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CHARACTERIZATION OF SERUM-INDEPENDENT POLYMORPHONUCLEAR LEUKOCYTE CHEMOTACTIC FACTORS PRODUCED BY *Propionibacterium acnes*

GUY F. WEBSTER[1] and JAMES J. LEYDEN[2]

Department of Pathology, School of Dental Medicine,[1] and Department of Dermatology, Duhring Laboratories, School of Medicine,[2] University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract—The size and production of polymorphonuclear leukocyte (PMN) chemotactic factors by *Propionibacterium acnes* has been studied. All eight strains of *P. acnes* which were tested liberated PMN chemotactic factors in their growth culture media. The factor(s) produced by one strain, 6919, were studied in greater depth. The PMN response was proportional to the dose of culture supernatant and chemotactic activity increased with the duration of *P. acnes* culture. The neutrophil migration towards culture supernatants was chemotactic, with a slight stimulation of random migration by 72-h supernatants. The size of the chemotactic molecules was studied through dialysis, ultrafiltration, and Sephadex G-25 chromatography of 72-h supernatants. Low-molecular-weight chemotactic factors were found to be predominant by each method of size determination.

INTRODUCTION

Propionibacterium acnes is thought to be the stimulus for inflammation in acne vulgaris. It has been shown to trigger lysosmal release (1); activate complement (2–4), producing complement-derived polymorphonuclear leukocyte (PMN) chemotactic factors; and to produce PMN chemotactic factors as a metabolic by-product (5–9). The nature of these complement-independent factor(s) is not certain. Lee and Shalita (8) found that the majority of chemotactic activity in *P. acnes* growth culture supernatants coincided with the gel filtration peak for lipase, a high-molecular-weight protein. Conversely, Puhvel and Sakamoto (9) found *P. acnes*-produced chemotactic factors to be completely dialyzable and, therefore, of a low molecular weight.

The size of the chemotactic factor would greatly affect its ability to

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diffuse through tissue and incite inflammation of the comedo. We have partially characterized *P. acnes* chemotactic factors with regard to their production by various strains, kinetics of production, and size.

MATERIALS METHODS

Microbiological Methods. *P. acnes* strains American Type Culture Collection 6919, 6922, 29399B, VPI (from Virginia Polytecnic Institute and State University, courtesy of C.S. Cummins) 3706, 6639, 0162, Duhring Laboratories 51022, and a fresh isolate from the face of an acne patient, LA, were maintained in chopped meat broth under CO_2. Culture supernatants for chemotaxis studies were prepared by inoculating 10 ml of medium 1066 (GIBCO, Grand Island, New York), pH 7.0, with a drop of a washed cell suspension in 1066. 1066 is a completely dialyzable tissue culture medium which supports vigorous *P. acnes* growth and has no inherent chemotactic activity. Culture tubes were flushed with CO_2, stoppered, and incubated at 37°C for the desired period, after which bacteria were removed by centrifugation, the supernatant was filtered through a 0.45 μm nitrocellulose filter (Millipore Corp., Bedford, Massachusetts) and frozen in small aliquots. All supernatants contained lipase activity, which was detected by production of clearing of a tributyrin emulsion in 1% Noble agar.

Chemotaxis. The chemotactic assay was performed in a Sykes-Moore chamber with a 3-μm nitrocellulose filter (Millipore) as previously described (3). PMNs were prepared from the buffy coat of heparinized blood and washed twice in physiological saline. PMN donors were all substantially acne-free, healthy adults who were not receiving medication. The concentration of PMN was adjusted to 5×10^6/ml 1066 with 10% heat-inactivated fetal calf serum (GIBCO), pH 7.0 After incubation at 37°C, filters were removed from the chambers, fixed in a graded series of alcohols, and stained with hematoxylin and eosin. Migration of PMNs was scored by counting the number of PMNs in the layer of cells which had migrated farthest from the cell side of the filter in 10 high-power fields. Results are expressed as this value minus the number of PMNs migrating in response to 1066 alone (chemotactic index). All tests were run in duplicate. After each test the coverslip at the bottom of the chamber was stained and examined for PMNs which had migrated completely through the filter and fallen into the attractant medium (drop-through). Before testing or treatment, the pH of all solutions was adjusted to 7.0.

The optimal chamber incubation time was determined in a preliminary study in which a 50% solution of a 72-h *P. acnes* 6919 supernatant was tested at 30, 60, 90, 120, 150, and 180 min. The maximal response occurred after a 180-min incubation and, although cells had migrated nearly the width of the filter, there was minimal to no drop-through. A 180-min incubation period was used in the balance of the experiments.

The ability of *P. acnes* strains to produce chemotactic factors was screened by testing 25% concentrations of 72-h supernatants of all strains with the cells from a single donor. Due to its easy availability and use in preceding studies, *P. acnes* 6919 was chosen for all subsequent studies. The effect of culture age and supernatant concentration on the migratory response of PMNs was tested by using 24- and 72-h supernatants at 6.25, 12.5, 25, and 50% concentrations. The size of the chemotractant(s) was investigated by subjecting 10-ml volumes of 72-h supernatant to dialysis against 2 liters of 0.85% saline at 4°C for one day and sterile distilled water for one day. Dialysis tubing with retention limits of less than 3500, 6000–8000, and 12,000–14,000 molecular weight was employed (Spectropore, Fisher Scientific, Pittsburgh, Pennsylvania). After dialysis the tubing contents were decanted and pooled with distilled water washes of the inside of the tubing and then lyophilized. Lyophilized dialysates were restored to their original concentration with 10 ml of 1066. An untreated control consisted of 10 ml of 72-h su-

pernatant which was kept at 4° C for 2 days and then lyophilized; this preparation was restored to its original concentration with 10 ml distilled water. The chemotactic activity of these preparations was tested at 25% concentrations in 1066.

Ultrafiltration of culture supernatants was also used to determine the size of the chemotactic factors. Three-day culture supernatants were filtered to 0.5 ml volume through Amicon UM2, UM10, and PM30 filters with retention limits of 2000, 10,000, and 30,000 mol wt. The material remaining within the ultrafiltration cell was adjusted to 10 ml volume with 1066, as was the filtrate. Both solutions were tested for chemotactic activity.

Sephadex Filtrations. Thirty ml of 72-h cultures of 6919 and 3706 were lyophilized and redissolved in 3 ml of pH 4.0 acetic acid, and applied to a 3 × 18-cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The column was eluted with pH 4.0 acetic acid at 0.4 ml/min and collected in 5-ml fractions. Acetic acid at pH 4 was employed to retard microbial contamination. Absorbance at 280 nm of the fractions was measured and fractions were frozen until chemotaxis assay. Molecular weight standards of Blue Dextran (2×10^6 mol wt, detected at 254 nm), RNAse A (13,500 mol wt, 280 nm), and riboflavin (366 mol wt, 280 nm) were immediately run through the column. It was not possible to assay all 80 fractions at the same time, and repetitive assay with different PMN donors was extremely impractical. Fractions to be assayed for chemotactic activity were selected to parallel closely the A_{280} curve. The fractions were suspended at 25% in 1066, adjusted to pH 7.0, and assayed for chemotactic activity.

Whether true chemotaxis was occurring was tested by incubating PMNs in chambers with equal concentrations of 24- and 72-h supernatant above and below the filter and comparing the response with that when a gradient was present.

The effect of chemotaxis preparations on the viability of PMNs was tested by trypan blue exclusion. PMNs were incubated with the highest concentration of each chemotactic preparation for 180 min and then tested for viability with a 1.2 dilution of 0.4% trypan blue in saline and compared to PMN which had been incubated in 1066. The release of lactate dehydrogenase (LDH), a marker of cell death, was also assayed by standard methods (10) in all preparations except the Sephadex-filtered fractions.

RESULTS

The 72-h supernatants of eight strains of *P. acnes* were tested for the ability to attract neutrophils. All strains were able to attract PMNs, but varied in their activity (Table 1). Strain LA was the most potent with a chemotactic index of 480; strain 0162 was less potent with an index of 155.

In order to test whether the PMN migratory response was chemotactic or chemokinetic, 24- and 72-h 6919 supernatants were assayed with equal concentrations of supernatant above and below the filter in the chemotactic chamber. The absence of a concentration gradient of supernatant across the filter produced a 99 and 83% diminution in chemotactic index, respectively (Table 2). With both 24- and 72-h supernatants, the absence of a gradient also greatly decreased the depth of penetration of the filter by PMN. A slight stimulation of random migration by 72-h supernatant was noted.

The effect of culture duration and supernatant concentration on the response of PMNs was tested using 24- and 72-h *P. acnes* 6919 supernatants

Table 1. Chemotactic Activity of *P. acnes* Strains

Strain	Chemotactic index[a]
6919	337
29399B	325
3706	333
0162	165
6922	261
6639	295
51022	155
LA	480

[a]Chemotactic index is defined as the number of PMNs in 10 high-power fields. The value shown is that of the test substance minus that of the buffer control.

in varying concentrations. PMNs from four donors were tested. The migratory response of PMN was proportional to the supernatant concentration and was greater for the 72-h supernatant at each concentration in all donors. The results of a representative experiment are presented in Figure 1. Variation between donors was less than 10%.

The nature of *P. acnes* 6919 chemotactic factors was investigated by dialyzing 72-h culture supernatants in tubing with retention cut-offs of 3500, 6000–8000, and 12,000–14,000 molecular weight and comparing their activity with undialyzed supernatant (Table 3). An average of 59.5 ± 1.5% of chemotactic activity was lost during dialysis in 3500 mol wt cut-off tubing; an additional 11.5 ± 9% was lost after 6000–8000 mol wt cut-off dialysis. The 12,000–14,000 mol wt cut-off dialysis tubing had approximately the same value, with a loss of 13 ± 6.5% of chemotactic activity over the 3500 cut-off tubing. PMNs from three donors were tested.

Table 2. Effect of Concentration Gradient on Migratory Response of PMN[a]

	Supernatant concentration (%)		
	Upper chamber	Lower chamber	Chemotactic index
24-hour	0	25	42
	25	25	0.3
72-hour	0	25	263
	25	25	45

[a]The 24- and 72-h supernatants were tested in single donors in separate experiments.

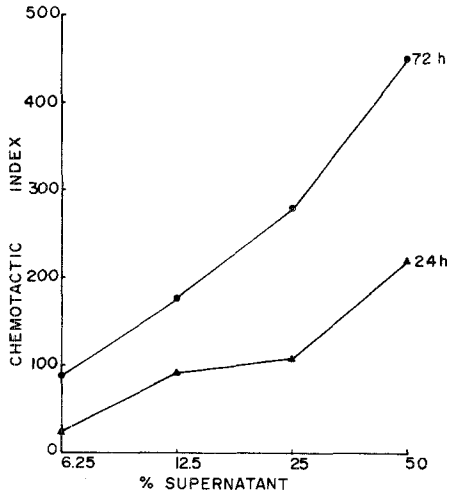

Fig. 1. Effect of the duration of *P. acnes* culture on the production of PMN chemotactic factors and the response of PMNs to varying doses of *P. acnes* culture supernatant.

Ultrafiltration with a filter retaining molecules greater than 2000 mol wt allowed 77 ± 5.5% chemotactic activity to pass into the filtrate; 38.5 ± 26.6 was retained within the cell. Filtration retaining molecules greater than 10,000 mol wt allowed 84.8 ± 11.9% activity to pass and retained 1 ± 1.73% activity (Table 4). In all cases there was a gummy residue on the cell side of the filtration membrane, which would not dissolve or suspend in buffer. It is possible that a portion of the chemotactic activity was trapped in this residue and was lost. PMNs from three donors were tested.

The results of Sephadex filtration are presented in Figure 2. There were several peaks of OD_{280} absorbing material, the greatest being at fraction 36. Chemotactic activity peaked at fraction 30, with an index of 80.5;

Table 3. Effect of Dialysis on Chemotactic Activity[a]

Attractant	Undialyzed activity (%)
Undialyzed	100
<3500	40.5 ± 1.5
<6000–8000	29 ± 5
<12,000–14,000	27.5 ± 6.5

[a]Results are ± standard deviation. PMNs from three donors tested.

Table 4. Effect of Ultrafiltration on Chemotactic Activity[a]

Attractant	Mol wt retained	Unfiltered activity (%)
Unfiltered		100
UM2		
Retained	>2000	38.5 ± 26.6
Filtered		77.0 ± 5.5
UM10		
Retained	>10,000	17.5 ± 13.8
Filtered		84.8 ± 6.5
PM30		
Retained	>30,000	1.0 ± 1.73
Filtered		80.0 ± 11.9

[a]Results are ± standard deviation. PMNs from three donors were tested.

fraction 36 had an index of 22. The remaining fractions had chemotactic indices of less than 24. A 72-h supernatant from strain 3706 also had low chemotactic activity prior to fraction 30.

All supernatant preparations were tested for PMN toxicity by trypan blue exclusion and LDH release. No supernatants exhibited PMN toxicity greater than that of 1066 buffer alone, which had greater than 98% viability by trypan blue exclusion. Likewise, no LDH release above that of 1066 buffer was noted.

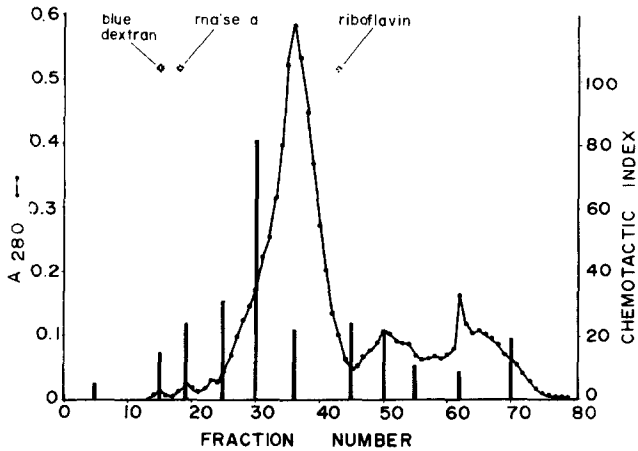

Fig. 2. Sephadex G-25 chromatography of *P. acnes* 6919 72-h culture supernatant. Chemotactic index of selected fractions is depicted by bars.

DISCUSSION

Production of serum-independent leukocyte chemotactic factors by *P. acnes* has previously been reported. Wilkinson et al. (11), using some of the same strains used in this study (6919 and 0162), showed that *P. acnes* cell suspensions are chemotactic for macrophages but not PMNs. The macrophage chemotactic activity was found in culture filtrates and was nondialyzable, lipid in nature, and heat-labile. Tucker et al. (7) found *P. acnes* cell-derived PMN and macrophage chemotactic factors. Lee and coworkers have found that *P. acnes* culture supernatants have attractant activity for PMN (6) and attributed this activity to the lipase which is copiously produced by the organism (8). They find purified lipase to be chemotactic and show that tetracycline, erythromycin, and a lipase inhibitor all inhibits this activity. In recent work, Puhvel and Sakamoto analyzed the chemotactic factors present in comedones and *P. acnes* culture supernatants (9). They found that both supernatants and comedonal lipids had true chemotactic activity for PMN. Their supernatant factors were entirely lost by dialysis and were heat stable, indicating that, in their systems, low-molecular-weight compounds are the sole detectable chemotactic factors produced by *P. acnes*.

In this study we found that a wide range of *P. acnes* strains produce diffusible serum-independent PMN chemotactic factors. Since no effort to ensure that the *P. acnes* strains were at the same stage of growth, no conclusions may be drawn regarding relative ability to produce chemotactic factors. The activity in culture supernatants increased with the duration of culture, and the response of PMN was proportional to the concentration of supernatant used as an attractant. A gradient of supernatant concentration was required for the PMN migratory response, indicating that true chemotaxis, not chemokinesis (stimulated random migration) was being measured.

Dialysis experiments showed that almost 60% of the chemotactic activity of 72-h 6919 supernatant was lost after dialysis in 3500 molecular weight cut-off tubing. An additional 11% was lost in tubing with a 6000–8000 cut-off and approximately 29% of the activity was retained by 12,000–14,000 molecular weight cut-off tubing. Since we did not confirm that the chemotactic activity which was dialyzed away appeared on the outside of the dialysis sac, it is possible that the loss in chemotactic activity which we attribute to dialysis could reflect binding of the factors to the membranes. Ultrafiltration of growth culture supernatants produced similar results to dialysis experiments. The majority (77%) of chemotactic activity was less than 2000 mol wt. Approximately the same amount of chemotac-

tic activity passed through membranes with retention limits of 10,000 and 30,000 mol wt. The 30,000 mol wt membranes retained very little chemotactic activity, possibly reflecting binding of factors to the membrane.

Sephadex G-25 filtration of growth culture supernatant revealed a peak of chemotactic activity at 150 ml of effluent. This peak did not coincide with the peak 280-nm absorbance, although it was close to it. This indicates that the chemotactic factor does not correspond to the most numerous species of OD_{280}-absorbing molecules. We feel, however, that in light of the peptide structure of other bacterial chemotactic factors (12, -14) it is probable that the *P. acnes* factor is also a peptide. Estimation of the molecular weight of this factor is between 13,500 and 366 mol wt. Purified *P. acnes* lipase has been shown to be chemotactic for PMN in vitro (8). This enzyme has a molecular weight of 32,000 (M. Christensen, personal communication) and would be eluted from the column roughly between fractions 15 and 19. Although the unfiltered supernatant had substantial lipase activity and thus contained proteins, the column effluent had a very low concentration of high-molecular-weight proteins and chemotactic activity was also low in these fractions.

In our hands, the chemotactic factors produced by *P. acnes* are predominantly low in molecular weight as determined by three methods and, in this respect, are similar to factors produced by *Escherichia coli* (12, 14), *Streptococcus pneumoniae* (13), and *Proteus mirabilis* (13). It is possible that with extended culture a greater proportion of high-molecular-weight chemotactic activity would be produced by *P. acnes*. Since *P. acnes* is in mid–late stationary phase at 3 days in 1066, however, any chemotactic factors first appearing later than 3 days would probably be cellular constituents liberated upon cell death.

Since in this and all foregoing studies the *P. acnes*-derived factors were produced in conditions quite different from the comedo, no firm statements about the size of the factors active in acne can be made solely on the basis of in vitro data. Puhvel et al. (15) have shown that nondialyzable factors from *P. acnes* supernatants can produce an immediate hypersensitivity response when injected into humans, indicating a possible role for high-molecular-weight *P. acnes* products in inflammation.

We have previously shown that *P. acnes* strains can activate serum complement to produce potent C_5-derived PMN chemotactic factors (3, 4). Since the uninflamed microcomedo is not in contact with interstitial fluid, complement-derived chemotactic factors might not be generated until after the rupture of the microcomedo. It is plausible that a low-molecular-weight *P. acnes*-derived chemotactic factor is the initiating factor in microcomedonal inflammation. As did Puhvel and Sakamoto (9), we found low-molecular-weight factors to be more copiously produced than high-molecular-weight factors. It would be expected that a low-molecular-weight

chemotactic factor would penetrate the follicular wall more readily than a large molecule and would be a more likely stimulus for inflammation in acne.

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