Cytotaxin Production by Comedonal Bacteria (Propionibacterium acnes, Propionibacterium granulosum and Staphylococcus epidermidis)

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The potential role of different species of comedonal bacteria as chemotactic stimuli in the inflammatory phase of acne vulgaris was investigated by comparing 12 strains of Staphylococcus epidermidis, 11 strains of Propionibacterium acnes, and 5 strains of P. granulosum for production of cytotaxin in vitro. Results indicated that not only were there marked differences in cytotaxin production between different strains of the same species grown under identical growth conditions, but there were often significant differences in cytotaxin activity of the same strain grown in different media. This finding is discussed in relation to development of inflammation in quiescent comedones in acne vulgaris.

This paper is an extension of our investigations of the comedonal factors which may be involved in initiating the change to the inflammatory phase in quiescent comedones in acne vulgaris. Recently we analyzed the relative chemotactic properties of different comedonal components for human polymorphonuclear (PMN) leukocytes and found that the dialysable, small molecular weight, cytotaxis produced by Propionibacterium acnes, represented potentially the most active chemotactic stimulus present in comedones [1]. In that study P. acnes UCLA strain #79 was the only species of bacterium tested as a representative comedonal microorganism. However, it is well recognized that comedones typically harbor combinations of three bacterial species, P. acnes, Propionibacterium granulosum, and Staphylococcus epidermidis [2]. Although production of cytotaxins by strains of S. epidermidis has been reported previously [3,4], little is known about the relative significance of these bacteria as potential chemotactic agents in acne.

The present study compared numerous strains of all 3 species of comedonal bacteria for cytotaxin production in vitro, in an effort to evaluate the potential significance of these species as possible instigators of inflammation in acne vulgaris.

MATERIALS AND METHODS

Eleven strains of P. acnes, 12 strains of S. epidermidis and 5 strains of P. granulosum were used in this study. All but one of the S. epidermidis strains were isolated in our laboratory, 8 from comedones extracted from subjects with acne, and 3 from facial skin of acne-free subjects. Strain BII, originally isolated from dust, was obtained from A. C. Baird-Parker, Unilever Research Laboratories, Sharnbrook, Bedford, England, and was a prototype strain of S. epidermidis biotype 1. All strains of S. epidermidis used were classified as belonging to biotype 1, according to standard procedures [5].

Of the P. acnes strains, 7 were isolated from comedones extracted from patients with acne, and 4 were isolated from nonacne skin. All were identified by biochemical tests [6] and by sensitivity to P. acnes specific bacteriophages.

Four strains of P. granulosum were originally obtained from Dr. J. G. Voss (formerly at the Procter and Gamble Company, Cincinnati, Ohio). The fifth strain was isolated in our laboratory from facial skin of a nonacne subject and was identified by biochemical tests [6] and by resistance to P. acnes bacteriophages.

In this paper the term "strain" is used synonymously with the term "isolate."

Media for Cytotaxin Production

Human callus extract (HCE) and Marshall-Kelsey broth were chosen as growth media for bacterial cytotaxin production. Human callus extract consisted of aqueous extracts of 10-50 g of tissue (of 0.4 to 5.5 ml) in a Coleman Jr. spectrophotometer of P. acnes and P. granulosum for cytotaxin harvest was 5 days, for S. epidermidis, 48 hr.

Preparation of Cytotaxin

Eleven strains of P. acnes and 5 strains of P. granulosum were grown for 5 days anaerobically under 90% nitrogen and 10% carbon dioxide. Each strain was grown in duplicate culture tubes containing 3 ml of HCE, and 3 ml of Marshall-Kelsey broth. Inocula consisted of suspension of identical turbidity optical density of 0.04 at 550 A in a Coleman Jr. spectrophotometer of P. acnes and P. granulosum strains which had been grown for 96 hours on brain heart infusion (BHI) slants and washed 3 times with sterile saline.

After 5 days incubation the optical densities and viable bacterial counts were established. Optical densities were read in a Coleman spectrophotometer at 550 A, and bacterial counts were made by serial 10-fold dilutions of bacterial suspensions in BHI broth, drop inoculate on BHI plates. Following sampling for quantification of bacterial growth, cultures were centrifuged, supernatants were decanted and stored frozen at -10°C until used for chemotactic assays.

Strains of S. epidermidis were processed in the same manner except that the inocula were prepared from 24 hr cultures, and incubation for cytotaxin production was carried out for 48 hr aerobically.

To establish the effect of dialysis on the chemotactic activity...
culture supernatants, one ml aliquots were removed from the cleared supernatants of 6 strains of *S. epidermidis*, 5 strains of *P. acnes* and 2 strains of *P. granulosum* prior to freezing. These aliquots were dialysed at 4°C for 48 hr against distilled water, and were then stored at −10°C until used in chemotactic assays.

Duplicate 3 ml samples of sterile Marshall-Kelsey broth and sterile HCE were always processed together with the inoculated cultures (i.e., incubated, dialyzed, frozen) for use as the negative controls in the chemotactic assays.

**Chemotactic Assays**

The methods used for chemotactic assays have been described in a previous publication [1]. PMN were obtained by Plasmagel sedimentation of heparinized blood from healthy volunteer donors. The final cell concentration in RPMI 1640 medium (GIBCO) was adjusted to 2.5 × 10^6 cells per ml. Zymosan activated serum (ZAS) was used as the positive control stimulus for chemotaxis with each set of PMN tested. Solutions of 10 mg of zymosan (Nutritional Biochemicals, Cleveland, Ohio) per ml of guinea pig serum were incubated at 37°C for 30 min, centrifuged, and the supernatants incubated for an additional 30 min at 56°C. Identical samples of guinea pig serum without the addition of zymosan underwent the same sequence of incubations and centrifugations as the ZAS, and served as the negative control solution in the ZAS chemotaxis assay.

The agarose method of neutrophil chemotaxis was used as previously described [1]. All test substances and the positive ZAS control were assayed in triplicate chambers. Following incubation for 3 hr at 37°C in a humidified atmosphere of 5% carbon dioxide, cell counts were made by counting the number of cells which had migrated toward the contralateral test substance. A 5 × 5 eyepiece grid aided in orientation of cells for counting. Results were calculated in terms of the average absolute number of cells which had migrated, which were then converted to percentages of the number of cells which had migrated in the corresponding positive ZAS control tests, which was taken to represent 100% migration.

It has been reported that extracts of heat inactivated suspensions of *P. acnes* may release chemotactic substances into aqueous solution upon storage [8]. For this reason, representative bacterial strains from each species were chosen at random and tested for the possibility that the chemotactic factor thought to be an actively secreted cytotaxin was instead a product of cell autolysis during incubation. Suspensions of *P. acnes* and *S. epidermidis* were harvested from BH agar slants, washed 3 times with sterile saline, heat inactivated at 60°C for 1 hr, and then added to 3 ml aliquots of duplicate tubes physiologic saline, HCE and Marshall-Kelsey broth, so that the final optical density of the suspensions approximated that of the cultures used for supernatant analysis. The suspensions of heat killed bacteria were incubated at 37°C for 3 days anaerobically, and for 48 hr aerobically for the *P. acnes* and *S. epidermidis* respectively. After incubation the supernatants were separated by centrifugation and tested for chemotactic activity directly.

**RESULTS**

The chemotactic activity of all supernatants tested was expressed as a percentage of activity elicited by ZAS which was the positive control with each set of PMN and was taken to represent the maximal response elicited.

Figure 1 summarizes the results of growth curve studies which correlated cytotaxin production by representative strains of the species studied, with bacterial growth. As can be seen, the curve for cytotaxin production followed the bacterial growth curve, reaching a peak at 5 days for the propionibacteria, and at 48 hr for *S. epidermidis*. When *S. epidermidis* cultures were incubated beyond 48 hr, the cytotaxin activity peaked to a second plateau at 96 hr, even though bacterial growth declined. This second peak was felt to represent release of cytotaxin material by cellular autolysis.

At the completion of our comparative studies, we repeated the growth curve studies with 2 strains of *P. acnes* and 2 of *S. epidermidis*. With each species one strain had been found to be a "high" and the other a "low" cytotaxin producer. Culture samples obtained at 24-hr intervals were assayed for bacterial growth and cytotaxin activity for a total of 5 days for staphylococci and 9 days for propionibacteria. Repetition of the preliminary growth curve studies confirmed the fact that 48 hr and 5 days were indeed the optimal times for cytotaxin harvest for staphylococci and propionibacteria respectively. These studies also demonstrated that cytotaxin curves paralleled the growth curves regardless of the activity of the strains.

To verify that cytotaxin production was an active process dependent on biologically viable bacteria and not the result of release of peptides of autolyzing bacteria, suspensions of washed heat killed *P. acnes* and *S. epidermidis* were "incubated" in saline in the media used for cytotaxin production under conditions identical to those used for cytotaxin production. Results of chemotaxis assays with supernatants of these experiments are summarized in Table I. No chemotactic factors were eluted from the heat killed bacteria during the "incubation," indicating that cytotaxin production was a function of viable cells.

The amount of cytotaxic activity exhibited by the different strains of the 3 species of comedonal bacteria varied considerably (see Table II). Of the 5 strains of *P. granulosum* tested, cytotaxic activity was low in 4 and high in 1. With *P. acnes* and *S. epidermidis* strains there were greater variations. As can be seen from Table II, growth of all 3 species was consistently heavier in Marshall-Kelsey broth than in HCE, but density of bacterial growth was not the prime determinant of the level of cytotaxin activity (see Fig 2 and 3). The medium itself appeared to be more significant in this regard. Thus the highest mean cytotaxon activity value was seen with *P. acnes* strains grown in HCE. With mean optical density of 0.210 the mean (± standard deviation) value for cytotaxin activity was 75.2 ± 38.6%. By contrast, the same strains grown in Marshall-Kelsey broth had a mean optical density of 0.997, but a mean (± standard deviation) cytotaxin activity of 56.3 ± 39.0%.

**Table I. Chemotactic activity of supernatants from viable vs. heat killed organisms**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>0.8% NaCl</th>
<th>HCE</th>
<th>M-K</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>not done</td>
<td>75.5%</td>
<td>60.5%</td>
</tr>
<tr>
<td><em>P. acnes</em> #79</td>
<td>2.7%</td>
<td>7.3%</td>
<td>3.9%</td>
</tr>
<tr>
<td><em>P. acnes</em> #79</td>
<td>not done</td>
<td>127.0%</td>
<td>90.0%</td>
</tr>
<tr>
<td><em>P. acnes</em> #79</td>
<td>3.5%</td>
<td>1.8%</td>
<td>2.3%</td>
</tr>
</tbody>
</table>

Chemotactic activity of supernatants of heat killed suspensions of *S. epidermidis* IX and *P. acnes* UCLA #79, in saline solution, human callus extract (HCE) and Marshall-Kelsey broth (M-K), compared to chemotactic activity of supernatants of viable cultures of the same organisms in the same media. Chemotactic activity is expressed as percentages of counts stimulated by zymosan activated serum controls.
relationship of optical density and cytotactin values for all 3 species is summarized in Table III. With P. acnes strains tested, HCE was clearly a superior medium for stimulation of cytotactin production. With S. epidermidis strains the results were not as clear cut. Out of 12 strains tested, Marshall-Kelsey had a stimulatory effect in 3 strains, HCE had a stimulatory effect in 2 strains and in 7 strains, the results were within the same range of activity with both media. By "stimulation" of cytotactin production we mean that the cytotactin activity of the supernatant from cultures grown in one particular medium had at least 30% more chemotactic activity than the same strain grown in the alternate medium.

We did not succeed in growing P. acnes and P. granulosum in defined media. For this reason we used HCE and Marshall-Kelsey broth. Because the water soluble extract from callus (HCE) is a relatively undefined medium, there existed the possibility that the cytotactic activity of the culture supernatants may have resulted from the production of chemotactically active peptides from the reduction of proteins in the media by bacterial proteases. If this would have been the case, then bacterial protease activity in vitro should have correlated with the cytotactic activity of the culture supernatants. We tested for this possibility by assessing the protease activity of all the strains involved, using the method of Sokol, Ohman, and Iglewski [9]. Protease activity could not be correlated with cytotactic activity of the specific strains.

Dialysis of culture supernatants resulted in significant loss of chemotactic activity with all supernatants tested (see Table II).

**DISCUSSION**

The stimulus for the initiation of inflammation in closed micro-comedones in acne vulgaris is poorly understood. Closed comedones can remain uninfamed for indefinite periods of time. Alternatively they can become inflamed while still in microscopic dimensions. The clinical observation that inflammatory acne can often be improved by administration of antibiotics and other antimicrobials, implicates sensitive micro-

![Figure 2](image_url)

**Fig 2.** Correlation between optical density of bacterial cultures in human callus extract (HCE) and cytotactic activity of culture supernatants.

![Figure 3](image_url)

**Fig 3.** Correlation between optical density of bacterial cultures in Marshall-Kelsey broth and cytotactic activity of culture supernatants.
organisms as having a role in the pathogenesis of the inflammatory phase of this disease. Until recently a widely advocated theory was that free fatty acids which accumulated in comedones as a result of bacterial lipolysis of sebaceous triglycerides were the prime stimuli for inflammation. More recent research however, suggests that this concept is improbable. Reduction of in vitro follicular fatty acid levels by administration of antilipase agents has proved to be clinically ineffective in alleviating acne vulgaris [10]. The concentrations of fatty acids which can be quantitated in comedones are considerably lower than concentrations required to produce inflammation in vivo in human skin [11,12] and purified sebaceous fatty acids were not effective chemotactic agents in in vitro assays [1]. Interest has instead focused on the comedonal bacteria themselves, particularly on P. acnes, as direct instigators of inflammation through chemotactic activity [13-16].

Several studies have shown that P. acnes is a potent cytotaxigen, capable of activating chemotactic serum factors, primarily C5, through both the classical as well as the alternate pathways of complement activation [1,15,16]. Thus exposure of P. acnes to complement in perifollicular tissues would result in C5 activation and would be a definite contributing factor in the inflammatory sequence in acne.

However, at the present time there is no evidence that complement is present in intact comedones. Assays in our laboratory for the presence of complement in open comedones have given negative results. Concentrated aqueous extracts of 8 different batches of open comedones (each batch consisting of 20 to 50 mg of comedonal material pooled from several patients) tested for the presence of C3, using commercial immunoplate radial immunodiffusion tests (with lower assay limits of 150 ug of C3/ml) have not detected C3. This despite the fact that the same extracts have given positive results for the presence of immunoglobulin G (in the range of 0.55 to 2.7 ug of IgG per mg of comedonal crude weight) in similar assays (Puhvel, unpublished observations).

Thus, until it can be demonstrated that complement is actually present in pre-inflammatory closed micro-comedones, cytotaxigenic activity of P. acnes does not appear to be a satisfactory explanation of initial bacterial instigation of inflammation in acne. The production of cytotaxins by comedonal bacteria appears to be a more likely possibility. Cytotaxins are substances which stimulate chemotactic activity independently, and do not require the additional presence of serum factors. Numerous bacteria are known to produce cytotaxins [3,4,17] and these cytotaxins of Escherichia coli have been analyzed in some detail [17].

It is conceivable that cytotaxins which are produced by viable, propagating bacteria in micro-comedones may accumulate and induce the migration of PMN to the quiescent lesion. This is more likely to occur in the closed rather than the open comedo, for in the open comedo comedonal contents are constantly eliminated through the patent opening to the skin surface [18] and soluble material (including cytotaxins) could theoretically be eluted by action of sweat and washing.

The present study demonstrated that there are significant variations in the capacity for cytotaxin production by different species and strains of comedonal bacteria. By using standardized inocula and comparative growth curves, we feel our results give fair estimation of the relative chemotactic potential of comedonal bacteria in so far as can be simulated by in vitro studies. Strains of all species of comedonal bacteria, but particularly strains of P. acnes and to a lesser degree S. epidermidis, were effective in the production of cytotaxins. The production of cytotaxin was affected by the substrates for bacterial growth and growth of P. acnes in human callus extract resulted in highest mean levels of cytotaxin production suggesting that for this species, the water soluble substances present in the epidermis form suitable substrates for cytotaxin production in vivo. The fact that certain strains of P. acnes (at densities of 2.6 to 3.1 x 10^8 organisms/ml) produced chemotactic activity which exceeded the chemotactic activity of zymosan activated serum by 26 to 27%, is strong indication that the potential for cytotaxin production by comedonal bacteria may have real significance in vivo in the pathogenesis of acne vulgaris.

The marked variation in cytotaxin production by different strains of the same species grown under similar growth conditions, together with the occasional variations in cytotaxin production by the same strains grown in different media, may offer a possible explanation for why comedones with seemingly similar bacterial flora differ in their propensity for inflammation.

We thank Jorge Mazo for helping us obtain blood samples for this study.

REFERENCES


<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Optical Density</th>
<th>Range of O.D.</th>
<th>Mean Chemo-tax</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>S. epidermidis (12 strains)</td>
<td>HCE</td>
<td>0.123</td>
<td>0.047-0.258</td>
<td>45.0%</td>
<td>11.0-121.0%</td>
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<td></td>
<td>M-K</td>
<td>0.66</td>
<td>0.450-0.810</td>
<td>52.2%</td>
<td>2.5-112.5%</td>
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<tr>
<td>P. acnes (11 strains)</td>
<td>HCE</td>
<td>0.210</td>
<td>0.114-0.331</td>
<td>75.2%</td>
<td>23.5-157.9%</td>
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<tr>
<td></td>
<td>M-K</td>
<td>0.997</td>
<td>0.730-1.100</td>
<td>56.3%</td>
<td>16.5-132.5%</td>
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<tr>
<td>P. granulosum (5 strains)</td>
<td>HCE</td>
<td>0.205</td>
<td>0.130-0.259</td>
<td>44.8%</td>
<td>15.5-120.8%</td>
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<tr>
<td></td>
<td>M-K</td>
<td>0.846</td>
<td>0.655-0.990</td>
<td>33.7%</td>
<td>2.0-5.5%</td>
</tr>
</tbody>
</table>

Mean and range of optical densities (at 550 λ) of 12 strains of S. epidermidis, 11 strains of P. acnes, and 5 strains of P. granulosum, grown in Human Callus Extract (HCE) and Marshall-Kelsey broth (M-K), related to the mean and range of cytotaxin activity exhibited by the supernatants of these cultures for human polymorphonuclear leukocytes, in the agarose assay for chemotaxis.