Bacterial Lipases and Chronic Blepharitis

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Eyelids and conjunctivae of 36 normal individuals and 60 patients from six clinical groups of chronic blepharitis were cultured for aerobic and anaerobic bacteria. The most common species isolated were coagulase-negative staphylococci (C-NS) and Propionibacterium acnes. All strains of these species, and all Staphylococcus aureus strains isolated were tested for the ability to break down triglycerides, cholesterol esters, and fatty waxes. Each strain was incubated independently with appropriate substrates in nutrient media. Each medium was then extracted and assayed for the presence of substrate hydrolysis products by thin-layer chromatography. The percentage of strains capable of hydrolyzing a particular substrate was determined for each individual. S. aureus was a consistent and strong lipase producer, able to hydrolyze all three substrates. P. acnes was able to hydrolyze triolein and behenyl oleate but not cholesteryl oleate. No differences were observed among groups for P. acnes or S. aureus. C-NS showed a high degree of strain variability. Eighty-three percent of C-NS strains could hydrolyze triolein, 82% behenyl oleate, and 40% cholesteryl oleate. Significant group differences were seen in the percentage of lipase positive C-NS strains isolated per individual. Patients in the mixed staphylococcal/seborrheic, meibomian seborrheic, secondary meibomitis, and the meibomian keratoconjunctivitis (MKC) groups harbored significantly more C-NS strains capable of hydrolyzing cholesteryl oleate than did normal individuals. Patients in the meibomian seborrheic, secondary meibomitis, and MKC groups harbored significantly more C-NS strains capable of hydrolyzing behenyl oleate than did normals. No group differences were seen among groups with triolein hydrolyzing C-NS strains.

We have been studying chronic blepharitis in order to define the mechanisms as well as elucidate any underlying predisposing factors which could contribute to the pathophysiology of this common and difficult disease. In the past, chronic blepharitis has been grouped as seborrheic, staphylococcal, or a mixture of both. Staphylococcus aureus was thought to have been the primary etiological agent in both the staphylococcal and mixed types. We have found that blepharitis is not so easily categorized, but instead consists of an array of several complex interacting and sometimes overlapping clinical entities: staphylococcal; seborrheic, alone, with mixed staphylococcal, meibomian sebaceous, or secondary (spotty) meibomitis components; and meibomian keratoconjunctivitis (MKC). S. aureus has indeed been found to be a possible etiologic agent in the staphylococcal and mixed groups, but not the others. In fact, no other bacterial or fungal pathogens were found to be unique to any of these groups.

There is a high degree of association of sebaceous gland dysfunction in all of the seborrheic groups. Inflammation of the meibomian glands is present in secondary meibomitis and MKC. MKC patients also have plugged meibomian glands and an unstable tear film. These observations led us to hypothesize that changes seen in the tear film and the meibomian glands could be a function of lipid abnormalities or alterations of meibomian lipids (Meibum). (The term “meibum” has been suggested to describe the meibomian gland secretion.) Data generated in this laboratory show significant changes in some minor free fatty acids of meibum collected from patients in all of the seborrheic groups and MKC but not the “sebaceously” normal staphylococcal group.

The possibility exists that these changes are endogenous, ie, present in the meibum before it is released to the surface of the lids and globe. An alternative, or additional pathway, could exist: that bacteria normally present on the lids and conjunctivae alter the meibum through production of lipases and subsequent release of hydrolysis products. These products would reflect the make-up of meibum and could include free fatty acids, cholesterol, fatty alcohols, mono- and di-glycerides, and glycerol. Changes in these components could...
have a marked influence on the physical characteristics of the tear film, through surfactant properties or changes in lipid melting points. Free fatty acids, in addition, are quite irritating and toxic.

The theory just described is not a new idea. A vast dermatologic literature has addressed the role of bacterial lipases in chronic acne but the question remains unresolved. (An excellent review is provided by Voss.)

However, regardless of their role in acne vulgaris, it is quite clear that triglyceride lipases are responsible for the "acid mantle" which is characteristic of human skin surface lipid. Cutaneous Propionibacterium acnes has been demonstrated to be a major source of triglyceride lipase, and the enzyme has been characterized.

We have found P. acnes, in concert with coagulase-negative staphylococci (C-NS) to be the most common lid and conjunctival organisms. Staphylococcus epidermidis is also known to produce triglyceride lipases, and these too, have been characterized. There have been no reports of a cholesteryl de-esterifying enzyme in P. acnes or the C-NS, neither have there been reports of fatty wax esterases in any of these organisms. Nevertheless, it seemed reasonable to suggest that bacteria present on the lids and conjunctivae could alter meibum in the same fashion as the cutaneous flora alter sebum. Therefore, the purpose of this investigation was to assess the differences in lipolytic capabilities of our isolates. We report here for the first time the ability of S. aureus, coagulase-negative staphylococci, and P. acnes to de-esterify fatty waxes, and the ability of C-NS to de-esterify cholesteryl esters.

Materials and Methods

Patient Selection and Evaluation

Sixty patients with chronic blepharitis were given careful ophthalmic evaluation. Patients had had symptoms for at least 6 months and had received no therapy for at least 2 wk. Signs and symptoms were graded and recorded, and the patients classified according to type of chronic blepharitis as previously described. Thirty-six normal volunteers, free of ocular disease, served as controls. All patients and controls were cultured using the techniques described previously. Informed consent was obtained from all subjects prior to their participation in the study.

Bacterial Strains

Organisms selected for this study represented the most frequently isolated aerobic and anaerobic bacteria cultured from the lids and conjunctivae of normals and blepharitis patients—C-NS and Propionibacterium acnes. We also included all strains of Staphylococcus aureus isolated from patients and normals. The C-NS were not further speciated; however, different strains were identified by a combination of colony morphology and antimicrobial susceptibility pattern (antibiogram). All representative strains from each individual were tested for lipase activity. Different strains of P. acnes were identified by colony morphology alone, and all representative strains were tested. Aerobic strains of bacteria not used immediately in the lipase assay were maintained on brain heart infusion agar (Baltimore Biological Laboratories; Cockeysville, MD) slants at room temperature. Anaerobes were stored frozen at −70°C in Brucella broth (Baltimore Biological Laboratories) supplemented with 20% glycerol.

Media and Reagents

The substrates were three different lipid classes representative of meibum: triglyceride (triolein) (Sigma Chemical Co.; St. Louis, MO), cholesterol ester (cholesteryl oleate) (Sigma Chemical Co.), and fatty wax (behenyl oleate) (Alltech Associates Inc.; Deerfield, IL). Lipid standards used for thin layer chromatography (TLC) included the three substrates listed above, plus oleic acid, free cholesterol, cetyl alcohol, mono- and diol (Sigma Chemical Co.). Internal standards for gas-liquid chromatography (GLC) included tripalmitin, cholesteryl palmitate, and arachidyl stearate (Sigma Chemical Co.). All solvents used were glass distilled, suitable for lipid work, and purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). TLC plates were either precoated with silica gel 60 (hard plates), or silica gel H (soft plates), and purchased from E. Merck (Darmstadt, Germany) or Analtech (Newark, DE) respectively. The plates were activated at 120° for 30 min prior to use.

Aerobes were subcultured to Brucella agar (Baltimore Biological Laboratories) supplemented with 5% sheep blood 24 hr prior to assay of lipase activity. The assay medium contained 10 g of peptone (Difco Labs; Detroit, MI), 9.0 g NaCl, and 2.4 g yeast extract (Difco) per 1000 ml of deionized H₂O, (final pH = 7.5). Anaerobes were subcultured 72 hr before assay to Brucella blood agar supplemented with vitamin K/hemin (GIBCO; Grand Island, NY), and yeast extract; and incubated anaerobically. The assay medium consisted of 30 g thiglycollate 153C (Baltimore Biological Laboratories), 250 ml glycerol, and 1000 ml deionized H₂O, (final pH = 7.0).

Substrates dissolved in chloroform were added to 16 × 125 mm screw-cap tubes; one substrate (4 mg) per tube. Substrates were dried down under nitrogen prior to the addition of media to the tubes. Medium was
added to each tube containing dried substrate to give a final volume of 10 ml for anaerobes, and 4 ml for aerobes. The media were autoclaved at 15-lb pressure for 15 min.

Lipase Assay

Aerobes were assayed by a modified procedure developed by Reisner and Puhvel.13 Anaerobes were assayed by a modified method developed by Kellum and Strangfeld.14 The procedures are similar, and a brief description will follow. Organisms obtained from blood agar plates were suspended in saline and diluted to an optical density equivalent to a McFarland #1 nephelometer standard. One drop of this suspension was used to inoculate a tube of each substrate and a blank containing only medium creating a series of four tubes for each strain. Inoculated blanks were used to check interference by endogenous bacterial acids. Cultures were incubated at 35°C, and vortexed daily for 7 days. A series of four uninoculated tubes were incubated as controls to check auto hydrolysis of the substrate. At the end of the incubation period, each tube was extracted with three 1-ml volumes of chloroform:methanol (3:1). The extracts were combined and dried down with nitrogen, then redissolved in 50 μl of chloroform. The extracted lipids from each series of four tubes were applied to TLC plates in 10 μl amounts and bracketed with appropriate reference standards. Plates were developed in hexane:diethyl ether:acetic acid (75:25:1) for 30 min.

After drying, silica gel 60 plates were sprayed with sulfuric acid:ethanol (1:1) then heated to 125°C for 20 min. Plates were viewed under normal laboratory lighting, and the presence or absence of lipolytic end products was noted. A positive reaction was noted for triolein if both oleic acid, diolein, and mono-olein were present. A positive reaction for cholesteryl oleate was noted when both oleic acid and behenyl alcohol were present. A negative reaction was noted for behenyl oleate when both oleic acid and behenyl alcohol were present. A negative reaction was noted when no breakdown products were observed. The presence of small amounts of free fatty acids without the other accompanying by-products was considered a negative reaction. The preceding methods were checked for reliability by assessing the disappearance of substrate by GLC.

Silica gel H plates were sprayed with 0.2% dichlorofluorescein (in 95% ethanol) and viewed under ultraviolet light. Bands of gel corresponding to the substrates were scraped from the plate into small screw-capped tubes. Appropriate internal standards were added at this point. The substrates were extracted from the gel with three 1-ml volumes of chloroform. The extracts were combined and taken to dryness with nitrogen then redissolved in 200 μl of chloroform. GLC analyses were performed isothermally in a Hewlett-Packard 5710A Gas Chromatograph (Hewlett-Packard, 1200B, Palo Alto, CA) equipped with dual flame ionization detectors. Columns were stainless steel (½" X 18") packed with Supelcoport (100/120 mesh) coated with 1% Dexsil 300 (Supelco Inc.; Bellefonte, PA). The carrier gas was Nitrogen at 20 ml/min. Injection ports were maintained at 350°C; the detectors at 400°C. Oven temperature varied with the substrate as follows: behenyl oleate, 275°C; cholesteryl oleate, 300°C; triolein, 325°C. Peak area was determined by integration relative to the internal standard with a Hewlett-Packard 3390A Reporting Integrator. Loss of substrate was confirmed by comparison of the inoculated series with the uninoculated control series.

Statistical Evaluation

For each substrate, the number of positive strains (of a given species) was divided by the total number of strains isolated from an individual. This gave the percentage of strains (of that species) capable of hydrolyzing a particular substrate for that individual. Individual percentages were collated by group and rank ordered. Differences between groups were analyzed by Mann Whitney U (one-tailed test).

Results

Patient Selection and Evaluation

Patients fell into six clinically distinct groups of chronic blepharitis as described in detail elsewhere.2 The number of patients from each group in this study was as follows: staphylococcal alone, 15; seborrheic, 7; mixed staphylococcal/seborrheic, 6; seborrheic with meibomian seborrhea, 11; seborrheic with secondary (spotty) meibomitis, 10; and MKC, 11.

Lipase Activity

Five hundred thirty strains of bacteria were tested for lipase activity: these included 22 strains of S. aureus; 324 strains of C-NS and 184 strains of P. acnes. S. aureus was a consistent and strong lipase producer, able to hydrolyze all three substrates. No negative strains were encountered. P. acnes was able to hydrolyze triolein and behenyl oleate but not cholesteryl oleate. (Ninety-nine percent of these strains were able to hydrolyze triolein, and 90% were able to hydrolyze behenyl oleate). No clinical group differences were observed with P. acnes. C-NS strains showed a high degree of strain variability. Approximately 83% of all strains tested were able to hydrolyze triolein; 82% were able to hydrolyze behenyl oleate; and 40% could break...
down cholesteryl oleate. Cholesteryl de-esterifying ability when present was weak compared to S. aureus. Forty-one percent of all C-NS strains were able to hydrolyze all three substrates. Also, forty-one percent were able to hydrolyze both triolein and behenyl oleate but not cholesteryl oleate. Seven percent were able to hydrolyze only behenyl oleate. Six percent were unable to hydrolyze any substrate.

Several group differences were apparent in the percentage of C-NS strains per individual capable of producing lipases. Patients in the mixed staphylococcal/seborrheic, meibomian seborrheic, secondary meibomitis, and MKC groups harbored a significantly greater percentage of C-NS strains capable of hydrolyzing cholesteryl oleate than did normal individuals, (P < 0.05) (Table 1). Patients in the meibomian seborrheic, secondary meibomitis, and the MKC groups harbored a significantly greater percentage of C-NS strains capable of hydrolyzing behenyl oleate than did normal individuals (P < 0.03) (Table 2). No significant group differences were seen among individuals harboring C-NS strains capable of hydrolyzing triolein (Table 3).

Gas chromatography confirmed the reliability of assessing lipase production by thin-layer chromatography. In all cases where hydrolysis products were detected on TLC, there was a concomittant decrease in substrate as measured on GLC. Controls showed no hydrolysis products on TLC and no loss of substrate on GLC.

**Discussion**

This is the first time a comparative study has been undertaken to assess the differential lipolytic capabilities of the ocular flora. It represents the first report of the cholesteryl de-esterifying ability of the coagulase-negative staphylococci, and the fatty wax de-esterifying abilities of S. aureus, P. acnes, and the C-NS.

Based on this study, it is apparent that the ocular flora possess an enormous potential for altering meibum. Whether these alterations occur in vivo, and to what extent, cannot be determined from this study. However, with the large quantity of lipid material available, and the bacterial lipase production found in this study, in vivo meibum alteration is probable. How much of a role these lipases play in the pathophysiology of chronic blepharitis is not known. However, the greatest bacterial lipolytic activity was found in those patients with meibomian gland abnormality, ie, chronic blepharitis with meibomian seborrhea, secondary meibomitis, and MKC. We have also found changes in
the free fatty acid fraction of expressed meibum in these patients. Bacterial lipases would probably have a more profound effect on the tear lipid layer. Therefore, it would be necessary to study tear film lipids directly to assess the full effects of lipase.

Lipases probably play some role in the normal state, since a high percentage of normal flora possess them. The constant flushing of the globe surface with aqueous tears and fresh meibum may prevent deleterious effects by these "normal" lipases. However, plugging of the meibomian glands or an abnormal preformed meibum may lead either to induction of increased lipase production, or changes in the lipolytic-capable flora.

There may be strains of pathogenic or opportunistic C-NS. Members of this group have been shown to cause disease. Newer methods of identification have been developed for this group, and in the future, it may be possible to identify the more virulent members of this genus. We have found a significantly increased number of tetracycline resistant strains of C-NS in the MKC group. Tetracycline resistance could be a marker for increased virulence in these strains. This may also have implications in the treatment of MKC, since it has been shown (at least with S. aureus) that tetracycline can inhibit the production of bacterial lipases at concentrations below the minimum inhibitory concentrations.

As we noted in the results, hydrolysis of cholesteryl oleate by C-NS strains apparently is far less vigorous than S. aureus. It has been shown that cutaneous S. epidermidis possess the ability to esterify cholesterol. Indirect evidence suggests that fatty acids from sebaceous triglycerides and free cholesterol from epidermal lipids are utilized for esterification by these bacteria. It may be that these enzymes are as important as deesterifying enzymes in contributing to the disease process. There is very little free fatty acid or cholesterol in meibum. However, bacterial interactions could take place in such a way that acids released by P. acnes were used by C-NS strains in esterification of free or epi-dermal cholesterol. Therefore the processes could be complicated by these bacteria-lipase interactions and difficult to assess totally in vitro.

Kellum has shown relative toxicity or irritancy of free fatty acids of different chain lengths, ie, fatty acids of intermediate chain lengths (C₈ to C₁₄) produce greater irritancy to human skin than shorter or longer fatty acids. It has also been shown that some strains of P. acnes have differential ability to hydrolyze different chain length triglycerides in vitro. If a differential ability exists, a primary abnormality of the meibum (for example a shift in chain lengths), may select for different lipolytic strains. Alternatively, the abnormality could be such that hydrolysis of the lipid classes released products of a chain length of greater irritancy or toxicity than that found in normals. Given the sensitivity of the ocular tissues, increased bacterial lipolysis alone or in concert with an abnormal secretion could contribute to the inflammation and irritation characteristic of this disease. This study showing an increase in bacterial lipolytic activity in patients with meibomian gland abnormalities and our previous work showing a shift in some of the free fatty acids support this view.

Key words: esterase, lipase, staphylococci, propionibacterium, blepharitis, meibomitis, meibomian lipids

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References


Table 3. Percentage of triolein hydrolyzing coagulate negative staphylococcal strains/individual

<table>
<thead>
<tr>
<th>Percentage positive Strains/individual</th>
<th>NORM</th>
<th>STAPH</th>
<th>SEB</th>
<th>MIX</th>
<th>MEIB</th>
<th>2° MEIB</th>
<th>MKC</th>
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<td>18</td>
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<td>18</td>
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<td>29</td>
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<td>71</td>
<td>50</td>
<td>73</td>
<td>80</td>
<td>64</td>
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* No significant differences among groups.

Key to Abbreviations: NORM = Normal controls; STAPH = Staphylococcal; SEB = Seborrheic; MIX = mixed staphylococcal/seborrheic; MEIB SEB = Meibomian seborrhea; 2° MEIB = Secondary (spotty) meibomitis; MKC = meibomian keratoconjunctivitis.