INFECTION AND IMMUNITY, Nov. 1978, p. 523-529
0019-9567/78/0022-0523$02.00/0

Vol. 22, No. 2
Printed in U.S.A.

Complement Activation in Acne Vulgaris: In Vitro Studies with *Propionibacterium acnes* and *Propionibacterium granulosum*

GUY F. WEBSTER,[1]* JAMES J. LEYDEN,[2] MICHAEL E. NORMAN,[1] AND ULF R. NILSSON[1]

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

Received for publication 10 May 1978

To better define the role of bacteria in inflammatory acne vulgaris, we have investigated the ability of four strains of *Propionibacterium acnes* and three strains of *Propionibacterium granulosum* to activate complement. Complement activation was assayed by incubating normal human serum with varying concentrations of each strain and measuring residual total hemolytic complement activity. When serum was tested unaltered, *P. acnes* strains were approximately threefold more potent than an equal weight of *P. granulosum* in consuming complement, which could reflect classical and/or alternative pathway activation. All strains also consumed complement in serum chelated with ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, which selectively assays alternative pathway activation. Incubation of unaltered serum with both *P. acnes* and *P. granulosum* resulted in immunoelectrophoretic conversion of C4, C3, and factor B of the alternative pathway. Incubation of chelated serum resulted in conversion of C3 and factor B. These data taken together suggest that both species can activate complement through either pathway. Serum incubated with *P. acnes* was chemotactic for polymorphonuclear leukocytes, and this chemotactic activity was largely C5 dependent as shown by antibody inhibition. It is suggested that complement activation may occur in vivo in acne, and the inflammatory response may be contributed to by the generation of C5-dependent chemotactic factors.

Acne vulgaris is a ubiquitous disease characterized by blockage of the sebaceous canal with tightly packed horny cells. This often leads to rupture of the follicle and discharge of its contents into the surrounding tissue, resulting in inflammation (7).

Propionibacterium acnes, an anaerobic diphtheroid which is numerous on the skin of normal and acne patients, lives in the follicle and has been assumed to play a role in the etiology of acne lesions (7, 9). Although few data implicating *P. acnes* in the initiation of follicular blockage exist, several lines of evidence point to its involvement in the inflammatory component of acne vulgaris. First, circulating anti-*P. acnes* titers are elevated proportionally to the extent of inflammatory involvement of the acne patient (13). Second, when *P. acnes* is injected into normally sterile sebaceous cysts, rapid rupture of the cyst occurs with the subsequent development of acneform inflammation (10). *Staphylococcus epidermidis*, another skin resident, will not produce this effect. Kligman has shown that dispersed comedones are phlogistic when injected intradermally and are more potent than an equivalent amount of *P. acnes* alone, implying the presence of an additional inflammatory factor (7). Recently, Puhvel and Sakamoto (15) studied the inflammatory capacity of purified comedonal components and found that keratinous material and live or killed *P. acnes* induced significant erythema and induration, while free fatty acids and other comedonal lipids had little effect.

Other than its association with *P. acnes*, the nature of the inflammatory stimulus in acne is ill-defined. Since the complement system is a key component of many inflammatory responses and has been shown to play a role in other diseases (6), we undertook to study its role in acne vulgaris. In the experiments to be reported, we show that *P. acnes* and *Propionibacterium granulosum*, a closely related species often found in inflammatory acne lesions, can activate complement by both the classical and alternative pathways. Moreover, serum complement

activated under these conditions liberates factors which are chemotactic for polymorphonuclear leukocytes (PMNs), the local accumulation of which is a hallmark of inflammatory acne (7).

MATERIALS AND METHODS

Bacteria. *P. acnes* type I strains ATCC 6919 and VPI 3706 (courtesy of C. S. Cummins), type II strains VPI 0162 and VPI 6583, and *P. granulosum* strains VPI 74-402 and Duhring Laboratory G-1 and G-2 were tested. Strains were grown in prereduced, anaerobically sterilized peptone-yeast-glucose broth with salts and lipid supplements (20). Cultures were incubated for 48 h at 37°C and washed repeatedly with 0.85% NaCl and finally with distilled water. Cell pellets were suspended in a small volume of distilled water and lyophilized. It was established that 1.2 mg of the lyophilized preparation was equivalent to 2.56×10^9 cells by performing lyophilized weight determinations and visual enumeration of cells in a counting chamber. There was no significant variation in this relationship among the seven strains. In this report the doses of bacteria employed are given as dry weight in micrograms per milliliter.

Serum. Sera from 10 healthy adults were pooled and stored in 1-ml aliquots at −70°C. The same lot of serum was used in all experiments. Agglutination titers of the serum were performed and found to be 1:128 for *P. acnes* 6919 and 0162 and 1:16 for *P. granulosum* G-1.

Antisera for complement components. Antisera to purified human C3, factor B (C3 proactivator [C3PA]), and C4 were prepared in rabbits according to the method of Goudie et al. (5). Goat anti-human C5 was obtained from Duane Shultz, and rabbit anti-human albumin was purchased from Miles Laboratories, Elkhart, Ind. Antisera produced a single band in immunodiffusion and electrophoresis gels against whole sera of the specific antigens.

Assays of complement activation. Bacteria at fixed concentrations of 300 to 18.8 μg/ml were suspended in an equal mixture of gelatin-Veronal buffer (8) and pooled serum. Preliminary kinetic studies indicated that an incubation of this mixture for 30 min at 37°C was optimum for complement activation. After incubation, bacteria were removed by centrifugation (2,400 rpm, 10 min), and residual hemolytic activity in the supernatant was assayed by the standard method of Mayer (11). Results were expressed as percentage of total hemolytic activity consumed. Controls included incubation of bacteria at the highest concentration employed in serum depleted of Ca^{2+} and Mg^{2+} with 0.01 M ethylenediaminetetraacetic acid (EDTA) to block complement activation.

The ability of the bacteria to consume hemolytic activity by the alternative pathway alone was also assayed. The experimental design was identical to the preceding experiment except that only Ca^{2+} was chelated by the addition of 0.01 M ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA). EGTA was supplemented with Mg^{2+} (0.05 M $MgSO_4$) before being exposed to bacteria (4). Serum was recalcified after removal of bacteria through the use of gelatin-Veronal buffer supplemented with 0.05 M Ca^{2+} for suspension of antibody-coated erythrocytes.

Activation and cleavage of complement was also assayed by the immunoelectrophoretic analysis (17) of serum which had been incubated for 30 min at 37°C with 300 μg of bacteria per ml. Samples of serum were subjected to electrophoresis for 90 min at 40 V in 1% agarose (Seakem, Marine Colloids, Rockland, Me.). Rabbit anti-human C3, C3PA, and C4 were used to develop the gels. Bacteria incubated with EDTA-treated serum served as control.

Chemotaxis. A modified Boyden chamber (1) with a 3-μm nitrocellulose filter (Millipore Corp., Bedford, Mass.) was used for all experiments. PMNs were retrieved from the buffy coat of heparinized blood from healthy adults and washed three times in 0.85% NaCl. PMNs in the buffy coat varied from 92 to 96%. PMNs were suspended at a concentration of 5×10^6 ml of 1066 medium (Grand Island Biological Co., Grand Island, N.Y.) with 10% heat-inactivated fetal calf serum (pH 7.0). After incubation at 37°C in a moist chamber, filters were removed from the units, inverted, fixed in a graded series of alcohols, and stained by hematoxylin and eosin. Migration was scored by counting the number of PMNs in the layer of cells that had migrated farthest from the cell side of the filter. The average value of 10 high-power fields was taken as the chemotactic index. The cover slip at the bottom of each chamber was stained and examined to determine if PMNs had migrated completely through the filter and into the attractant medium (drop-through). All tests were run in duplicate. Before testing, all sera were heat inactivated at 56°C for 30 min and, excepting the serum dilution experiment, all sera were tested at a final concentration of 10% in 1066.

The optimum incubation time for chemotactic factor generation was determined in a preliminary experiment in which serum was activated with an equal volume of 300 μg of *P. acnes* 6919 per ml for 30 to 120 min at 37°C and then tested for chemotactic activity.

The ability of *P. acnes* 6919 to generate serum-derived chemotactic factors was tested in dose-response fashion by incubating 6×10^2 to 3×10^{-4} μg of cells per ml with an equal volume of serum for 30 min at 37°C. Bacteria were removed by centrifugation, and the serum was diluted and tested for chemotactic activity. The chemotactic activity of whole *P. acnes* cells was tested by suspending 3×10^2 to 3×10^{-2} μg of *P. acnes* 6919 per ml in 1066 and applying directly to the attractant chamber. The effect of the concentration of serum on the generation of chemotactic activity was tested by incubating 300 μg of *P. acnes* 6919 per ml with serum in concentrations of 50, 25, 12.5, and 6.25%. After incubation, bacteria were removed by centrifugation, and the serum was tested for chemotactic activity after diluting to final concentrations of 5, 2.5, 1.25, 0.63 and 0.37%, respectively, in 1066.

Inhibition of chemotaxis. Equal volumes of serum and 300 μg of *P. acnes* 6919 per ml were incubated at 37°C for 30 min. After bacteria were removed by centrifugation, the serum was heat inactivated and then incubated with 50 μl of monospecific anti-C3, anti-C5, or anti-human albumin for 15 min at 37°C

and 30 min at 25°C. Serum was then finally diluted to 10% in 1066 and tested for chemotactic activity. Controls included *P. acnes* 6919-activated serum without antibody, antiserum alone, and buffer alone.

RESULTS

Consumption of hemolytic activity in normal serum. Lyophilized cells of *P. acnes* and *P. granulosum* at final concentrations of 300, 150, 75, 37.5, and 18.8 μg/ml were incubated with normal human serum, which was then assayed for residual hemolytic activity. All seven strains tested activated complement in a dose-dependent manner. The averaged results for *P. acnes* I, *P. acnes* II, and *P. granulosum* strains are presented in Fig. 1. *P. acnes* I strains were the most potent, consuming an average of 93.7% of total hemolytic activity at a concentration of 150 μg of cells per ml. Type II strains consumed 47.3% at this concentration, and *P. granulosum* strains averaged only 11.7% consumption.

Consumption of hemolytic activity in EGTA-treated serum. The same concentrations of bacteria were incubated with serum which had been decalcified with EGTA and supplemented with Mg^{2+}. The averaged results for *P. acnes* I, *P. acnes* II, and *P. granulosum* are presented in Fig. 2. All strains consumed complement in a dose-dependent manner under these conditions, although to a lesser degree than in unchelated serum. At 150 μg/ml, *P. acnes* type I strains were slightly more active than type II strains, consuming 25.5 and 18.5%, respectively. *P. granulosum* consumed 6.7% of the hemolytic activity at this concentration.

Electrophoretic modification of complement components. See Fig. 3. Lyophilized cells were incubated with normal serum for 30 min at 37°C. The serum then was analyzed electrophoretically, employing monospecific antisera to C3, C3PA, and C4. All strains of *P. acnes* and *P. granulosum* tested were able to induce the cleavage of C3, C4, and C3PA. C3 and C4 were partially cleaved, as indicated by the appearance of their electrophoretically fast conversion products, C3b-c and C4d, respectively. C3PA was partially split into its fast- and slow-migrating fragments upon incubation with bacteria. No

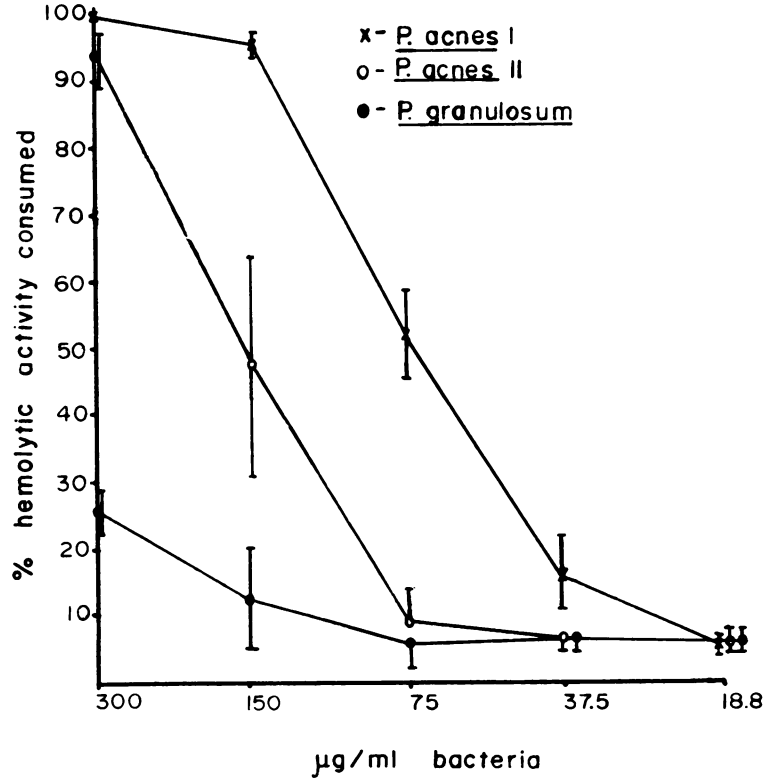

FIG. 1. *Consumption of complement in unchelated serum. The results presented are the averaged results for strains of P. acnes I and II and P. granulosum from a single experiment. Similar results were obtained in other experiments, with variation being less than 10% for each strain.*

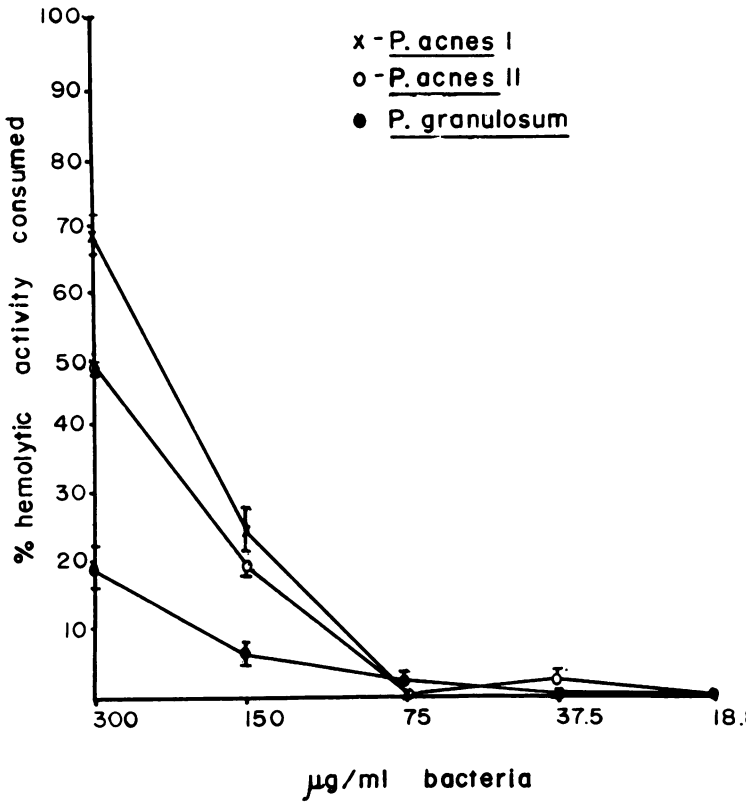

FIG. 2. *Consumption of complement in EGTA-chelated serum. The results presented are the averaged results from strains of P. acnes I and II and P. granulosum. Similar results were obtained in other experiments, with variation being less than 10% for each strain.*

significant difference in potency between any of the strains was noted. When incubated in serum which had been chelated with EGTA, all strains tested were able to cleave C3 and C3PA. No cleavage occurred in EDTA-treated serum.

Chemotaxis. Initial experiments. *P. acnes* 6919 at 300 μg/ml was used to activate normal serum, which was diluted to 10% and tested for chemotactic activity. Initial chamber incubation periods varied from 90 to 120 min. An incubation period of 90 min was chosen for further experiments because it provided the maximum migration of PMNs to the attractant surface of the filter without having drop-through into the attractant medium. Subsequently, *P. acnes* 6919, from 3×10^{-4} to 3×10^{3} μg/ml, was used to generate chemotactic activity in normal serum. The mean responses of three experiments are presented in Fig. 4. Serum chemotactic activity for PMNs was generated by increasing doses of *P. acnes* in a dose-response fashion. Absolute counts for PMN migration were low despite massive influx of cells into the filter, because the short chamber incubation period of 90 min was required to avoid drop-through of PMNs into the attractant medium. When varying concentrations of whole *P. acnes* cells alone were tested for chemoattractant activity, only the highest concentration of cells (300 μg/ml) was capable of eliciting migration of PMNs. In these latter experiments (not shown), very few layers of cells were shown to penetrate the filter.

Varying dilutions of serum were then incubated with 300 μg of *P. acnes* 6919 per ml and tested for chemotactic activity over a dose range of 5 to 0.37%. PMN migration was directly proportional to the amount of activated serum in the attractant medium. The maximum chemotactic response was seen with 5% serum (11.5 cells per high-power field) and the minimum response at 1.25% (3.4 cells per high-power field).

Inhibition of chemotactic activity in activated serum. Serum was activated with 300 μg of *P. acnes* 6919 per ml and reacted with antiserum to C3, C5, and human albumin. The results from two experiments are presented in Table 1. No inhibition of migration was produced by incubation with either anti-human al-

bumin or anti-C3. Anti-C5 produced a mean of 75.5% inhibition in the chemotaxis of PMNs. The chemotactic activity of each antiserum alone was not different from buffer 1066 alone.

DISCUSSION

A body of literature exists on the immunological capacities of strains labeled *Corynebacterium parvum*, a heterogeneous group of anaerobic diphtheroids now shown to be composed of *P. acnes*, *P. granulosum*, and *Propionibacterium avidum* (2). Some strains in the *C. parvum* group have been shown to activate complement (8), stimulate the reticuloendothelial system (3), and produce complement-independent chemotactic factors for PMNs (10) and mononuclear cells (10, 16). The interpretation of these studies in relation to acne vulgaris is complicated by the heterogeneity of the organisms used and the fact that many investigations used a commercially produced Formalin-treated vaccine as stimulant.

The experiments reported here demonstrate that *P. acnes* and *P. granulosum* whole cells are capable of consuming complement by both the classical and alternative pathways. Several lines of evidence lead to the conclusion that both pathways of complement are activated. First, all strains consumed hemolytic activity in both normal and EDTA-treated serum. In normal serum hemolytic consumption may proceed by either pathway, whereas EGTA treatment permits selective activation to proceed via the alternative pathway by removing Ca^{2+} and thereby inhibiting the activity of Clr and Cls of the classical sequence (4). Second, all strains induced the electrophoretic conversion of C3, C3PA, and C4 when incubated with normal serum. Cleavage of C4 only occurs in response to antibody-antigen complexes (12) and is thus a marker for activation via the classical pathway. C3 may be cleaved by both pathways, and C3PA may be cleaved either through the alternative pathway or through the amplification loop of the classical pathway (12). Therefore, to determine if cleavage of these could also occur through the selective activation of the alternative pathway, bacteria were incubated in EGTA-treated serum, which was then analyzed immunoelectrophoretically. That all strains converted C3 and C3PA is further evidence for their ability to activate complement by the alternative pathway.

Our data suggest that there is a difference in the ability to consume hemolytic activity between *P. acnes* I and II and *P. granulosum*. In

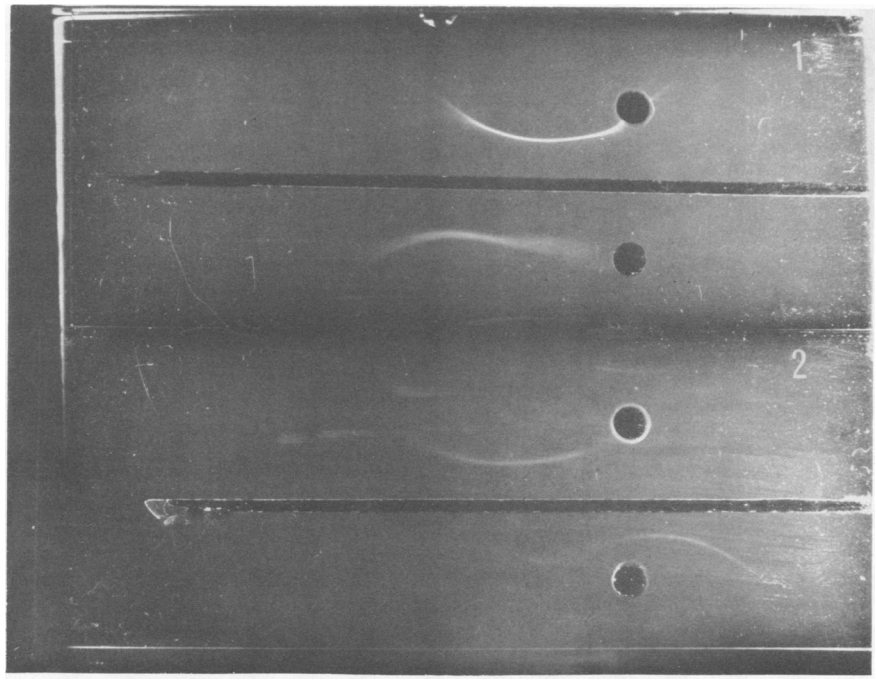

FIG. 3. *Immunoelectrophoresis of serum incubated with P. acnes 6919. Slide 1 was developed with antiserum to C3. The top well contained EDTA-treated serum and the bottom well EGTA-treated serum. Slide 2 was developed with antiserum to C3PA. The top well contained EDTA-treated serum and the bottom well EGTA-treated serum. P. acnes was also able to initiate the cleavage of C4 in fresh serum. Treatment with EGTA prevented this cleavage.*

normal serum, *P. acnes* I was able to consume 98% of total hemolytic activity and *P. acnes* II 47% at a concentration of 150 μg/ml, whereas *P. granulosum* could only consume 12%. In this system, the differences in complement consumption could have been due either to differences in specific antibody titer of the serum or to innate differences in potency of the cells. Support for the role of antibody is found in the sixfold-higher antibody titers against *P. acnes* I and II than against *P. granulosum* in the test serum pool. Attempts at specific antibody absorption were unsuccessful, as hemolytic complement activity was reduced during the absorption process. To better define the source of differences between strains, hemolytic complement consumption was also assayed in EGTA-treated serum, which blocks antibody-mediated complement activation. Overall, complement consumption was diminished in chelated versus nonchelated serum, although *P. acnes* I consumed four times as much hemolytic activity as *P. granulosum* (i.e., 25 versus 7%) at concentrations of 150 μg/ml.

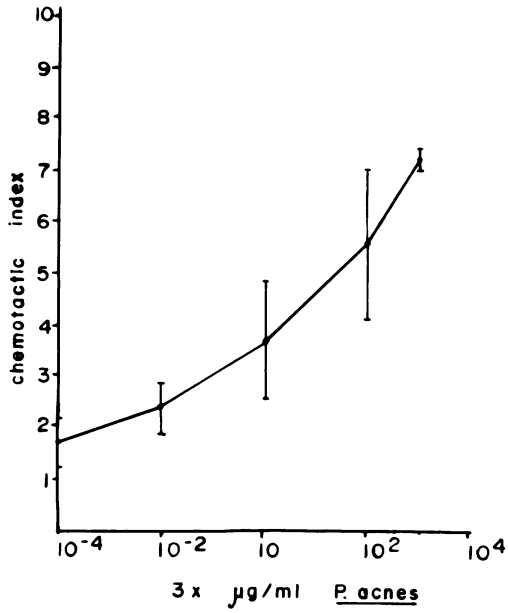

FIG. 4. *P. acnes-generated serum chemotactic activity. The averaged results of three separate experiments are shown. Buffer which had been incubated with 300 μg of P. acnes per ml and then diluted 10-fold had the same chemotactic index (expressed as the average number of cells per high-power field) as untreated buffer.*

The differences between strains in EGTA-chelated serum probably reflects innate differences in potency of the strains, with *P. acnes* being the more potent. The reason for this is not apparent, although one possible explanation might relate to differences in cell size and the amount of surface area exposed to complement. At the present time this hypothesis has not been tested. A second explanation relates to the amount of teichoic acid per cell and its accessibility to extracellular complement, since teichoic acid has been shown to be the most potent bacterial activator of the alternative pathway (21). Russle and co-workers (16) have identified a lipid fibril from *P. avidum*, a closely related species, which appears to cover the organism's surface. Recently we (G. F. Webster and J. J. Leyden, Clin. Res. **26**:578A, 1978) have found a similar compound on the surface of *P. acnes*. When purified, it fails to activate complement and could conceivably occlude sites for complement activation. If a similar structure is more plentiful on the surface of *P. granulosum*, a decrease in anticomplement activity might be noted relative to *P. acnes*.

Activation of the complement cascade results in the elaboration of factors which play important roles in inflammation, including increased vascular permeability, chemotaxis of leukocytes, enhanced phagocytosis, and release of lysosomal enzymes (6). Our studies reveal that *P. acnes* 6919 (a strain similar to commonly isolated types) (20), in activating both the classical and alternative pathways, generates C5-dependent chemotactic factors from human serum. Of interest is the observation that complement acti-

TABLE 1. *Inhibition of serum chemotactic factors*

Sera	Expt I		Expt II		Avg	
	Chemotactic index[a]	% Inhibition	Chemotactic index	% Inhibition	Chemotactic index	% Inhibition
AS[b]	9.8	<0	7.2	<0	8.5	<0
Anti-C5 + AS	3.1	68.2	0.9	88.1	2.0	78.2
Anti-C3 + AS	12.1	<0	7.5	<0	9.8	<0
Anti-HSA[c] + AS	10.1	<0	7.3	<0	9.7	<0

[a] Average number of cells per high-power field.
[b] AS, Serum activated with *P. acnes*.
[c] HSA, Human albumin.

vation leading to production of chemotactic factors was accomplished with 3 μg of *P. acnes* per ml, a dose 10-fold lower than that required to consume hemolytic complement activity (37.5 μg/ml).

Could these in vitro observations provide an explanation for the mechanism of neutrophil infiltration so typical of inflammatory acne? Puhvel et al. (14) have quantified the numbers of *P. acnes* in isolated sebaceous follicles and found the average density of viable *P. acnes* to approach 1.7×10^7 per follicle. Since *P. acnes* 6919 generated complement chemotactic factors at a concentration of 3 μg/ml (6.4×10^8/ml), it is conceivable that sufficient organisms are present in a follicle, whose volume is estimated at 0.02 ml, to produce a chemotactic gradient. Moreover, the serum source of chemotactic factors in our experiments produced a chemotactic gradient for neutrophils at concentrations 1:200 of whole serum (0.5%) when incubated with the organism. Although no direct data exist on this point, it is conceivable that the perifollicular tissue would contain transudated serum at this concentration. Studies of the ratios of various serum proteins in serum and gingival crevice fluid of patients with frank periodontitis (18) provide evidence to support this argument from another localized inflammatory model in humans.

Finally, Kligman in 1974 (7) observed that the microscopic rupture of the sebaceous follicle is the event that precedes the clinical inflammatory lesion. At times it is possible to identify invasion of "intact" follicular epithelium by neutrophils; occasionally pockets of neutrophils can also be identified in the lumen of the follicles at these points, suggesting increased permeability of the epithelium. This observation implies that there are chemotactic factor activators or direct chemotactic factors which leach out of microscopically intact follicles and attract neutrophils into the site. The role of these neutrophils in inflammatory acne, whether tissue protective or destructive, remains a matter of speculation.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Arlene Taylor and the criticism of C. C. Tsai.

This work was supported in part by Public Health Service grants 1-RO1-DE-04809-01 from the National Institute of Dental Research and AM-13515 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

LITERATURE CITED

1. **Atkins, P. C., M. Norman, H. Weiner, and B. Zweiman.** 1977. Release of neutrophil chemotactic activity during immediate hypersensitivity reactions in humans. Ann. Intern. Med. **86**:415-418.
2. **Cummins, C. S., and J. L. Johnson.** 1974. *Corynebacterium parvum*: a synonym for *Propionibacterium acnes*? J. Gen. Microbiol. **80**:833-992.
3. **Cummins, C. S., and D. M. Linn.** 1977. Reiculostimulating properties of killed vaccines of anaerobic coryneforms and other organisms. J. Natl. Cancer Inst. **59**:1696-1708.
4. **Fine, D. P., S. R. Marrey, D. G. Colley, J. S. Sergeant, and R. M. DesPrez.** 1977. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109**:807-809.
5. **Goudie, R. B., C. H. W. Hawe, and P. C. Wilkinson.** 1966. A simple method for the production of antibody to a single selected diffusable antigen. Lancet **ii**:1224.
6. **Johnston, R. B.** 1977. Biology of the complement system with particular reference to host-defense vs. infection: a review, p. 295-307. *In* R. M. Suskind (ed.), Malnutrition and the immune response. Raven Press, N.Y.
7. **Kligman, A. M.** 1974. An overview of acne. J. Invest. Dermatol. **62**:268-287.
8. **McBride, W. H., D. M. Weir, A. B. Kay, D. Pearce, and J. R. Caldwell.** 1975. Activation of the classical and alternative pathways of complement by *Corynebacterium parvum*. Clin. Exp. Immunol. **19**:143-147.
9. **McGinley, K. J., G. F. Webster, and J. J. Leyden.** 1978. Regional variations of cutaneous propionibacteria. Appl. Environ. Microbiol. **35**:62-66.
10. **Majeski, J. A., and J. D. Stinnet.** 1977. Brief communication: chemoattractant properties of *Corynebacterium parvum* and pyran copolymer for human monocytes and neutrophils. J. Natl. Cancer Inst. **58**:781-783.
11. **Mayer, M. M.** 1961. Complement and complement fixation, p. 133-240. *In* E. A. Kabat and M. M. Mayer (ed.), Experimental immunochemistry, 2nd ed. Charles C. Thomas, Springfield, Ill.
12. **Müller-Eberhard, H. J.** 1975. Complement. Annu. Rev. Biochem. **44**:697-724.
13. **Puhvel, S. M., M. Barfatani, M. Warnick, and T. H. Sternberg.** 1964. Study of antibody levels to *Corynebacterium acnes*. Arch. Dermatol. **90**:921-927.
14. **Puhvel, S. M., R. M. Reisner, and D. A. Amirian.** 1975. Quantification of bacteria in isolated sebaceous follicles. J. Invest. Dermatol. **65**:525-531.
15. **Puhvel, S. M., and M. Sakamoto.** 1977. An *in vivo* evaluation of the inflammatory effect of purified comedonal components in human skin. J. Invest. Dermatol. **69**:401-406.
16. **Russle, R. J., R. J. McInroy, P. C. Wilkinson, and R. G. White.** 1976. A lipid chemotactic factor from anaerobic coryneform bacteria including *C. parvum* with activity for monocytes and macrophages. Immunology **30**:935-949.
17. **Scheidegger, J. J.** 1955. Une micro-methode de l'immunoelectrophorese. Int. Arch. Allergy Appl. Immunol. **7**:103-110.
18. **Shillitoe, E. J., and T. Lehner.** 1972. Immunoglobulins and complement in crevicular fluid, serum and saliva in man. Arch. Oral Biol. **17**:241-247.
19. **Walker, W. S., R. L. Barlet, and H. M. Kurtz.** 1969. Isolation and partial characterization of a staphylococcal leukocyte cytotaxin. J. Bacteriol. **97**:1005-1008.
20. **Webster, G. F., and C. S. Cummins.** 1978. Use of bacteriophage typing to distinguish *Propionibacterium acnes* types I and II. J. Clin. Microbiol. **7**:84-90.
21. **Winklestein, J. A., and A. Tomasz.** 1978. Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid. J. Immunol. **120**:174-178.