent of children had nasopharyngeal streptococcal isolates that were inhibitory to strains of one or more of the AOM pathogens. Six children (17%) had streptococci that demonstrated strong BLIS activity against strains of at least three of the pathogenic species. Three of these inhibitory isolates were *Streptococcus salivarius*, two were *S. pneumoniae* and one was *S. pyogenes*. The inhibitory *S. salivarius* and *S. pyogenes* were shown to have structural genes for known streptococcal bacteriocins. No statistically significant difference was found between the two groups of children with respect to the presence of inhibitory streptococci in their nasopharyngeal floras. The finding of *S. salivarius* with strong inhibitory activity against several AOM pathogens in the nasopharyngeal flora of children is unique. Although there is no clear evidence from the present study that these organisms protect against AOM, their low pathogenicity and strong *in-vitro* BLIS production capability indicate that they should be incorporated in future trials of bacteriotherapy for recurrent AOM.

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INTRODUCTION

Acute otitis media (AOM) is the most common bacterial infection in young children. It is thought that the bacteria that infect the middle ear do so via the Eustachian tube, from the nasopharynx (Faden *et al.*, 1997). The carriage of AOM pathogens in the flora of the nasopharynx increases during the first year of life, particularly in those children who are prone to recurrent AOM (Faden *et al.*, 1991). This corresponds to the peak incidence of the disease in this age group.

The ability of the normal flora of the upper airways to inhibit growth of potential pathogens *in vitro* has been well described (Bernstein *et al.*, 1994; Brook & Gober, 1998, 2000; Grahn *et al.*, 1983; Tano *et al.*, 1999). Most of this inhibitory activity

has been attributed to α -haemolytic streptococci. In the oral cavity, the presence of *Streptococcus salivarius* producing the bacteriocin salivaricin A has been shown to reduce the frequency of acquisition of *Streptococcus pyogenes* in school-children (Dierksen & Tagg, 2000). Bacteriocins are proteinaceous antimicrobials produced by bacteria that kill closely related bacteria but not the producer strain itself, which exhibits a degree of specific immunity to that bacteriocin (Jack *et al.*, 1995). The term bacteriocin-like inhibitory substance (BLIS) is used to describe bacterial products that have inhibitory effects like those of bacteriocins, prior to the isolation and characterization of the active agent. BLIS-producing streptococci, staphylococci and enterococci have been shown to inhibit the growth of *Streptococcus mutans*, the species most commonly implicated in the aetiology of dental caries (Chikindas *et al.*, 1997).

Several studies have shown that α -haemolytic streptococci isolated from the nasopharynx are capable of inhibiting the growth *in vitro* of pathogens that frequently cause AOM

Abbreviations: AOM, acute otitis media; BLIS, bacteriocin-like inhibitory substance.

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(Bernstein *et al.*, 1994; Brook & Gober, 2000; Tano *et al.*, 1999). Children who are prone to AOM have significantly fewer α -haemolytic streptococci in their normal flora and these are less likely to be inhibitory to AOM pathogens than those from children who are not prone to AOM (Faden *et al.*, 1991; Bernstein *et al.*, 1993, 1994; Brook & Yocum, 1999; Fujimori *et al.*, 1996; Long *et al.*, 1983; Stenfors & Raisanen, 1989). Recently, Roos *et al.* (2001) completed a clinical trial in which inhibitory α -haemolytic streptococci were sprayed into the noses of children with recurrent AOM. Children in this test group had fewer episodes of AOM when compared with placebo-treated children. It has been assumed that this inhibition is related to the production of bacteriocins by these bacteria, although the actual nature of the inhibitory agents has not been well documented.

The present study compares the BLIS activities against potential AOM pathogens of streptococcal isolates from the nasopharyngeal microfloras of children who do or do not have recurrent AOM. Comparison is made between the BLIS activities of streptococcal isolates from their nasopharyngeal and oral microfloras to determine whether any BLIS-producers appear to have a particular tropism for the nasopharynx.

METHODS

Patients. Thirty-five children (20 male and 15 female) were enrolled in the study, 20 in the recurrent AOM group and 15 controls. They were all between the ages of 12 months and 6 years. Children were included in the recurrent AOM group if they had been treated for six or more episodes of AOM in the previous year. Control children had experienced no more than one episode of AOM. Children in either group were excluded from the study if they had any of the following: (i) taken antibiotics in the preceding 2 weeks, (ii) previous surgery to their upper airway, including adenotonsillectomy, (iii) anatomical abnormalities of their upper airway or (iv) known immune deficiency. Consent was obtained from a parent of each child in accordance with the requirements of the Otago Ethics Committee, who approved the study.

Sampling technique. The children were sampled while under general anaesthetic for either surgical or dental procedures. A nasopharyngeal swab was taken from each nostril using a sterile calcium alginate swab. A catheter enclosing the swab was passed through the anterior nares to minimize contamination. The tip of the swab was then pushed through the end of the catheter and rubbed against the posterior wall of the nasopharynx. Before the catheter was removed from the nose, the tip of the swab was retracted. The catheter tip was then cut off with sterile scissors before re-exposing the swab and plating the microflora sample directly onto the appropriate agar media. A tongue swab was also taken at the time to provide a representative sample of the subject's oral streptococcal population. All of the freshly inoculated agar plates were transported to the lab for incubation within 2 h.

Microbiology. The nasopharyngeal specimens were plated directly onto (i) mitis-salivarius agar (Difco), a selective medium for streptococci and (ii) chocolate agar [Columbia agar base (Gibco) plus 5% human blood, heated to lyse the erythrocytes prior to pouring into Petri dishes] to provide a 'total microflora' population. The cultures were grown anaerobically at 35 °C for 18 h. From each mitis-salivarius agar culture, up to five morphologically distinct colonies were selected and plated onto blood agar [Columbia agar base (Gibco) plus 5% human blood]. These cultures were grown overnight at 37 °C in an atmosphere

of 5% CO₂ in air. The tongue swabbings were plated directly onto mitis-salivarius agar and grown anaerobically at 35 °C for 18 h and representative colonies were tested for BLIS production. Samples of the total nasopharyngeal and tongue microflora cultures were resuspended in sterile skimmed milk and snap-frozen at -20 °C prior to storage at -70 °C.

Testing for BLIS production. Each of the subcultures of the morphologically distinct colonies from the mitis-salivarius agar nasopharyngeal cultures was evaluated for BLIS production using a deferred antagonism test on trypticase/soy/yeast extract/calcium (TSYCa) agar [Trypticase soy broth (Becton Dickinson) plus 2% yeast extract (Difco), 1.5% Davis agar (Davis Gelatin) and 0.1% CaCO₃]. In addition, the mixed bacterial populations recovered from the tongue and nasopharyngeal swabbings were also tested by this method as a screen for BLIS activity. The deferred antagonism method has been described previously (Tagg & Bannister, 1979). It involves first incubating a 1-cm-wide diametric strip of the test bacteria anaerobically at 35 °C for 18 h on the test agar plate. The streak culture is then scraped from the surface of the medium and the agar surface is sterilized by inversion for 30 min over a chloroform-infused cloth. The agar surface is then exposed to the air to remove residual chloroform, following which, 18 h 35 °C Todd-Hewitt broth cultures of the bacterial strains to be tested for BLIS sensitivity are streaked across the agar at right angles to the test streak. The plate is then returned to the incubator and, after 18 h, is examined to determine the degree of inhibition of the indicator organisms. For the purposes of the present study, bacterial inhibition was considered significant if the zone of inhibition of the indicator streak growth was at least twice the width of the original test streak. Isolates that significantly inhibited the growth of either one or both of the two representative strains of each species of AOM pathogen were considered to be inhibitory to that species.

The indicator strains used as representative of species commonly associated with AOM were *Streptococcus pneumoniae* PK2 and PK34, *S. pyogenes* FF22 and 71-698, *Moraxella catarrhalis* 4 and 22 and *Haemophilus influenzae* 30 and 33. The *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* indicator strains were clinical isolates taken from the middle ear of children with AOM. The *S. pyogenes* strains were reference isolates used commonly in this laboratory as indicators of streptococcal BLIS (Tagg & Bannister, 1979). In addition, the inhibitory isolates were tested to determine their patterns of inhibitory activity [referred to as BLIS production (P)-types] against a set of nine standard indicator strains (I1–I9) to enable comparison of their BLIS activities with those of previously documented BLIS-positive streptococci (Tagg & Bannister, 1979).

Six nasopharyngeal streptococcal isolates (each from a separate subject) that showed significant inhibitory activity against at least three of the species of AOM pathogens were each tested by PCR for the presence of structural genes previously shown to encode bacteriocin production in the oral streptococcal species *S. salivarius* [salivaricin A (Upton *et al.*, 2001) and salivaricin B (Tagg *et al.*, 2001)] and *S. pyogenes* [streptococcin A-FF22 (Hynes *et al.*, 1993) and streptin (Karaya *et al.*, 2001)]. The procedures for DNA extraction and PCR analysis were described previously (Upton *et al.*, 2001). The primers used were as follows (sequences are 5'–3'): salivaricin A (SalA), SrtAfwd (AAGACTTTGATCTCGATTGAA) and SrtArev (AAACTAATTTCCAACAAGAACCA); salivaricin B (SalB), SalBfwd (GTGAATTCTCTTCAAG AATTGACTCTT) and SalBrev (AAAAATATTCATACCGCTCTTCC); streptococcin A-FF22 (Scn), ScnAfwd (GCACCTATCCTTCTGAAGA AAG) and ScnArev (GCACCTAGGCACATTTTTTCTTCC); and streptin (Srt), SrtAfwd (AAGACTTTGATCTCGATTGAA) and SrtArev (AAACTAATTTCCAACAAGAACCA).

DNA extracts from the mixed microfloras from the mitis-salivarius

tongue cultures were also tested by PCR using the salivaricin A and salivaricin B primers as a means of detecting the presence of small numbers of salivaricin-producers in the oral microbiota.

BLIS-producing streptococcal isolates were characterized initially on the basis of their colony appearance on mitis-salivarius and blood agar media and on their biochemical profiles (API 20 Strep; bioMérieux).

RESULTS

Twenty-three (65 %) of the AOM-prone and control children sampled had bacterial strains isolated on mitis-salivarius agar from their nasopharynx that were inhibitory to AOM pathogens in the deferred antagonism test on TSYCa. The numbers of children in each group with isolates inhibitory to each of the pathogens are listed in Table 1. The inhibitory isolates were found in children from both groups and there was no significant difference between the groups with respect to the total number of inhibitory organisms isolated.

Streptococcal isolates from six children (three controls and three AOM group) were inhibitory to both representative strains of at least three of the species of AOM pathogens (Table 2). Of these six inhibitory isolates, two were *S. pneumoniae*, one was *S. pyogenes* (emm-type 11) and three were *S. salivarius*. Both direct culture and PCR analysis of the extracted DNA failed to demonstrate the presence of BLIS-producing *S. pyogenes* or *S. pneumoniae* in the total tongue microflora samples from subjects B, D and E. *S. pyogenes* strain D was shown by P-typing (and confirmed by PCR amplification of the appropriate structural gene) to produce the bacteriocin streptin. Neither of the BLIS-positive *S. pneumoniae* was PCR-positive for any of the tested streptococcal bacteriocin structural genes. Although *salA* was amplified from the tongue microflora of subject E, no corresponding salivaricin A-producing bacterium could be recovered in culture from that subject's tongue swab. Strong BLIS-producing *S. salivarius* nasopharyngeal isolates that were PCR-positive for both *salA* and *salB* were obtained from subjects A and C (both in the control group). In neither of these subjects, however, could the same inhibitory *S.*

Table 1. Numbers of subjects having streptococcal isolates inhibitory to strains of AOM pathogens in deferred antagonism tests on TSYCa

| AOM species | Recurrent AOM (%) (n = 20) | Controls (%) (n = 15) | P value |
|-----------------------|----------------------------|-----------------------|---------|
| <i>S. pneumoniae</i> | 5 (25) | 2 (13) | 0.67 |
| <i>S. pyogenes</i> | 5 (25) | 2 (13) | 0.67 |
| <i>M. catarrhalis</i> | 10 (50) | 7 (47) | 0.88 |
| <i>H. influenzae</i> | 2 (10) | 2 (13) | 0.81 |

salivarius be detected in the corresponding tongue sample by either direct culture or PCR. An apparently unrelated population of *salA*⁺ *salB*⁻ *S. salivarius* was detected both by PCR and by direct culture in the tongue sample from subject C. *S. salivarius* strain F had inhibitory activity consistent with production of the bacteriocin salivaricin A and was positive by PCR for the appropriate structural gene, *salA*. What appeared to be the same strain of *S. salivarius* was detected by PCR and was also isolated directly in culture from the tongue flora of subject F.

DISCUSSION

The finding of bacteria that are inhibitory to AOM pathogens *in vitro* in the nasopharynx of children is common. The present study, however, has demonstrated that *S. salivarius* is present in the nasopharynx in some children and that some strains produce BLIS that is strongly inhibitory *in vitro* to AOM pathogens.

Several previous studies have shown that there are significant differences between the numbers of inhibitory organisms in children prone to AOM and those who are not (Bernstein *et al.*, 1994; Brook & Gober, 2000; Tano *et al.*, 1999). The nature of this inhibition has been thought to be through BLIS production by these organisms or other factors such as competition for essential nutrients (Brook, 1998; Long *et al.*,

Table 2. Detection of *salA* and *salB* by PCR in strongly inhibitory nasopharyngeal streptococcal isolates and in samples of the tongue microflora from these subjects

| Inhibitory strain* | <i>salA</i> | | <i>salB</i> | |
|------------------------|-------------------|-------------------|-------------------|-------------------|
| | Inhibitory strain | Tongue microflora | Inhibitory strain | Tongue microflora |
| <i>S. salivarius</i> A | + | - | + | - |
| <i>S. pneumoniae</i> B | - | - | - | - |
| <i>S. salivarius</i> C | + | + | + | - |
| <i>S. pyogenes</i> D | - | - | - | - |
| <i>S. pneumoniae</i> E | - | + | - | - |
| <i>S. salivarius</i> F | + | + | - | - |

*Sources of strains are indicated: A, C and E are control subjects, B, D and F are recurrent AOM subjects.

1983; Van Eldere, 2000). The results of the present study do not support the hypothesis that BLIS production by the streptococcal component of the normal nasopharyngeal flora is an important factor in protection against recurrent AOM. There was no statistically significant difference between the two groups of children with respect to the presence of inhibitory streptococci. It remains possible that species other than streptococci produce BLIS with activity against AOM pathogens or that some strains of streptococci capable of producing BLIS *in situ* fail to produce inhibitory activity under the present laboratory test conditions.

A recent clinical trial by Roos *et al.* (2001) found that, by administering a nasal spray containing organisms with significant inhibitory activity against AOM pathogens, they were able to protect against recurrent AOM. One possible explanation for this is that, by introducing large numbers of the organisms into the nasopharynx following a course of antibiotics, it may be possible to increase the proportion of bacteria in the normal flora with inhibitory activity.

The deferred antagonism test used in this study has been used in this laboratory for over 20 years to test for BLIS production by streptococci. It is unique in that the two bacteria being tested are never in direct contact with each other. This excludes substances that are integral components of the cell wall of the producer strain as the cause of inhibition. The BLIS proteins secreted by streptococci are generally relatively small compounds that can diffuse through agar and produce inhibition well away from the location of the organism that secreted them. This kind of inhibition, at a distance from the original organism, makes other causes of inhibition less likely and suggests that the activity is due to BLIS.

The isolation from the nasopharynx of several strains of BLIS-producing *S. salivarius* is unique. Bernstein *et al.* (1993) found that the predominant streptococci in the nasopharyngeal flora of children were *Streptococcus mitis* and *Streptococcus sanguinis*. BLIS production by *S. salivarius* has been well documented (Dierksen & Tagg, 2000; Jack *et al.*, 1995). The bacteriocins salivaricin A and salivaricin B have been shown previously to be strongly inhibitory to the growth of most *S. pyogenes* (Upton *et al.*, 2001). The streptococci isolated in the present study have inhibitory activity *in vitro* not only against *S. pyogenes*, but also against a range of other pathogens including the Gram-negative bacteria *M. catarrhalis* and *H. influenzae*. It is of special interest that *S. salivarius* producing both salivaricin A and salivaricin B were recovered from the nasopharyngeal specimens, but not from the tongue swabbings, of two of the control subjects. Furthermore, none of the AOM-prone subjects appeared to have these double-BLIS-producing *S. salivarius* in their nasopharyngeal microfloras. Could this perhaps indicate that *S. salivarius* strains producing both salivaricin A and salivaricin B are particularly well adapted to growth in the nasopharynx? Although *S. salivarius* is not usually considered to be a predominant member of the nasopharyngeal flora of adults (Rasmussen *et al.*, 2000), in children under the age of 2 it is the second-most-frequently isolated strepto-

coccal species at this site after *S. mitis* 1 (Könönen *et al.*, 2002). Since *S. salivarius* is considered to be essentially non-pathogenic, it could be an excellent candidate for possible introduction into the normal nasopharyngeal flora as protection against recurrent AOM. Future clinical trials introducing inhibitory bacteria into the nasal cavity to protect against recurrent AOM should include BLIS-producing *S. salivarius*.

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