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A Method for the Assay of Inflammatory Mediators in Follicular Casts

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A method is presented whereby inflammatory mediators may be detected and quantified in individual follicular casts. Lysozyme, lactoferrin, IgG, IgM, C3 and material reacting with antiserum to polymorphonuclear leukocytes (PMN) were assayed by functional and immunologic methods. By these techniques, lysozyme, IgG and anti-PMN reactive material were detected in clinically uninflamed follicular casts from acne subjects.

Inflammation in acne vulgaris may begin long before the physical rupture of the involved follicle. Kligman has observed neutrophils around the periphery of intact closed comedones and suggested that the arrival of these leukocytes may presage the rupture of the follicle and the development of clinical inflammation [1]. In 1977, Lees and co-workers [2] analyzed the soluble proteins in pooled comedones and found that comedones from 3 individuals contained material which had the same electrophoretic mobility as serum alpha and beta globulins. They theorized that this material might be IgA which has been directly secreted into the lumen of the pilosebaceous follicle. Knop and Oleffs [3] reported that IgG and, in some cases, IgA could be identified in open and closed comedones. Most recently, Hernandez and Puhvel [4] analyzed the soluble proteins in a single pool of 713 open comedones, using antisera to IgA and numerous other serum and nonserum proteins. They found only material reacting with antisera to human serum albumin and Zn-alpha-2-glycoprotein, a minor serum protein which is also found in callous. IgA was not detected nor were complement components. These data taken together provide evidence that there may be serum components in mature comedones.

In order to see if early evidence of inflammation can be found in clinically normal sebaceous regions we have developed a microassay system for individual follicular casts by which the presence of inflammatory mediators can be quantitatively detected. A follicular cast is that material which adheres to some of the hairs which are removed by the skin surface biopsy technique [6]. Plewig and Kligman [7] have suggested that they may be considered to be pre-comedones. Holmes, Williams, and Cunliffe [8] have found these structures to be more numerous in acne patients. We assayed for proteins IgG, IgM, C3, lactoferrin and lysozyme and material reacting with antiserum to human polymorphonuclear leukocytes (PMN). Lysozyme is an enzyme found in the primary and secondary granules of PMN and macrophages [5] which may be released in response to phagocytic or leukotoxic stimuli [9]. Lysozyme concentrations have been used as a marker for inflammation and found to increase in synovial fluid [10], gingival crevice fluid [11], and tuberculosis lesions [12] in proportion to the degree of inflammation present. Similarly, lactoferrin is found exclusively in neutrophil granules [13] and has been shown to be a marker for inflammation [10].

MATERIALS AND METHODS

Collection of Acne Lesions

Follicular casts were collected from the cheeks of 5 acne patients, using the method of Marks and Dawber [6]. A total of 890 follicular casts was examined. The areas from which the casts were taken showed no evidence of comedones or inflammation. In no cases was there any evidence of hemorrhage or erythema after the removal of the cement. Follicular casts were harvested individually under a dissecting microscope, using a needle, and then plunged directly into the assay agars.

Antisera

Antihuman lysozyme was purchased from Miles Laboratories. Human lactoferrin and rabbit antihuman lactoferrin were provided by Dr. C. -C. Tsai. Antilactoferrin gave a single band on immunodiffusion and immunoelectrophoresis against purified human lactoferrin and PMN lysate. Anti-PMN serum was prepared by the following method.

PMN were isolated from heparinized blood by a modification of the method of Leffel and Spitznagel [14]. Briefly, buffy coat cells were prepared by dextran sedimentation of erythrocytes and then purified by ficoll-hypaque (lymphocyte separation medium Litton Bionetics) gradient centrifugation. The PMN fraction was removed, contaminating erythrocytes were lysed with hypotonic saline, and the cells washed. PMN were than suspended to a concentration of 5×10^6/ml in Hanks buffer, frozen and thawed, and emulsified with an equal volume of Freund's complete adjuvant (Difco). Male white rabbits were given 8 weekly injections of 0.5 increasing to 1 ml by the third week, divided between each flank. Ten days following the final injection, rabbits were bled and serum was collected and inactivated at 56°C for 30 min. The specificity of this serum was tested by several methods. First, it failed to agglutinate human type AB+ erythrocytes, and would not cause their lysis when fresh rabbit serum was added. Immunoelectrophoresis (IEP) against human serum, which was rapidly separated from blood (in order to minimize PMN degranulation), detected no cross-reactivity. Immunoelectrophoresis against PMN lysate developed with anti-PMN serum and antilysozyme serum showed that this preparation of antiserum does not detect PMN lysozyme (Figure). Buffy coat cells were incubated with the anti-PMN serum, treated with fluorescent antirabbit IgG (Miles), and then examined under a fluorescence microscope. All PMN fluoresced strongly and some monocytes showed weak staining. Since this cross-reactivity might be due to the presence of common molecules on both cell types, no effort was made to absorb out with monocyte preparations. Washed horny cells, hair, buccal mucosa cells, and epidermal cells retrieved from a biopsy of normal skin were incubated with anti-PMN serum, washed, and then reacted with fluorescein labeled antirabbit IgG. Fluorescence microscopy failed to demonstrate antibody binding on any of the test tissues.

Immunoelectrophoresis

IEP was performed after the method of Scheidegger [15] in 1.0% Agarose (Seakem) with 0.01 M ethylenediamine-tetraacetic acid. Samples were electrophoresed for 60 min at 40 v. After development, gels were washed, dried, and stained with 1.0% amido black.

Assay System

Follicular casts were assayed for the presence of lysozyme by a functional assay [16] in which they were individually embedded in agar containing *Micrococcus lysodeicticus* cells (Worthington). After an 18-h incubation at 30°C, evidence of lysozyme activity is seen as a zone of clearing around the cast, the size of which is proportional to the amount of enzyme present. Standard solutions of egg white lysozyme (grade 1, Sigma) in concentrations of 0.5, 0.1, 0.05, and 0.01 mg/ml were run on

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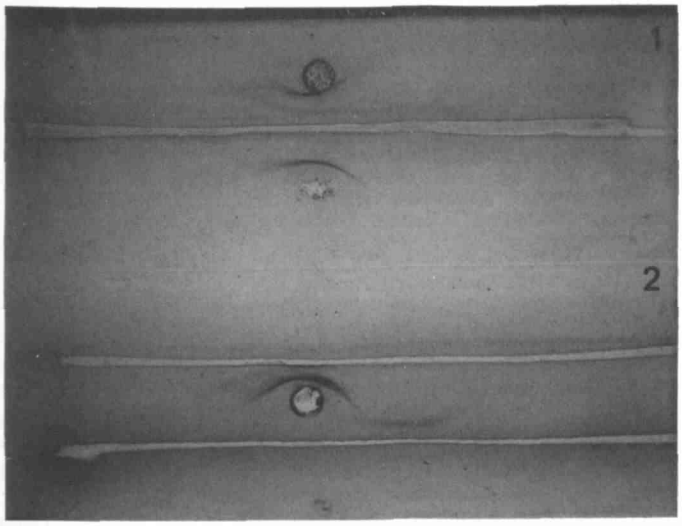
Abbreviations:
IEP: immunoelectrophoresis
PMN: polymorphonuclear leukocytes



Immunoelectrophoresis of anti-PMN serum and follicular casts. *Slide 1* contains polymorphonuclear leukocytes (PMN) lysate in the *top well* and follicular casts in the *bottom well*. The slide was developed with anti-PMN serum. *Slide 2* contains PMN lysate in the *center well*, anti-PMN serum in the *upper trough* and antilysozyme serum in the *lower trough*. Cathod is to the left.

each assay plate. Quantitative radial immunodiffusion assays for IgG, IgM, and C3 were similarly performed on embedded follicular casts according to the manufacturer's instructions. IgG and IgM were assayed on RID plates with lower sensitivity limits of 3-13 mg/dl (IgG ultra-low level) and 12.5-50 mg/dl (IgM low level) (ICL Scientific). C3 was assayed on RID plates with a lower sensitivity limit of 35 mg/dl (Hyland). Anti-PMN assay agar was prepared by incorporating 0.5% anti-PMN serum in 1.0% Noble agar (Difco). Sensitivity levels were not determined since the anti-PMN serum contained antibody of a multitude of specificities against a wide range of antigens. The PMN antigen assays were allowed to proceed for 48 h at 30°C in a humid chamber. Quantification in all assays was achieved by comparison with standardized amounts of the antigen or enzyme. On all plates, areas of precipitation or clearing from the standard solutions were calculated and the area of the sample well subtracted from each value. This was done in order that the standard solutions might be compared to a solid embedded follicular cast.

RESULTS

Antisera raised against human PMN was used to develop electrophoresis of PMN lysate and 30 follicular casts embedded in agarose. As shown in the Figure, the follicular casts shared 2 antigens with the PMN lysate. One follicular cast antigen produced a more intense precipitation than the corresponding antigen in the PMN lysate which contained 5×10^6 PMN/ml. Neither follicular cast antigens had similar mobilities to lysozyme.

The results of assays of 890 follicular casts for lysozyme, lactoferrin, IgG, IgM, C3 and PMN antigens are presented in Table I. No casts contained detectable levels of lactoferrin, IgM, or C3. Twenty-four out of 150 (16%) casts assayed for lysozyme had measurable activity, and 32 out of 90 (35.6%) casts tested had measured IgG. PMN antigens were detected in 25 out of 150 (16.5%) casts tested. No individual had more than 25% of casts positive in the PMN antigen assay.

In a second experiment, follicular casts from one subject were washed immediately after collection for 2 min in physiologic saline and then assayed for IgG along with unwashed casts. In this experiment IgG was used as a marker for possible contamination of the cast with serum during removal from the cheek. Presumably IgG which resulted from brief contact with serum would be easily washed away, while a truly positive follicular cast would be impregnated with serum. The results in Table II show that there was little difference in the IgG content of washed and unwashed casts. The washed casts had a higher mean IgG content which appeared to reflect a loss of positive casts with low levels of IgG during washing. This argues against the trauma of collection producing hemorrhage and thereby positive follicular casts.

DISCUSSION

This paper presents a methodology whereby individual follicular casts or acne lesions may be quantitatively assayed for components of the inflammatory response. The use of agar diffusion assays permits rapid quantitative testing of many samples. The use of follicular casts provides data which may be more relevant to the genesis of inflammatory acne than that gleaned from the study of end-stage comedones.

In this study we detected lysozyme, IgG, and PMN antigens in many follicular casts and failed to detect lactoferrin, IgM, and C3. The failure to detect the latter compounds may be due to their absence in the cast, lesser sensitivity of the assays, or to a binding, trapping, or degradation of the molecules in the cast. Since we detected PMN antigen and lysozyme, the negative findings in the lactoferrin assay initially seem surprising. Lactoferrin, however, is in lower amounts in PMN than lysozyme and strongly binds to membranes and acidic macromolecules [17]. Thus, the low level of lactoferrin which might be present in a cast could easily be bound in a form which would not diffuse out of the cast.

That no individual had greater than 25% of casts giving positive results in the PMN antigen assay indicated that the antiserum is not detecting antigens which might be common to all of an individual's casts, such as histocompatibility antigens. The similar incidence of lysozyme-containing casts and PMN antigen-containing casts, 16% and 16.5% respectively, further supports our contention that the anti-PMN serum detects inflammatory activity and not some tissue-borne antigen.

It has been proposed that PMN may migrate to uninflamed acne lesions and through release of hydrolases promote rupture of the follicle leading to clinical inflammation [1]. In support of this hypothesis recent work has shown that there are neutrophil chemotactic factors within comedones [18] and that *Propionibacterium acnes* can activate complement systems to produce PMN chemotactic factors [19]. Furthermore, neutrophils have been observed around the borders of closed uninflamed comedones [1]. It is probable that a PMN in this evironment would rapidly release its hydrolases in response to the plentiful bacterial substances. (In support of this we have shown that, *in vitro*, PMN will release lysozyme in response to *P. acnes* cells

TABLE 1. *Inflammatory mediators in follicular casts*

	No. positive/ No. tested	% Positive	Mean +/− SD[a]
Lysozyme	24/150	16	0.024 +/− 0.006 mg/follicular cast
IgG	32/90	35.6	0.022 +/− 0.01 mg/follicular cast
IgM	0/250	0	0
C3	0/250	0	0
PMN antigen	25/150	16.5	N.A.[b]
Lactoferrin	0/90	0	0

[a] SD = standard of deviation.
[b] NA = not applicable.

TABLE II. *Effect of washing on follicular cast IgG content*

	No. positive/ No. tested	% Positive	Mean +/− SD[a]
Washed	4/30	13	0.0224 +/− 0.026 mg/follicular cast
Unwashed	6/30	20	0.014 +/− 0.014 mg/follicular cast

[a] SD = standard deviation.

[20]. In this paper we have presented data which confirm the presence of PMN products within clinically uninflamed sebaceous follicles.

At present, it is not clear why some individuals have inflammatory acne and others remain purely comedonal. It is possible that those with inflammatory acne have follicles which are more permeable than normal follicles, readily allowing the egress of bacterial proinflammatory factors. Conversely, all follicles may be of similar leakiness, but the comedonal individual may be more vigorous in repair of breached follicles. Whichever is the case, the techniques presented here should help to answer this question. However, until a large rigorously controlled population of acne patients and normal individuals is studied, no conclusions may be drawn regarding the initiation of inflammation in acne. Our data do confirm that inflammation may begin before a clinically detectable comedo is formed.

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