Relation of Cholesterol-Stimulated *Staphylococcus aureus* Growth to Chronic Blepharitis

Ward E. Shine, Robert Silvany, and James P. McCulley

**Purpose.** Many types of chronic blepharitis have been believed to be primarily microbial in origin; however, it was proposed that differences and changes in lipid composition of meibomian secretion may be the initiating factor in some of these. It was recently reported that there are two subgroups of normals, those whose meibomian secretions contain high levels of cholesterol esters and those whose secretions contain very low levels of these esters. Thus, these subgroups of normals were defined on the basis of detailed lipid analyses of meibomian secretions from individuals showing no clinical signs of chronic blepharitis. All secretions from patients in the various disease groups contain high levels of these esters. Based on previous observations that in some chronic blepharitis disease groups certain *Staphylococcus* species were capable of hydrolyzing cholesterol esters, the authors tested the hypothesis that the resulting cholesterol might affect growth of *Staphylococcus aureus*.

**Methods.** *Staphylococcus aureus* growth stimulation in Mueller-Hinton broth by cholesterol was determined by colony forming units. Growth stimulation by cholesterol and other additives was also determined by the optical density 650 nm method. Statistical analyses included analysis of variance and the Student’s *t* test.

**Results.** Cholesterol stimulated *Staphylococcus aureus* growth was significant during the first 24 hr period (20% increase at 25 μM cholesterol, *P* < 0.02), and for the total 48 hr period (40% increase at 400 μM cholesterol, *P* < 0.005) when compared to the respective control. Growth stimulation, determined by OD at 650 nm, in the presence of cholesterol was significantly greater (*P* < 0.02) than that in the presence of either sitosterol or cholestanol when the sterol concentration was 190 μM.

**Conclusion.** These results suggest that the presence and hydrolysis of cholesterol esters of meibomian secretions may contribute to the proliferation of *Staphylococcus* spp, especially *Staphylococcus aureus*, observed in some chronic blepharitis disease groups. Invest Ophthalmol Vis Sci 1993;34:2291-2296.

We previously reported that coagulase-negative *Staphylococcus* spp. (C-NS), *Propionibacterium acnes*, and coryneform bacteria were the most commonly isolated bacteria from eyelids of normal subjects as well as all clinical groups of chronic blepharitis patients; in contrast, *Staphylococcus aureus* was frequently found only in the staphylococcal and staphylococcal/seborrheic disease groups.\(^1\) We subsequently reported that eyelids and conjunctivae of patients in the staphylococcal/seborrheic, meibomian seborrheic, secondary meibomitis, and meibomian keratoconjunctivitis disease groups contained significantly more coagulase-negative *Staphylococcus* strains capable of hydrolyzing cholesteryl oleate than did normal persons.\(^2\) Furthermore, we recently reported the inhibition of lipase production by low levels of the antibiotic tetracycline, which also has been used successfully to treat the meibomian keratoconjunctivitis disease group.\(^3\) The possi-
ble role of cholesterol esters and free cholesterol in the development of chronic blepharitis disease signs and associated microbial populations has been further investigated in our laboratory. We observed that meibomian gland secretions of normal subjects were of two types: those with very low levels of both cholesterol esters and esterified unsaturated fatty acids, N(CA) subgroup, and those with high levels of cholesterol esters and esterified unsaturated fatty acids, N(CP) subgroup; all disease groups' secretions contained high levels of cholesterol esters and esterified unsaturated fatty acids. Finally, we have observed that the N(CP) subgroup contained twice as many Staphylococcus strains as the N(CA) subgroup. Thus, we have suggested that the composition of lipid secretions from the meibomian glands may contribute to the development of many of the disease signs associated with chronic blepharitis. The composition may also affect the bacterial populations present in some disease groups. With only a few exceptions, however, prokaryotic bacteria do not require cholesterol for growth. Furthermore, cholesterol usually is not found in membranes of prokaryotic bacteria. Nevertheless, we observed a significant increase in the growth rate of the prokaryotic bacterium S. aureus in the presence of cholesterol.

METHODS

Source of Organism

Isolates of S. aureus were obtained from patients with extraocular disease signs indicative of an infection process. Identification was confirmed by a positive coagulase test and by use of the API Staph-Ident (Analytab Products, division of Sherwood Medical, Plainview, NY) profile tests, which gave a profile number of 3700. Isolates were maintained on blood agar (Brucella Agar, BBL; Becton Dickinson, Cockeysville, MD), 43 g/l, containing 5% defibrinated sheep blood. Before conducting growth assays the culture was subcultured into Mueller-Hinton (M-H) broth (Difco, Detroit, MI), 21 g/l, and then diluted with normal saline to a concentration that yielded a McFarland number of 0.5 (approximately $1.5 \times 10^8$ organisms/ml).

Assay Conditions

The assay system consisted of a 96-well (flat bottom) polystyrene microplate with lid (Corning #2586, Corning, NY). The sterols, dissolved in absolute ethanol, were first added to the well (25 microliters/well); wells of the controls contained an equal volume of ethanol. After evaporating the ethanol with a stream of filtered nitrogen at room temperature, 100 $\mu$L of M-H broth (21 g/l, pH 7.3) was added to each well and the plates vibrated for 5 min to disperse the sterols. To determine if the ethanol affected growth another set of wells had no ethanol addition before adding the M-H broth. Finally 2 $\mu$L of bacterial inoculum (0.5 McFarland number) was added to each well; this resulted in a bacterial concentration of $1.35 \times 10^6$ to $1.50 \times 10^6$ colony forming units (CFU) per milliliter (0.135–0.150 $\times 10^6$/well) as determined at time 0. The plates were covered and then incubated at 35°C in the dark.

Sterol Additives

The effect of various sterols on growth was tested by using the same concentration ranges of sterols in all microplate assays. Preliminary studies had indicated a growth stimulation by cholesterol; therefore, the structurally closely related sterols sitosterol (with an ethyl group on the side-chain) and cholestanol (with no double bond) were selected for comparative purposes. Specifically, the sterols tested were cholesterol (5-cholesten-3β-ol), sitosterol (5-cholesten-24β-ethyl-3β-ol) and cholestanol (5-cholestan-3β-ol). The amount of sterol added to the wells was 0.57, 7.2 and 70 $\mu$g; the corresponding concentrations (in 102 $\mu$L of final volume) were 5.8, 74 and 720 $\mu$g/ml or 15, 190, or 1850 $\mu$M for cholesterol and cholestanol, and 14, 180, or 1730 $\mu$M for sitosterol. When only cholesterol was tested, the three concentrations used were 1.5, 25, and 400 $\mu$M.

The native M-H broth was also assayed for free (unbound) cholesterol. The M-H broth powder was extracted with chloroform-methanol (1:1) at 25°C. After centrifugation, the solvent was assayed by gas-liquid chromatography for cholesterol as previously described.

Fatty Acid Additives

For comparative purposes, the effect of free fatty acids on S. aureus growth was also investigated. Oleic, stearic, and palmitic acids were assayed at the same level (0.57, 7.2, and 70 $\mu$g) as the three sterols and growth was determined by the OD (650 nm) method.

Growth Rate Determination (OD Method)

In comparative experiments (sterols or fatty acids) the amount of growth was determined by the use of a THERMOMax microplate reader (Molecular Devices, Menlo Park, CA). Plates were read at 650 nm after vibration. A standard curve using known dilutions on the Staphylococcus culture was prepared to correlate absorbance (OD) with actual bacterial count. Culture plates were read at 0, 24, and 48 hr.

Confirmation of Cholesterol Growth Stimulation (Colony Forming Unit Method)

Growth was also determined by actual colony count at three cholesterol concentrations. Growth in the presence of 1.5, 15, and 400 $\mu$M cholesterol was compared to the ethanol control and also to growth when no
ethanol addition was made. Growth stimulation was tested using five replicates for each cholesterol concentration as well as 15 replicates for the ethanol (controls) and 15 replicates where no ethanol addition was made, at 0, 24, and 48 hr. Aliquot portions of each replicate (3 μl) were appropriately diluted and plated with M-H agar (38 g/l) by the pour plate method to determine CFU. All colonies on each plate were counted manually, using a colony counter (Darkfield Quebec Colony Counter, American Optical, Buffalo, NY).

Statistical Analyses

Statistical analyses were performed using CSS:STATISTICA (StatSoft, StatSoft Inc., Tulsa, OK) software. Statistical evaluations included analysis of variance, and a two-tailed t test.

Research protocol followed the tenets of Declaration of Helsinki, informed consent of patients was obtained and the study was approved by the institutional human experimentation committee.

RESULTS

The addition of each of the three sterols, cholesterol, β-sitosterol and cholestanol, to the M-H medium resulted in increased S. aureus growth during the first 24-hr period; however, only at the highest sterol concentration (1850 μM) were growth increases statistically significant (Table 1, Figure 1), when determined by the OD method. In contrast, growth during the total 48 hr period was significantly increased by all three sterols at all three concentrations (except for the lowest [15 μM] sitosterol concentration). At the medium (190 μM) concentration, however, this growth increase (total 48 hr) in the presence of cholesterol was also significantly greater than for either sitosterol or cholestanol. An apparently significant decrease in growth was noted at 48 hr compared to 24 hr in the no addition (none) treatment. The broad effectiveness of cholesterol to stimulate S. aureus growth was tested in further detail using the CFU assay. When growth stimulation by cholesterol was determined by actual CFU, much more dramatic differences were noted, (Table 2, Figure 2). As determined by analysis of variance, these growth differences were significant for cholesterol concentration (P < 0.0001), growth period (P < 0.0001) and cholesterol concentration/growth period interaction (P < 0.0001). Thus, both cholesterol concentration and time of growth affected the final number of CFU. The significance of the interaction among these factors suggests that the optimum cholesterol concentration depends on growth period and/or actual concentration of bacteria present.

TABLE 1. Effect of Specific Sterols and Sterol Concentrations on Staphylococcus aureus Growth During a 48-hr Time Period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μM/l)</th>
<th>Growth Period†</th>
<th>24-hr</th>
<th>48-hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>167 ± 13</td>
<td>148 ± 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>150 ± 21</td>
<td>138 ± 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>148 ± 10</td>
<td>198 ± 11†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>166 ± 18</td>
<td>230 ± 20‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>201 ± 31†</td>
<td>244 ± 32§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitosterol</td>
<td>173 ± 14</td>
<td>181 ± 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitosterol</td>
<td>160 ± 10</td>
<td>180 ± 10†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitosterol</td>
<td>212 ± 21‡</td>
<td>247 ± 30§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholestanol</td>
<td>172 ± 4</td>
<td>191 ± 12†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholestanol</td>
<td>162 ± 22</td>
<td>163 ± 6†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholestanol</td>
<td>205 ± 15‡</td>
<td>239 ± 16§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Growth values (X10^6) are the average number of organisms/culture well (organisms/100 μl) ± SD for the time period indicated.
† The decrease in none (no additions) at 48 hr was significant (P < 0.02) versus the none 24-hr value. Significantly different (P < 0.02) for the time period indicated versus the following: ethanol control, §none (no additions), or ‡both 190 μM/l sitosterol and 190 μM/l cholestanol.
In the total 48 hr growth period the highest cholesterol concentration (400 μM) resulted in the most significant (P < 0.005) growth increase (40%) over the control. In contrast, neither the lowest cholesterol concentration (1.5 μM) nor the untreated medium (no addition) were significantly different from the ethanol control. All 48-hr growth values were significantly greater than the corresponding 24-hr values, with the exception of the lowest cholesterol concentration (no significant difference).

The amount of cholesterol (free sterol) present as a component in the native M-H broth was also determined. The concentration of this M-H broth derived free cholesterol was determined to be less than 0.015 μM in the final incubation medium.

Finally, growth was not significantly affected by the free fatty acids tested. Thus, oleic, stearic, and palmitic acids neither inhibited nor stimulated S. aureus growth significantly under the conditions employed in these experiments.

**DISCUSSION**

The data presented demonstrate that sterols can stimulate the growth of S. aureus. In the first set of assays, using different sterols and determining growth by OD, significantly increased growth was observed, especially during the total 48-hr incubation (Table 1, Figure 1). The apparent significant loss of organisms in the no addition (none) treatment may be attributable to clumping or some other factor that caused small changes in the OD. Significant growth stimulation was noted for all three sterols tested. However, at the medium concentration tested (190 μM) cholesterol was significantly (P < 0.02) more stimulatory than the other two sterols. Thus sterol structure appears important since cholestanol has exactly the same structure as cholesterol except that it is completely saturated; similarly, sitosterol's structure differs only in the side-chain that has an added ethyl group. It should be noted that we have previously determined that all meibomian gland samples analyzed contain cholestanol esters, but at very low levels; in contrast, both the N(CP) subgroup and all disease groups' meibomian secretions contain high levels of cholesterol esters. No sitosterol has been detected in meibomian secretions. We did not observe a significant growth effect in the presence of free fatty acids, although free fatty acids are present in meibomian secretions. This was unexpected, because free fatty acids, especially unsaturated ones such as oleic acid, were reported to have a bactericidal effect on S. aureus. One explanation for this lack of inhibition we observed is suggested by reports that pigmented strains of S. aureus are less susceptible to oleic acid inhibition than nonpigmented strains. Furthermore, oleic acid is much more inhibitory when cultures are grown in the light rather than the dark; our incubations were carried out in the dark.

**TABLE 2. Effect of Cholesterol Concentration on Staphylococcus aureus Growth During a 48-hr Time Period**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μmol/l)</th>
<th>Growth Period†</th>
<th>24-hr</th>
<th>48-hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>138 ± 31</td>
<td>238 ± 29</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>—</td>
<td>145 ± 18</td>
<td>232 ± 36</td>
<td></td>
</tr>
<tr>
<td>Cholesterol 1.5</td>
<td>149 ± 34</td>
<td>198 ± 49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol 25</td>
<td>178 ± 34*</td>
<td>292 ± 93‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol 400</td>
<td>167 ± 24</td>
<td>330 ± 82§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Growth values (X10⁶) are the average number of organisms/culture well (organisms/100 μl) ± SD for the time period indicated and were determined by actual count of colonies.
† All 48-hr values are significantly greater (P < 0.02) than the corresponding 24-hr values, except for the lowest level of cholesterol (no significant difference). Significantly different (P < 0.02) for the time period indicated from the ethanol (control) or none (no additions) treatments.
Thus, even if the only energy source were starch, the cholesterol-stimulated growth was significant at both the medium and highest (400 μM) concentrations. For the total 48-hr period growth stimulation by cholesterol was greater at the highest concentration (40%) than at the medium concentration (20%). The data suggest that a cholesterol concentration of at least 25 μM but less than 400 μM optimally stimulates. No stimulation was noted at the lowest cholesterol concentrations (1.5 μM).

The reason for this cholesterol induced growth stimulation does not appear to be related to its possible use as an alternate energy source. M-H broth powder contains 7% soluble starch (the remaining 93% is mainly beef extract and casein hydrolysate). Thus, even if the only energy source were starch, the amount of cholesterol added at the three levels used was 0.05%, 0.6% or 10% that of starch. Therefore, cholesterol does not appear to be serving as a significant energy source.

Another possible explanation for the cholesterol effect could be that growth stimulation is the result of esterification of inhibitory endogenous free fatty acids. However, as previously mentioned, in experiments run concurrently with the assay of the three sterols no significant inhibition of S. aureus growth was noted in the presence of similar concentrations of oleic, stearic, or palmitic acid.

A suggestion for the growth stimulating effect of cholesterol can, however, be found in the literature. Various strains of S. aureus have been shown to accumulate cholesterol from growth medium and this cholesterol then becomes associated with the membrane fraction as free cholesterol and cholesterol esters. In fact, it has been demonstrated that stable L-forms, derived from S. aureus and which can grow without cell walls, are capable of synthesizing large amounts of cholesterol. Thus, it appears that certain prokaryotic bacteria, such as S. aureus, are capable of altering the cholesterol composition of their cytoplasmic membrane to adapt to unusual conditions. The data presented suggest that changes in the growth rate of ocular microflora, such as Staphylococcus spp, can be greatly influenced by the presence or absence of cholesterol esters in glandular secretions of the eyelid. If the strain(s) of Staphylococcus present are capable of hydrolyzing cholesterol esters then free cholesterol could be produced if these strains produce enzymes which hydrolyze cholesterol esters. Certain Staphylococcus spp. may then become the predominant microflora associated with the lid and conjunctiva through the action of these esterase enzymes, which hydrolyze cholesterol esters and release free cholesterol from meibomian gland secretions. It is a common observation that many blepharitis patients have the same types of bacteria present as do normal subjects, but the numbers present are much greater in the disease state. Thus, it is the formation of free cholesterol by cholesterol ester lipases that may be a necessary precondition for significantly increased S. aureus populations, resulting in at least some of the signs associated with the chronic blepharitis disease groups, such as in the staphylococcal and staphylococcal/seborrheic groups.

The significantly increased populations of Staphylococcus spp that we observed in most chronic blepharitis disease groups could have been a direct result of the high concentrations of cholesterol esters in meibomian gland secretions. We have recently reported the observation that even in the two normal subgroups, the normal-cholesterol present subgroup, N(CP), contained twice as many coagulase negative Staphylococcus strains and Staphylococcus strains showing cholesterol ester lipase activity as the normal-cholesterol absent subgroup, N(CA). Thus, colonizing Staphylococcus spp, capable of hydrolyzing cholesterol esters, may liberate cholesterol if they produce active cholesterol ester lipase enzymes, which could in turn stimulate growth of certain Staphylococcus spp such as S. aureus. This may be particularly true in two chronic blepharitis disease groups, staphylococcal and staphylococcal/seborrheic, where high populations of S. aureus do occur.

Key Words: cholesterol, Staphylococcus aureus, chronic blepharitis

References

7. Russell NJ. Function of lipids: structural roles and


