The Microbial Content and Complement C3 Cleaving Capacity of Comedones in Acne vulgaris

J. P. LEEMING,[1] E. INGHAM[2] and W. J. CUNLIFFE[3]

Departments of [1]Microbiology, [2]Immunology and [3]Dermatology, University of Leeds, Leeds, Yorkshire, England

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Complement C3 deposition around lesions is an early event in the inflammation of acne vulgaris. The aims of this study were to determine the relationship between the capacity of individual comedones to cleave complement C3 and their microbial flora. The contents of 48 open comedones were expressed from the upper back of acne vulgaris patients and each comedo was homogenized individually and assayed for microbial content and capacity to induce cleavage of complement C3 in an in vitro assay system. An association between *Propionibacterium* population size and extent of C3 cleavage was found, but *Staphylococcus* and *Pityrosporum* population sizes did not appear to have an appreciable influence. A strong association between the weight of expressed material and C3 cleavage was apparent, irrespective of microbial population size. This observation suggests that comedones contain non-microbial material having the capacity to induce complement cleavage and hence initiate inflammation.
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J. P. Leeming, Department of Microbiology, Bristol Royal Infirmary, Bristol BS2 8HW, Avon, England.

The majority of inflamed acne vulgaris lesions are believed to arise from comedones (open, closed and clinically inapparent micro-comedones) (1). Although the factors responsible for eliciting inflammation around these lesions have not been identified, most investigations have concentrated on micro-organisms and products of their metabolic activities such as free fatty acids (2–4). One of the earliest histologically apparent manifestations of an inflammatory response around comedones is the deposition of complement C3 at the basement membrane zone of many affected pilosebaceous units and in the walls of adjacent dermal blood vessels (5, 6). The capacity of pooled samples of expressed comedonal material to consume complement-mediated haemolytic activity has been demonstrated (7). However, the nature and distribution of factors amongst comedones responsible for complement activation has not been elucidated.

The three groups of micro-organisms most frequently found in acne lesions (propionibacteria, coagulase-negative staphylococci and *Pityrosporum spp.*) have all been shown to cleave complement in in vitro assay systems (8–10). However, the abundance of these microbial groups varies widely amongst comedones (11, 12). This investigation was undertaken to determine whether there is heterogeneity amongst individual open comedones with respect to their capacity to cleave complement in vitro and, if so, whether a high cleavage capacity is associated with a particular microbial profile.

MATERIAL AND METHODS

Comedo extraction

Forty-eight open comedones were extracted from the upper back of 19 volunteer acne vulgaris patients attending the outpatient department of Leeds General Infirmary. None of the subjects had received treatment for their acne or any other anti-microbial therapy for at least 4 weeks. The skin surface was

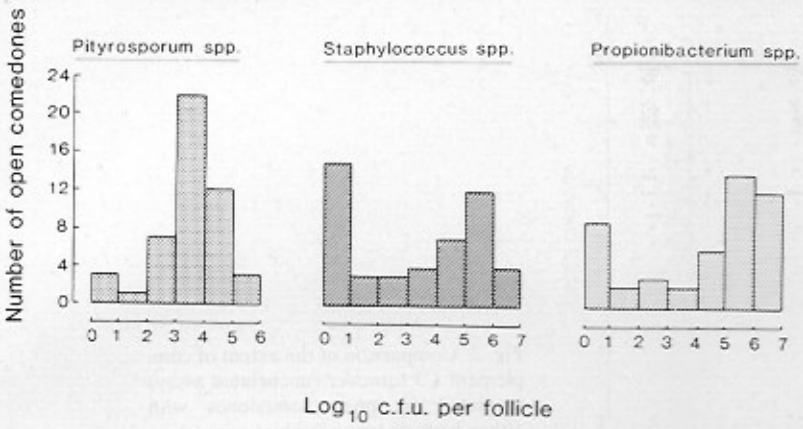

Fig. 1. Distribution of microbial densities in 48 expressed open comedones. c.f.u. = colony-forming units.

lightly swabbed with a propanol-impregnated swab (Sterets; Schering-Prebbles Ltd.) and a maximum of 3 comedones expressed from each subject, using sterile Unna comedo extractors. Each comedo was weighed and homogenized individually in 125 µl phosphate-buffered (pH 7.9) 0.1% Triton X-100 solution ('wash fluid') (13).

Estimation of micro-organisms

Ten-fold dilutions of each homogenate were made in half-strength wash fluid (wash fluid diluted 1 : 1 with distilled water). Aliquots of these dilutions were inoculated onto the surfaces of:

i) reinforced clostridial agar (RCA; Oxoid) supplemented with 0.1 ml/l Tween 80 and 6 mg/l furazolidone for the counting of propionibacteria;

ii) heated blood agar (HBA, Oxoid) for the counting of aerobic and facultative bacteria; and

iii) a *Pityrosporum* recovery medium composed of (/1)-bacteriological peptone (10 g; Oxoid), yeast extract (0.1 g; Oxoid), glucose (40 g), glycerol (1 ml), glycerol monostearate (0.5 g; BDH), Tween 60 (0.5 ml), whole fat cow's milk (10 ml), agar no. 1 (12 g; Oxoid), chloramphenicol (50 mg), cycloheximide (0.2 g). Colonies were counted after incubation at 37°C, RCA anaerobically for 6 days, HBA aerobically for 2 days and *Pityrosporum* medium for 10 days. Microbial groups were differentiated on the basis of gross colony and microscopic morphology. *Pityrosporum* counts were also made microscopically as described previously (14). *Pityrosporum* counts presented are a combination of microscopic and cultural data; the cultural techniques used being more sensitive than microscopy but occasionally yielding unreliable results when fastidious *Pityrosporum* isolates are encountered.

Estimation of complement C3 cleavage

A pool of normal human serum (NHS) was obtained from 10 healthy volunteers and stored in aliquots at −70°C. Seventy-five µl of the ten-fold dilution of each comedo homogenate was incubated at 37°C for 3 h with (i) 100 µl NHS plus 60 µl 0.9% (w/v) saline to measure C3 cleavage by the classical and alternative pathways combined, and (ii) 100 µl of NHS plus 30 µl 0.2 mol/l ethylene glycol *bis*-(aminoethyl)-tetra-acetic acid (EGTA; Koch Light, Colnbrook, Bucks) and 30 µl 0.3 mol/l Mg_2SO_4 to measure C3 cleavage by the alternative pathway only. Positive controls in which zymosan (10 mg/ml; Sigma) replaced the comedo homogenate and negative controls in which half-strength wash fluid replaced the homogenate were included with each batch of incubations. Cleavage of C3 was stopped by adding 20 µl 0.2 mol/l ethylenediaminetetra-acetic acid (EDTA) to each reaction mixture at 3 h. Reaction mixtures were stored at −70°C until assayed for C3 cleavage by standard 2-dimensional electrophoresis as described previously (15). The extent of C3 cleavage was calculated as the percentage of total C3 (β1c plus β1A peaks) migrating as cleavage product (β1A peak). The percentage C3 cleaved by the corresponding negative control was subtracted from each test value prior to data analysis.

Complement cleavage by laboratory cultures of micro-organisms

To assess the capacity of micro-organisms to mediate cleavage of complement C3 in the assay system described, known densities of washed, laboratory cultured cells were added to the test system in place of the comedo homogenates. *Propionibacterium acnes* strain P37 and *Staphylococcus epidermidis* strain NCTC 11047 were grown in brain–heart infusion broth (Difco; supplemented with 3 g/l glucose) for 72 h and 12 h respectively; a fresh isolate of *Pityrosporum* was cultured in an ox-bile-supplemented nutrient

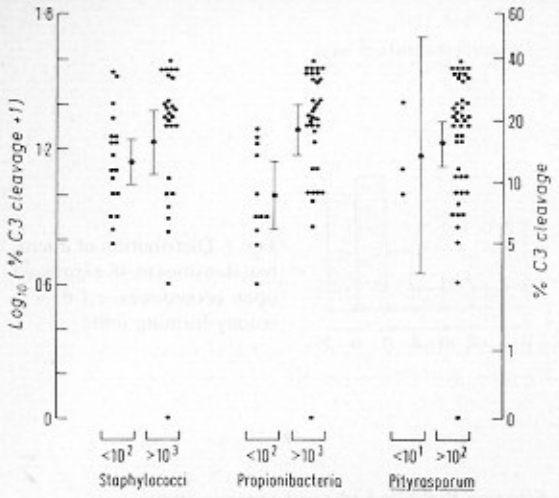

Fig. 2. Comparison of the extent of complement C3 turnover (unchelated assay) mediated by open comedones with either high or low microbial population sizes. Vertical range bars represent 95% confidence limits.

broth for 48 h. Cells were harvested by centrifugation and washed and resuspended to a density of 10^{10} cells/ml in PBS. Aliquots of various dilutions of these cell suspensions were assayed for their unchelated complement cleaving capacity in a similar manner to that described above.

Statistical methods

Statistical analyses were performed according to the recommendations of Sokal & Rohlf (16).

RESULTS

Comedo composition

The weights of extracted comedones were lognormally distributed with a range of 0.065–2.880 mg and a median value of approximately 0.5 mg. The great majority of microbial isolates were members of the genera *Staphylococcus, Propionibacterium* and *Pityrosporum* (*Malassezia*). The distribution of densities of these groups or organisms are displayed in Fig. 1.

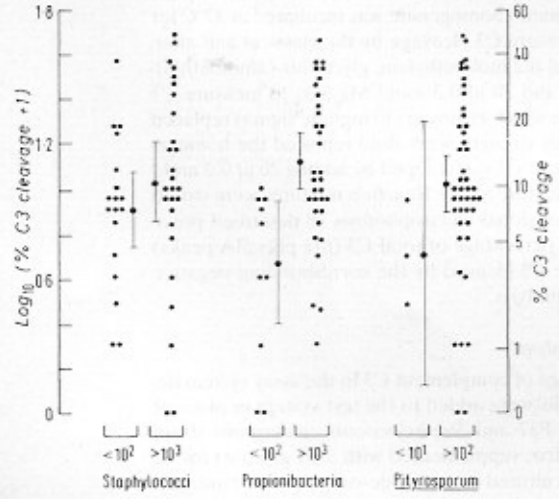

Fig. 3. Comparison of the extent of complement C3 turnover (alternate pathway assay) mediated by open comedones with either high or low microbial population sizes. Vertical range bars represent 95% confidence limits.

Complement cleavage by comedo homogenates

Complement C3 was cleaved to an extent by all the comedonal homogenates tested in either one or both assays (0 to 48%, median 8% in the alternate pathway assay; 0 to 38%, median 18% in the unchelated assay). There was a highly significant correlation between the alternate pathway and the unchelated assay results ($p<0.0001$; Spearman Rank).

Associations between complement cleavage and comedo composition

Complement C3 cleavage was found to correlate positively with comedonal weight and population sizes of all three microbial groups described previously (Table I). However, these simple two-variable comparisons were complicated by the observation that both staphylococcal and pityrosporal (but not propionibacterial) counts were also closely correlated with comedo weight. For this reason, partial correlation analyses were performed on these variables, revealing that when co-correlations were compensated for, complement C3 turnover was correlated to a highly significant extent with both propionibacterial counts and comedonal weight, but not with staphylococcal or pityrosporal counts (Table I). These observations were confirmed by comparing complement turnover in groups of comedones containing either low or high counts of each group of micro-organism (Figs. 2 and 3). Comedones colonized by high numbers ($>10^3$) of propionibacteria cleaved significantly more complement than did those yielding low ($<10^2$) counts. Similar differences associated with staphylococcal and pityrosporal colonization did not achieve significant levels.

Complement cleavage by laboratory cultures of micro-organisms

Complement cleavage was not detectable unless $>10^6$–10^7 cells/ml were present in the test system. This was the case with each of the strains tested, whether the cells were live or formalized. To achieve a cleavage equivalent to that obtained with the most potent comedo homogenates, 10^8–10^9 cells/ml were required.

Table I. *Analysis of the associations between complement cleavage, weight and microbial content of comedones by Spearman's rank correlation and partial correlation analyses*

Correlation coefficients and statistical probabilities are displayed

	Spearman's rank correlation			Partial correlation analysis*	
	Unchelated C3 turnover	Alternate pathway C3 turnover	Comedo weight	Unchelated C3 turnover	Alternate pathway C3 turnover
Pityrosporum counts	0.296 $p=0.0410$	0.303 $p=0.0365$	0.507 $p=0.0002$	0.284 $p=0.029$	0.204 $p=0.090$
Staphylococcus counts	0.421 $p=0.0029$	0.355 $p=0.0133$	0.609 $p<0.0001$	−0.007 $p=0.483$	−0.2453 $p=0.052$
Propionibacterium counts	0.453 $p=0.0012$	0.308 $p=0.0331$	0.115 $p=0.436$	0.535 $p<0.001$	0.497 $p<0.001$
Comedo weight	0.570 $p<0.0001$	0.654 $p<0.0001$		0.357 $p=0.008$	0.548 $p<0.001$

* Partial correlation coefficients of C3 cleavage with each of the variables were calculated whilst controlling for the co-correlations of the other three variables. All data were transformed to obtain approximately normal distributions. Calculations were made using the "SPSS-X" software package (SPSS Inc., Chicago, USA).

DISCUSSION

The results presented demonstrate strong associations between the capacity of comedo homogenates to cleave complement C3 in vitro and both propionibacterial content and the weight of the comedo (independent of the microbial load). This suggests that both propionibacterial-derived material and non-microbial components of comedones have C3 cleavage potential. Although the results of the partial correlation analyses on the microbial data should be interpreted with some caution due to the departure from normality of their distributions (see Fig. 1), these conclusions were supported by very high statistical confidence values and by the results of independent analyses (see Figs. 2 and 3).

The nature of the material affecting the observed complement cleavage is not obvious. Although cleavage correlated with propionibacterial counts, the results obtained using washed cells in the test system suggest that at least 100-fold more microbial cells than were ever found in comedones would be necessary to exert a direct effect in the system used. Webster et al. (17) also reported that greater than 10^7 *Propionibacterium spp.* cells/ml were required to obtain detectable effects in a complement haemolytic assay. Thus, unless cells grown in vivo are considerably more potent in triggering complement cleavage than are laboratory cultured cells, it is likely that any causal relationship is due to the metabolic activities of propionibacteria. This could be due to products released into the environment, or to the microbial enzyme activity on structural components of the comedo. It is unlikely to be the direct result of exocellular enzyme cleavage of C3, as it has been demonstrated previously that purified propionibacterial exocellular enzymes (including proteinases) fail to cleave complement proteins in normal human serum in vitro (18). Further investigations have been undertaken to isolate and characterise the factors within comedones which cleave C3 in vitro.

It is important to note that the identification of comedo components which cleave C3 in vitro takes no account of factors such as the permeability of the pilosebaceous follicle wall to molecules of interest. Only compounds capable of passing through comedo walls will be capable of initiating the complement deposition observed in vivo around apparently intact comedones (5, 6). This is another important area for future investigations.

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REFERENCES

1. Kligman A. An overview of acne. J Invest Dermatol 1974; 62: 268–287.
2. Holland K, Ingham E, Cunliffe W. The microbiology of acne. J Appl Bacteriol 1981; 51: 195–215.
3. De Young L, Young J, Ballaron S, Spires D, Puhvel S. Intradermal injection of *Propionibacterium acnes:* a model of inflammation relevant to acne. J Invest Dermatol 1984; 83: 394–398.
4. Allaker R, Greenman J, Osborne R, Gowers J. Cytotoxic activity of *Propionibacterium acnes* and other skin organisms. Br J Dermatol 1985; 113: 229–235.
5. Scott D, Cunliffe W, Gowland G. Activation of complement—a mechanism for the inflammation in acne. Br J Dermatol 1979; 101: 315–320.
6. Dahl M, McGibbon D. Complement C3 and immunoglobulin in inflammatory acne vulgaris. Br J Dermatol 1979; 101: 633–640.
7. Webster G, Leyden J, Nilsson U. Complement activation in acne vulgaris: consumption of complement by comedones. Infect Immun 1979; 26: 183–186.
8. McBride W, Weir D, Kay A, Pearce D, Caldwell J. Activation of the classical pathways of complement by *Corynebacterium parvum*. Clin Exp Immunol 1975; 19: 143–147.
9. Forsgren A, Quie P. Influence of the alternate complement pathway on opsonization of several bacterial species. Infect Immun 1974; 10: 402–404.