Report

Propionibacterium acnes: interaction with complement and development of an enzyme-linked immunoassay for the detection of antibody

Craig G. Burkhart, MSPH, MD, John Cantrill, PhD, Cynthia L. Butcher, MD, and Paul F. Lehmann, PhD

From the Departments of Medicine and Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio, and Ribi Immunochem Research, Inc., Hamilton, Montana

Correspondence
Craig G. Burkhart, EMDE
5600 Monroe Street
Suite 106B
Sylvania, OH 43560

Abstract

Objective To characterize the immune response to *Propionibacterium acnes* in acne patients.

Design Comparison of serologic responses in acne and normal patients using counterimmunoelectrophoresis for antibody and an enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin G (IgG) antibody.

Setting The serum of acne and nonacne patients from the Dermatology Clinic at the Medical College of Ohio was utilized for analysis.

Results Using counterimmunoelectrophoresis, antibody was detected in 13 of 20 acne patients. The antigen was detectable as an anion in the barbital buffer at pH 8.2, strongly suggesting a carbohydrate component. By ELISA, the antibody proved to be IgG, and the bacteria and its water-soluble fractions were capable of fixing complement.

Conclusions The primary instigator of inflammation in acne vulgaris is an immunologic reaction to extracellular products of *P. acnes*. The immunologic response involves both humoral and cell-mediated pathways. The antibodies to *P. acnes* have not been characterized fully, although they are largely of the IgG class. We have further characterized the dominant antigen to have a carbohydrate component.

Propionibacterium acnes is overwhelmingly the predominant microorganism in the normal pilosebaceous follicle as well as in the acne state. This bacterium is of central importance in the etiology of acne.[1] *P. acnes* produces chemotactic factors and proinflammatory mediators that lead to inflammation.[2–4] The precise conditions responsible for the susceptibility of certain individuals to severe acne, or for the development of the acne lesion itself, are not fully understood. Circulating immune complexes have not been detected in acne patients.[5] Nevertheless, complement activation via the alternative pathway has been demonstrated for *P. acnes in vitro*,[6] and deposits containing complement have been observed in tissues surrounding comedones *in vivo*.[7] *P. acnes* has been studied by immunologists for whom its synonym, *Corynebacterium parvum*, is better recognized. Among additional activities are its behavior as an adjuvant for the reticuloendothelial system (RES), as a stimulant of natural killer (NK) cell activity, and as a producer of tumor necrosis factor.[8–10] In contrast, there is only limited information available concerning the immune responses to *P. acnes* in acne patients. The availability of a water-soluble extract of the organism, which no longer retained its activity as a stimulant of the RES,[11] allowed us to study the serologic responses in patients and normal persons. For this, we utilized counterimmunoelectrophoresis for antibody and an enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin G (IgG) antibody. In addition, we investigated the capacity of the extract to activate complement *in vitro* and, finally, we studied the role of complement in the RES stimulation found in mice following their intraperitoneal injection with *P. acnes*.

Methods

Antigen

The antigen preparation was the water-soluble fraction of a pyridine-insoluble extract of whole cells of *P. acnes*, Paris

strain. The preparation is described below. In some cases, extracts of *P. acnes* type II were used for comparative purposes.

Source of organisms and culture method
Propionibacterium acnes type II, strain VPI 0204, was kindly provided by Dr C.S. Cummins, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA, and *Corynebacterium parvum* type I, Paris strain, was obtained from Dr P. Minden, National Jewish Hospital, Denver, CO. Organisms of each strain, which were received as lyophilized cultures, were grown in thioglycollate broth (BBL, Baltimore, MD) at 37 °C. The cells were harvested at mid-logarithmic phase of growth (66 h) by centrifugation (6000 × *g*, 30 min, 4 °C). The organisms were washed in sterile distilled water and a portion (approximately 10 g wet weight) of each strain was dialyzed for 4 days against distilled water and then lyophilized. This preparation was used in studies involving whole cells. The remaining cells of each strain (90–110 g wet weight) were stored at –20 °C as paste.

Preparation of aqueous soluble fraction
Ninety grams (wet weight) of washed *P. acnes* or *C. parvum* whole cell paste was added to 200 mL of neat pyridine, reagent grade (J.T. Baker Chemical Co., Phillipsburg, NJ). The extraction mixture was stirred for 18 h at 37 °C. Following this, the mixture was centrifuged (17,000 × *g*, 1 h, 4 °C), and the pelleted materials were extracted twice more in the original volume of pyridine under identical conditions. Following filtration (Whatman No. 1 paper), the pyridine-insoluble residue was washed three times with sterile distilled water, suspended n distilled water, dialyzed for 4 days, and lyophilized. The water washings (aqueous soluble fraction) were pooled, dialyzed against distilled water for 4 days, and lyophilized. Yields of the pyridine-soluble extract ,obtained after evaporation of the solvent, the aqueous-soluble fraction, and the insoluble residue for each strain were 8–10%, 2–4%, and 80–86%, respectively.

Counterimmunoelectrophoresis
The procedures were similar to those described by Lehmann and Reiss.[12] Agarose solution (10 mL) (HE agarose; Marine Colloids, Rockland, ME; –mr = 0.24; 0.075 M barbital buffer; pH 8.2) was allowed to gel on 10.2 × 8.3 cm plates and wells cut 3 mm apart. Serum was placed on the side of the anode and the antigen preparation (0.1 mg/mL buffer) placed into the well on the cathodic side. After electrophoresis (25 m, 45 min), the plates were held over at 4 °C and precipitates were observed. For increased sensitivity, the gels were stained using the method of Crowle and Cline.[13]

Enzyme-linked immunosorbent assay
The ELISA was developed using microtiter plates having U-shaped wells (U-1-221-24; Dynatech Labs, Alexandria, VA). The procedure was similar to that described earlier for detecting antibody to *Candida albicans* mannan.[14] A 100 μL aliquot of antigen solution in buffer (0.01 M sodium carbonate buffer, pH 9.2) was added to each well. After incubation (3 h, 25 °C), the wells were washed three times with saline–Tween solution (0.15 M NaCl, 0.05% (v/v) Tween 20). The wells were then filled with 150 μL saline–Tween solution and 50 μL serum was added to the first well. Serial, fourfold dilutions were prepared by transferring 50 μL of the diluted serum from the first to the second well, then from the second to the third well, and so on. After incubation (2 h, 25 °C), the plates were washed three times with the saline–Tween solution. A 0.1 mL aliquot of horseradish peroxidase-labeled goat anti-human IgG (Miles-Yeda, IN) in saline–Tween was added to each well. The plates were incubated (2 h, 25 °C), and were again washed three times with saline–Tween solution. The enzyme substrate (0.2 mL) was then added to each well. For the substrate, we used the *o*-phenylenediamine reagent.[14] The plates were allowed to sit in the dark until the color change was prominent. Fifty microliters of 12 M HCl was added to each well to stop the reaction. Reactions were read visually. A positive result was recorded when the color in a well was darker than the control wells in which either no serum or no antigen had been added.

Complement fixation
Complement activation by bacterial fractions was determined by immunoelectrophoresis on a 10.2 × 8.3 cm plate (25 mA per plate) using a commercial rabbit anti-human C3 antiserum (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The procedure was similar to that described by others,[15] and involved incubation (37 °C, 30 min) of a 50 μL fraction dissolved or suspended at 1 mg/mL in complement fixation buffer (BBL, Cockeysville, MD) with 50 μL serum which had been stored at –70 °C. Following incubation, 20 μL of 0.1 M ethylenediaminetetraacetic acid (EDTA) was added to stop the reaction and the conversion of C3 to C3b was determined. When cells were used, the suspension was then centrifuged in a microcentrifuge. For controls, fixation was determined in the presence of 0.02 M EDTA, 0.02 M ethyleneglycol tetraacetic acid (EGTA), and cobra venom factor (CVF; Cordis Labs, Miami, FL).[15]

Activation of the reticuloendothelial system (RES)
RES activation was determined via the measurement of spleen enlargement. Female, 6-week-old C57BL/6J mice were injected with 100 mg *P. acnes* (dry weight). Eight days later, the spleens were removed, blotted on filter paper, and weighed. When complement-depleted animals were used, mice were injected intravenously (i.v.) with 5 U CVF one day before their injection with *P. acnes*. One day after injection (day +1), a further 5 U CVF was injected i.v. On three further occasions, days +3, +5, and +7, 2.5 U CVF was injected i.v. At sacrifice,

no C3 was detectable by single radial immunodiffusion[16] in sera collected from mice which had been treated by this schedule.[17]

Results

Antibody in patients and blood donors

Using counterimmunoelectrophoresis (CIE), antibody was detectable in 14 of 20 blood donors and 13 of 20 acne patients. A single line of precipitin was seen for the blood donors, while more than one line was seen in five of the acne patients, four showing two lines and one, who presented as a severe case, with four lines. The specificity of the antibody was studied by immunodiffusion in gel using sera from three blood donors and three acne patients. It was found that the precipitin produced by the donors' sera formed a line identical with one of the lines formed by the patients' sera, and in a case where only a single line was seen for a patient's serum, the same antigenic species was detected as that found for the blood donors.

The antigen that was detectable with each serum was found to behave as an anion in 0.075 M barbital buffer at pH 8.2. Preincubation with 0.05 M sodium periodate (24 h, 4 °C) resulted in a modification of the antigen, although not all the antigenic reactivity was destroyed. The same treatment completely destroyed the serologic reactivity of *Candida albicans* mannan. The results suggest the presence of a carbohydrate component in the *P. acnes* antigen.

By ELISA, all persons were found to have IgG antibody for the antigen preparation. There was no significant difference in the titers observed for sera from the blood donors or from the patients.

Complement fixation

Both the whole bacteria and the water-soluble fractions were capable of fixing complement. Fixation via the alternative pathway occurred with the fractions from the Paris strain and the type II strain of *P. acnes*.

Requirement for complement in RES stimulation

The RES stimulation, observed in complement-depleted animals following intraperitoneal injection of *P. acnes*, was not significantly different from that found for untreated mice (Table 1), with $P > 0.05$.

Table 1 Splenomegaly in complement-depleted and normal mice injected with 100 mg *P. acnes* intraperitoneally

Treatment	No. of mice	Spleen wt.* (mg)
Saline	5	77.8 ± 10.2
Saline + CVF	5	65.4 ± 9.9
Saline + *P. acnes*	5	139.3 ± 17.2
Saline + *P. acnes* + CVF	5	184.4 ± 20.6

*Weight of spleen ± standard deviation, 8 days after injection of *P. acnes*.

Conclusions

Humoral responses to *P. acnes* have been studied by Puhvel *et al.*[18] They reported that titers of complement fixing antibodies were raised in acne patients. In our study, we found no evidence of a significantly higher titer of IgG antibody in patients versus the blood donors. Indeed, high titers of antibody were detected in some donors with insignificant acne. Both the immunoglobulin isotypes and antigen preparation for the two assays were different and this might explain the difference between our results and those of Puhvel *et al.*[18] Furthermore, elevated IgG levels have been demonstrated in severe acne cases.[19] IgG subclasses specific to *P. acnes* have been investigated, and IgG and IgG_3 were significantly elevated in cystic acne.[20]

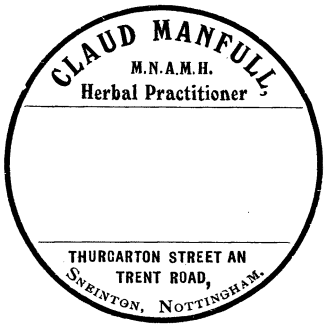
The soluble extract of *P. acnes* contained several antigenic species; however, it appears that one of these is dominant antigen detected by patients' and donors' sera. The composition of the antigen is under study. From preliminary findings concerning its behavior following exposure to sodium periodate, it appears to have a carbohydrate component. The demonstration by ELISA that all persons have antibody to *P. acnes*, and probably to this antigen, is not unexpected. IgG antibody to other colonizers, such as *Candida albicans*, is found in all normal individuals.[14]

The availability of a water-soluble extract will provide a useful tool for the study of immunologic reactions to *P. acnes*. We wish to purify further the dominant antigenic moiety and to determine whether it is an antigen which induces skin test responses and lymphocyte transformation in acne patients.[21,22] In addition, we wish to determine whether the same material is responsible for complement fixation and whether it has any activity as an adjuvant. Finally, a purified antigen will allow us to monitor both cell-mediated and humoral responses to *P. acnes* during therapy and in relapses following treatment.

References

1 Leyden JJ. New understandings of the pathogenesis of acne. *J Am Acad Dermatol* 1995; **32:** S15–25.
2 Vowels BR, Yang S, Leyden JJ. Induction of proinflammatory cytokines by a soluble factor of *Propionibacterium acnes*: implications for chronic inflammatory acne. *Infect Immun* 1995; **63:** 3158–3165.
3 Akamatsu H, Nishijima S, Takahashi M. Effects of subminimal inhibitory concentration of EM, TC,

CLDM, MINO on the neutrophil chemotactic factor in *Propionibacterium acnes* biotype 5. *J Dermatol (Tokyo)* 1991; **18:** 247–251.
4 Akamatsu H, Niwa Y Kurokawa I. Effects of subminimal inhibitory concentrations of minocycline on neutrophil chemotactic factor production in comedonal bacteria, neutrophil phagocytosis and oxygen metabolism. *Arch Dermatol Res* 1991; **283:** 524–528.
5 Burkhart CG, Lehmann PF. Absence of circulating immune complexes in acne vulgaris. *Br J Dermatol* 1982; **110:** 120–121.
6 Webster GF, Leyden JJ, Norman ME, Nilsson UR. Complement activation in acne vulgaris: in vitro studies with *Propionibacterium acnes* and *Propionibacterium granulosum*. *Infection and Immunity* 1978; **22:** 523–528.
7 Dahl MGC, McGibbon DH. Complement in inflammatory acne vulgaris. *Br Med J* 1976; **2:** 1383–1385.
8 Senaldi G, Yin S, Shaklee CL, *et al*. *Corynebacterium parvum* and *Mycobacterium bovis* Bacillus Calmette–Guerin-induced granuloma formation is inhibited in TNF Receptor I Knockout Mice and by treatment with soluble TNF-RI. *J Immunol* 1996; **157:** 5022–5026.
9 Adlam C, Scott MT. Lymphoreticular stimulatory properties of *Corynebacterium parvum* and related bacteria. *J Med Microbiol* 1973; **6:** 261–263.
10 Herberman RB. Natural killer cells and their possible roles in resistance against disease. *Clin Immunol Rev* 1981; **1:** 1–14.
11 Cantrell JL, Wheat RW. Antitumor activity and lymphoreticular stimulation properties of fractions isolated from *Corynebacterium parvum*. *Cancer Res* 1979; **39:** 3554–3555.
12 Lehmann PF, Reiss E. Comparison by ELISA of serum anti-*Candida albicans* mannan IgG level of a normal population and in diseased patients. *Mycopathologia* 1980; **70:** 89.
13 Crowle AJ, Cline LJ. An improved stain for immunodiffusion tests. *J Immunol Methods* 1977; **17:** 379–381.
14 Lehmann PF, Reiss E. Detection of *Candida albicans* mannan by immunodiffusion, counterimmunoelectrophoresis, and enzyme-linked immunoassay. *Mycopathologia* 1980; **70:** 83.
15 Lachmann PJ. Complement technology. In: Weir DM, ed. *Handbook of Experimental Immunology*, 3rd edn. Oxford: Blackwell Scientific Publications, 1978: 5–10.
16 Mancini G, Carbonara AO, Heremens JF. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 1965; **2:** 235–238.
17 Laurell CB. Antigen–antibody crossed electrophoresis. *Anal Biochem* 1965; **10:** 358–362.
18 Puhvel SM, Hoffman IK, Sternberg THE. *Corynebacterium acnes*. Presence of complement fixing antibodies to *Corynebacterium acnes* in the sera of patients with acne vulgaris. *Arch Dermatol* 1966; **93:** 364–369.
19 Holland DB, Ingham E, Gowland G, Cunliffe WJ. IgG subclasses in acne vulgaris. *Br J Dermatol* 1986; **114:** 349–351.
20 Ashbee HR, Muir SR, Cunliffe WJ, Ingham E. IgG subclasses specific to *Staphylococcus epidermidis* and *Propionibacterium acnes* in patients with acne vulgaris. *Br J Dermatol* 1997; **136:** 730–733.
21 Gowland G, Ward RM, Holland KT, Cunliffe WJ. Cellular immunity to *Propionibacterium acnes* in the normal population and patients with acne vulgaris. *Br J Dermatol* 1978; **99:** 43–47.
22 Kersey P, Sussman M, Dahl M. Delayed skin test reactivity to *Propionibacterium acnes* correlates with severity of inflammation in acne vulgaris. *Br J Dermatol* 1980; **103:** 651–655.



From the collection of
Lawrence Charles Parish, MD,
Philadelphia, Pennsylvania.