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Functional Evidence for Complement-activating Immune Complexes in the Skin of Patients with Bullous Pemphigoid

W. RAY GAMMON, CAROLYN C. MERRITT, DANIEL M. LEWIS, W. MITCHELL SAMS, JR., C. E. WHEELER, JR., AND JAIME R. CARLO

Department of Dermatology, University of North Carolina, Chapel Hill, North Carolina, U.S.A.

Previous immunofluorescent studies showing deposits of immunoglobulin and complement at the cutaneous basement membrane zone have provided evidence supporting a role for immune complexes in the pathogenesis of bullous pemphigoid. In this study the functional activity of the deposits has been examined using leukocyte attachment, a method for detecting and quantitating the biological activity of complement-activating immune complexes in tissues. When peripheral blood leukocytes suspended in serum complement were incubated with cryostat sections of lesional and adjacent normal-appearing skin from 9 patients with pemphigoid, skin from 11 normal controls and lesional skin from 14 nonpemphigoid disease controls there was significantly greater attachment of leukocytes to the basement membrane zone of lesional bullous pemphigoid skin compared to normal-appearing pemphigoid skin and skin of both control groups. A significant reduction in attachment in the absence of serum complement suggested the reaction was dependent on activation of complement by tissue-deposited complexes. Although leukocyte attachment was greater in lesional than normal-appearing pemphigoid skin, a comparison of the incidence and intensity of cutaneous IgG and complement immunofluorescence between the 2 groups showed no significant differences. Furthermore, no correlation between leukocyte attachment and serum titers of immunoglobulin G or complement-binding anti-basement membrane zone antibodies was observed. These results suggest that immune reactants in lesional pemphigoid skin are functional complement-activating immune complexes, that differences exist between the activity of complexes in lesional and normal-appearing pemphigoid skin and may explain why lesions develop at some sites and not others.

There is abundant indirect evidence supporting the proposal that complement (C)-activating immune complexes may be important in the pathogenesis of cutaneous inflammation in bullous pemphigoid (BP) [1]. Results of studies using immunofluorescent methods for the detection of immune reactants have shown deposits of immunoglobulin (Ig) and C at the basement membrane zone (BMZ) in both normal-appearing and lesional skin [2–4], C-binding anti-BMZ antibiodies in sera [5] and biologically active C fragments in blister fluids [6–8]. Although these observations support a role for immune complexes in the disease, it has not been directly shown that the immune reactants at the BMZ are components of functional complexes nor has it been determined why inflammation develops at some sites of Ig and C deposition and not others. These questions, we feel, are critical to the proposed pathogenesis of the disease.

In this study we used a recently described method, leukocyte attachment (LA) which has been shown effective in detecting and quantitating C-activating, immune complexes deposited in tissues *in vivo* and *in vitro* [9–11]. The method was used to determine: (1) if biologically functional immune complexes are present at the BMZ in skin of patients with active BP and (2) whether or not quantitative differences in function can be detected between lesional and adjacent normal-appearing skin. Furthermore, an attempt has been made to determine the relationship between functional immune complex activity and results of immunofluorescent studies of immune reactants in tissue and sera.

MATERIALS AND METHODS

Skin

Four groups of skin samples were used in these studies. Two groups consisted of paired biopsies obtained simultaneously from the prebullous macular or papular erythematous lesional skin (BPL) and adjacent, 2 cm away, clinically normal-appearing skin (BPN) from 9 patients with BP. All patients were diagnosed by established clinical, histologic and immunofluorescent criteria [12]. Skin samples were also obtained from 2 control groups. One control group consisted of skin from 11 normal humans (NS). Skin in this group was obtained from adult volunteers (5) and from neonatal foreskin (6). A second control group consisted of lesional macular or papular erythematous skin from 14 patients with a variety of active inflammatory diseases (DS). Included in this group were biopsies from patients with eczema (5), erythema multiforme (2), hypersensitivity vasculitis (2), urticaria (1), dermatomyositis (1), papular dermatitis of pregnancy (1), Sweet's syndrome (1) and immunofluorescent-negative chronic bullous disease of childhood 1. All samples were obtained after informed consent by 3–4 mm punch biopsies under 1% xylocaine anesthesia or routine circumcision. Samples were immediately frozen in liquid N_2, mounted in OCT compound (Ames, Co., Elkhart, IN) and stored frozen at −70°C.

Pemphigoid Sera

Serum from all 9 BP patients was obtained at the time of biopsy, heat-inactivated (56°C × 30 min) and stored forzen in 1.0 ml aliquots at −70°C.

Serum Complement

Fresh frozen, platelet-poor normal human serum (NHS) from a single blood group AB, type Rh positive donor was used as a source of serum C in all studies. Blood was collected under sterile conditions and platelet-poor serum prepared as previously described [10]. NHS was divided into 1.0 ml aliquots and stored frozen at −70°C. In some experiments heat-inactivated (56°C × 30 min) NHS was prepared from fresh NHS immediately before use.

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Reprint requests to: Dr. W. Ray Gammon, Department of Dermatology, University of North Carolina, Chapel Hill, NC 27514.

Abbreviations:
BMZ: basement membrane zone
BP: bullous pemphigoid
BPL: bullous pemphigoid lesional
BPN: normal appearing skin
C: complement
DS: disease control skin
FITC: fluorescein isothiocyanate
Ig: immunoglobulin
LA: leukocyte attachment
NHS: normal human serum
NS: normal skin
PBL: peripheral blood leukocytes

Peripheral Blood Leukocytes (PBL)

PBL from normal human volunteers were prepared by dextran sedimentation of heparinized whole blood using a previously described method [10]. Following dextran sedimentation for 30 min at 37°C, the leukocyte-rich supernate was removed and cells washed ×2 in 20.0 ml Geys balanced salt solution (Flows Labs, McLean, VA) containing 2% bovine serum albumin (GBSS + 2%) BSA). Residual red cells were removed by hypotonic lysis with distilled water. Total leukocytes and viability were determined by counting trypan blue-stained cells in a hemocytometer. Viability was consistently greater than or equal to 98%. The percentage of granulocytes determined by differential counts on 200 cells was consistently between 70 and 85%.

Immunofluorescent Technique

Direct immunofluorescence for IgG and C3 was performed on all BP skin using the methods of Beutner and Nisengard [13]. Indirect immunofluorescence for IgG anti-BMZ antibodies and indirect complement-binding immunofluorescence for C3-binding anti-BMZ antibodies were performed using normal human skin as substrate according to the method of Jordon, Sams, and Beutner [5]. Antisera used were fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG and C3 (Cappel Labs, Cochranville, PA). Total protein concentration and molar fluorescein to protein (F/P) ratio for anti-C3 were 3,000 μg/ml and 2.0 respectively. For anti-IgG, specific antibody concentration and F/P ratio were 610 μg/ml and 2.6. Antisera were used at a working dilution of 1:10. The intensity of direct immunofluorescent staining was graded by using an arbitrary scale of 0–4+. Anti-BMZ antibody titers in BP sera were the highest serum dilutions producing 1+ BMZ fluorescence. Sera were not diluted beyond 1:1280. All immunofluorescence was performed using a Leitz Orthoplan fluorescent microscope equipped with epi-illumination.

Leukocyte Attachment Method

Three-four 8 μm sections of skin were cut from each skin sample at −20°C on a Harris model WRC cryostat (Harris Manufacturing CO., Inc., Cambridge, MA) and placed approximately 4-mm apart in the center of gelatin-coated microscope slides. The tissue was allowed to dry briefly at 25°C and then washed for 10 min at 4°C in 0.15 M NaCl buffered with 0.01 M Na_2HPO_4 and 0.01 M NaH_2PO_4, pH 7.2 (PBS). Excess buffer was blotted from the slides and tissue chambers prepared as previously described [9]. Briefly, the tissue-slide was covered by a second slide to which 2 thicknesses of vinyl tape (Fisher Scientific Co) had been attached at each end. This prevented the covering slide from making direct contact with the tissue sections and provided a chamber between the slides 0.2 mm thick and 0.4 ml volume. The tissue and covering slides were then taped together using Highland 6200 cellophane tape (3M Co., St. Paul, MN), and immediately placed in a humidity chamber at 25°C to prevent tissue sections from drying out. Slides were removed from the chamber as needed and each was injected with a supsension of 10×10^6 PBL/ml in 5% NHS or 5% heat-inactivated (56°C × 30 min) NHS diluted in GBSS + 2% BSA. The suspension was injected slowly over tissue sections with a tuberculin syringe equipped with a 27-gauge needle taking care to avoid air bubbles, to avoid disturbing tissue sections and to completely fill chambers. Slide chambers were then incubated for min at 37°C in a humidified ambient air incubator. Following incubation slides were disassembled, excess serum and nonadherent cells removed by rinsing slides for 10 min in PBS and air-dried briefly at 25°C. Tissues were then fixed in 100% ethanol, stained with hematoxylin and eosin (H & E) and examined at 250× with a Zeiss light microscope equipped with an eyepiece linear micrometer (American Optical Corp., Buffalo, NY).

Assessment of Leukocyte Attachment and Computation of Results

Leukocyte attachment was performed on at least 2 separate occasions for each of the 29 skin samples in groups BPL, BPN, and NS. It was performed twice on 12 and once on 2 samples in group DS. For each assay, the 2 best skin sections on each slide (those showing minimal distortion of the BMZ) were counted. Frequently 1 or 2 of the 3–4 sections originally placed on slides were torn or distorted preventing reliable counting of cells. For the 2 selected sections, 2–3 1000 μm segments of BMZ per section were selected at random and the number of PBL attached to the BMZ counted. An average LA value per 1,000 μm of BMZ was then computed for each assay by totaling the number of cells counted on sections and dividing by the number of 1,000 μ segments counted. An average LA value for each skin biopsy was then calculated by adding the average for each assay and dividing by the number of assays performed on that biopsy. Mean attachment values for each group of biopsies were calculated on the basis of the average LA value for each biopsy in the group. Statistical analysis of data was performed using the paired *t*-test, analysis of variance, correlation coefficients and chi-square analysis.

RESULTS

Immunofluorescence

The results of direct immunofluorescence for IgG and C3 at the BMZ in paired lesional and normal-appearing BP skin are shown in Table I. IgG was detected at the BMZ in 8 of 9 biopsies in group BPL and 5 of 9 in group BPN. Chi-square analysis of the incidence of IgG and C3 between groups showed no significant differences ($p > .1$). A comparison (paired *t*-test) of the mean intensity of immunofluorescent staining for IgG and C3 between groups also showed no significant difference. Results of indirect immunofluorescence for IgG and C3-binding anti-BMZ antibody titers for BP patients are also shown in Table I. Six of 9 sera had IgG anti-BMZ antibody activity at titers of 1:40 to 1:1280. Five of the 6 also had complement-binding anti-BMZ antibodies at titers of 1:20–1:640.

Results of Leukocyte Attachment

Table II shows the number of LA assays performed (column A) the number of 1000 μm segments of skin examined (column B) and the average LA value (column C) for each of the 43 biopsies in groups BPL, BPN, NS, and DS. The mean number of leukocytes attached per 1,000 μm BMZ for lesional BP skin (BPL) was 26.5, for adjacent normal-appearing skin (BPN)-8.9, for the normal control group (NS)-6.6 and for nonpemphigoid disease control group (DS)-8.2. Analysis of variance between the means of the 4 groups showed significantly greater attachment in group BPL than in groups BPN, ($p < .02$) NS ($p < .01$) and DS ($p < .02$). No significant differences were observed between the means of groups BPN, NS, and DS.

An analysis of the distribution of LA values for biopsies in group BPL and corresponding values for paired biopsies in group BPN (Fig 1) showed that in all patients, attachment was greater in lesional skin than in paired normal-appearing skin.

Evidence supporting a requirement for serum complement in attachment of leukocytes to BP lesional skin: The LA assay was performed on lesional BP skin sections from 5 biopsies on 4 patients (patients number 5, 6, 7, 9) using cells suspended in either 5% NHS or 5% heat-inactivated (56°C × 30 min) NHS). These experiments were performed in duplicate. The results (Fig 2) showed a significant reduction in attachment in the presence of heat-inactivated (56°C × 30 min) NHS compared to NHS ($p < .05$).

Inhibition of LA by $F(ab)_2$ antibody to the Fc fragment of human IgG. In duplicate experiments skin sections from the lesional skin of 3 BP patients were preincubated for 30 min at 4°C with a 1:10 dilution of the $F(ab')_2$ fragment of goat IgG with specificity for the Fc fragment of human IgG (Cappel Labs). The anti-human IgG Fc was used at a concentration of 0.5 mg/ml antibody protein. Control sections received incubation with buffer alone. Following incubation, sections were rinsed in PBS and the LA procedure performed. Results, Table III, showed significant inhibition of LA in sections treated with antibody to human IgG (Fc) compared to sections treated with buffer alone ($p <$s .02).

Comparison between LA and Results of Direct and Indirect Immunofluorescence

To determine if a relationship between LA values and intensity of immunofluorescence was present in BP biopsies, LA in each of the biopsies in groups BPL and BPN was compared with the respective intensity (0–4+) of direct immunofluorescence for both IgG and C3. The results showed no correlation for any of the comparisons. Furthermore, no correlation was observed between the level of LA and titers of IgG or C3-binding anti-BMZ antibodies.

TABLE I. *Results of immunofluorescence for IgG and C3 deposits in skin and IgG anti-BMZ antibodies and complement-binding antibodies in sera of pemphigoid patients*

Patient	Lesional skin IgG	(BPL) C3	Normal skin IgG	(BPN)[a] C3	IgG Anti-BMZ antibody titers	C3-Binding titers
1	3+	4+	3+	4+	>1280[b]	160
2	3+	4+	0	2+	<10	<10
3	2+	4+	0	2+	80	<10
4	4+	4+	3+	2+	160	40
5	4+	4+	2+	2+	>1280	640
6	2+	2+	2+	3+	320	320
7	0	3+	0	0	<10	>10
8	1+	2+	0	1+	40	20
9	3+	1+	3+	2+	<10	10
Mean	2.4	3.1	1.4	2.0	251	131
Incidence	89%	100%	56%	89%		

[a] Biopsies of normal skin were taken approximately 2 cm from the site of lesional skin biopsies.
[b] Titers 10 or 1280 were not determined.
[c] 0 = no staining; 1+ = trace discontinuous staining; 2+ = trace continuous; 3+ = moderate staining; 4+ = heavy staining.

TABLE II. *Results of leukocyte attachment in bullous pemphigoid lesional (BPL) and paired normal-appearing skin (BPN), normal skin (NS) and disease control skin (DS) groups*

Sample #	BPL			BPN			NS			DS		
	A[a]	B[b]	C[c]	A	B	C	A	B	C	A	B	C
1	2	18	17.0	3	19	9.5	2	12	5.2	2	12	14.6
2	2	9	10.8	2	12	6.6	2	12	4.4	2	12	6.6
3	3	18	34.2	2	12	10.6	2	12	4.0	2	12	12.4
4	3	15	30.9	3	18	6.4	2	12	3.4	1	6	5.0
5	3	20	24.4	2	9	16.1	2	12	6.6	2	12	8.8
6	2	12	23.8	2	12	10.4	2	12	6.8	2	12	7.9
7	2	11	32.7	2	12	4.6	2	12	8.7	2	12	6.6
8	2	9	36.4	2	12	9.0	2	12	10.4	2	12	6.9
9	2	12	28.2	2	13	7.1	2	12	5.5	2	12	7.2
10							2	12	5.1	2	12	7.1
11							2	12	12.1	2	12	7.1
12										2	12	5.2
13										2	12	7.2
14										1	6	12.0
Totals	22	124	238.4	20	119	80.3	22	132	72.2	26	156	115.
Means	2.4	13.8	26.5	2.2	13.2	8.9	2	12	6.6	1.8	11.1	8.2

[a] Number of assays.
[b] Total number of 1,000 μm segments of BMZ counted.
[c] Average number of leukocytes/1000 μm BMZ for all sections.

DISCUSSION

Since the initial reports that patients with BP have immune reactants at the BMZ, there has been speculation that they are components of immune complexes and important in causing cutaneous inflammation. Supporting the hypothesis is: (1) the similarity of BMZ deposits to those seen in animal models of experimental antibasement membrane nephritis [1], (2) the finding that most patients have anti-BMZ antibiodies that will activate and deposit multiple components of the classical and alternative C pathways in the identical ultrastructural location occupied by *in vivo* deposited Ig and C [14–20], (3) the presence of multiple components of C at the BMZ in patients skin which strongly suggests sequential activation rather than random or nonspecific deposition [6,12,21,22] and studies of C components in blister fluids which have reported consumption and activation of C and production of biologically-active factors with chemotactic and possibly anaphylatoxic properties [6–8,23,24].

Nevertheless, there has been difficulty in providing functional support for an immune complex mediated pathogenesis of BP. For example, it has not been possible to passively transfer blisters or inflammation to animals with BPS containing high titer anti-BMZ antibodies and to date no in vitro model of the disease has been reported [1]. Furthermore, difficulties in accepting an immune complex pathogenesis have been due in part to the inability to directly show that immune reactants in BP skin possess functional properties that could contribute to inflammation.

Until recently, methods for demonstrating immune complex function in tissue had not been described and thus their presence was implied primarily by labeled antibody methods such as immunofluorescence. Although the methods can identify "components" of complexes, e.g., Ig, C and in some cases antigen and have been extremely useful in disease diagnosis and pointing out possible pathologic mechanisms, they have 2 important limitation: inability to (1) detect and (2) quantitate immune complex activity.

Recently, Yamamoto et al described a method for detecting and quantitating C-activating complexes deposited in animal tissue *in vivo* [11]. These workers used cryostat sections of kidneys from rats with experimental immune complex glomerulonephritis as a source of tissue deposited complexes. When sections of normal kidney and kidney containing glomerular deposits of IgG and C3 were incubated with rat peritoneal leukocytes suspended in fresh rat serum, cells attached specifically only to kidney sections containing immune reactants. Although the mechanism of LA was not completely defined, it was shown that cells attached to the exact site (glomeruli) of immune reactant deposition, only to glomeruli containing Ig and C and demonstrated that a source of exogenous (serum) complement was required. When rat serum was heat-inactivated (56°C × 30 min) or absorbed with anti-C3 globulin, attachment did not occur. Furthermore, these authors showed that the number of cells attached was proportional to the extent of morphologic glomerular damage and, to some extent, the intensity of IgG and C3 immunofluorescence.

Using a modification of the method, we recently reported that human PBL suspended in fresh NHS would attach to the BMZ of cryostat sections of normal human skin which had been treated with BPS containing C-binding anti-BMZ antibodies [9,10]. Evidence that cells were attaching to complement-activating immune complexes was supported by the findings that (1) only BPS or IgG fractions of BPS containing C-binding anti-BMZ antibodies mediated the reaction; (2) a strong correlation existed between titers of C-binding anti-BMZ antibodies and LA titers and (3) attachment occurred in the presence of fresh NHS, and C2-deficient serum reconstituted with purified C2 but not with heat-inactivated NHS, decomplemented NHS or C2-deficient serum alone. Furthermore, using a modification of the method, it was possible to show that the mechanism of attachment involved the complement-dependent directed migration of cells to the BMZ followed by immune adherence. The studies on the detection of *in vivo* deposited immune complexes by Yamamoto et al. and studies on human skin noted above suggested LA would be an effective method for evaluation of *in vivo*-deposited immune reactants in BP.

Using the method to investigate the activity of immune reactants in the skin of patients with BP, the following observations were made: (1) there was significantly greater attachment of leukocytes to the BMZ of lesional BP skin compared to normal BP skin, normal skin and skin from patients with a variety of inflammatory diseases not associated with immune deposits at the BMZ; (2) LA in lesional BP skin was significantly reduced when cells were suspended in heat-inactivated NHS rather than fresh NHS and when sections were pretreated with anti-human IgG (Fc) to block the complement activating site of complexes and (3) of possible importance, LA was not significantly different in normal BP skin compared to the 2 control groups.

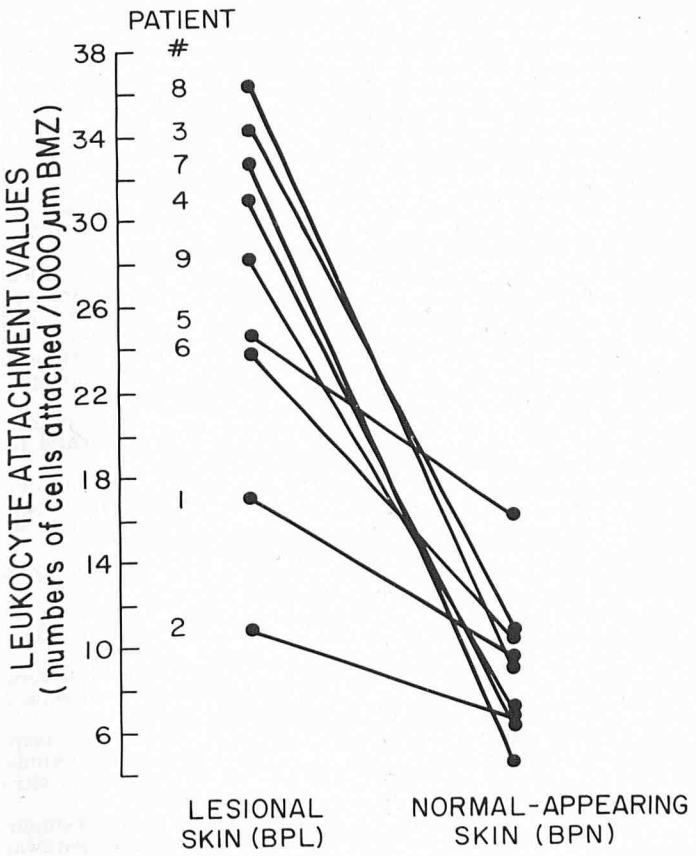

FIG 1. A comparison of leukocyte attachment to the BMZ in paired lesional (*BPL*) and normal-appearing (*BPN*) skin in 9 patients with active BP. The *points connected by lines* represent LA results in BPL and BPN skin from the same patient.

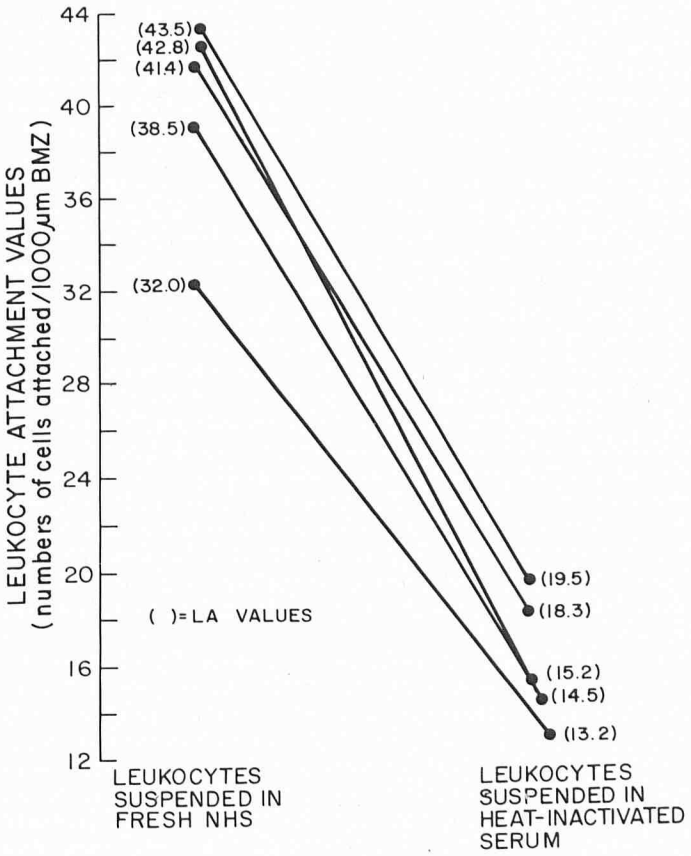

FIG 2. The effect of suspending leukocytes in heat-inactivated (56°C × 30 min) serum on leukocyte attachment to the BMZ. The *points connected by lines* represent the same biopsy assayed for LA with NHS and with heat-inactivated NHS.

TABLE III. *Results of leukocyte attachment in lesional BP skin following pretreatment of tissue sections with Fab goat anti-human IgG Fc*

BPL skin	LA (PFL/1,000 μm BMZ)	
	Fab goat/human IgG Fc	Buffer
1	20.0	36.0
2	19.0	35.7
3	19.3	30.3
Mean ± SD	19.4 ± 0.4	34.0 ± 2.3

BPL: Lesional BP Skin.

The first two observations provided evidence for the presence of C-activating IgG immune complexes in the lesions of patients with BP. This conclusion is supported by the presence of IgG and C3 at the BMZ, the apparent requirement for serum C in the attachment reaction and inhibition of LA by antibody to human IgG (Fc). Although IgG immune complex-mediated C-activation is the most likely mechanism of attachment, other possibilities were considered.

Factors other than C-activating IgG immune complexes and present only in or at higher activity in lesional BP skin could have caused LA. Tissue proteases with serine esterase activity such as plasmin which can cleave Clr, Cls, C3 and C5 [15] and an epidermal cell protease which has been shown to cleave C5 releasing C5a chemotactic activity could presumably cause leukocyte attachment [26]. Although these enzymes could account for the requirement for C, we feel this mechanism is unlikely because: (1) all tissues were washed in buffer prior to incubation with cells and serum, (2) there is no known reason why the activity of these proteases should be localized to the BMZ and (3) most importantly, significant attachment was not observed in either normal or nonpemphigoid inflammed skin.

This observation suggests proteases released or activated in damaged (freeze-thawed and cut) normal skin or present in inflammed skin are not sufficient to account for the attachment observed in lesional BP skin. Furthermore, this mechanism of attachment would not be expected to be inhibited by anti-human IgG Fc.

Another possibility is that immune complexes other than those containing IgG were present at the BMZ. This could account for C activation and attachment of leukocytes to the BMZ and could explain the lack of a correlation between results of LA and IgG immunofluorescence. Although this possibility cannot be excluded it has been shown in several studies that Ig deposits other than IgG are relatively uncommon in BP skin [6,21,22,27]. Furthermore, in 6/9 cases examined here, IgM and IgA immunofluorescence were negative in both lesional and normal skin (results not shonw). Against this possibility also is the finding that LA was blocked by anti-human IgG (Fc). It is, of course, conceivable that antibody not detectable by immunofluorescence accounted for attachment since attachment was seen in the lesional skin of case 7 in which no IgG was observed. In this regard it is known that anti-BMZ antibodies including those present in BP and herpes gestationis sera can bind C to the BMZ *in vitro* in the absence of detectible antibody binding [28,29].

Functional components of the C system deposited at the BMZ *in vivo* could also account for the attachment of cells to the BMZ. If, for example, functional C3 or C5 convertases such as C42, C423b or C3bBb were present in lesional skin, they could presumably utilize exogenous C3 or C5 as substrate to form additional convertase activity, deposit membrane bound C3B, generate C5 cleaving activity and cleave C5 to C5a causing the migration and attachment of cells [25]. Supporting this concept is a report suggesting that C3b may be present in lesional but not normal BP skin [30].

Although LA was significantly greater in lesional compared to normal BP skin, no significant differences in either the intensity or incidence of IgG or C3 immunofluorescence between the groups was observed. Furthermore, no correlations were found between the level of LA and the intensity of immunofluorescence in any of the BP tissues. These results strongly suggest that conclusions about the presence of functional immune complexes in BP cannot be based on immunofluorescent results alone.

The lack of agreement between the immunofluorescence and LA assays was somewhat unexpected but not totally surprising since they appear to measure or detect different things. Immunofluorescence detects Ig or C simply as an antigen, the intensity of which is determined by the total amount of antigen present. LA on the other hand presumable detects physiological activity which is determined not by the total amount of Ig but the amount that is functional (C-activating). Since immune complexes may contain C-binding and non C-binding subclasses of Ig, differences in the relative amounts of C-binding IgG in BP tissues could explain the lack of agreement between the methods.

It is possible that as yet undetermined factors which could enhance the immune complex-mediated activation of C in lesional skin or alternatively inhibit, block or saturate this activity in normal BP skin could account for the discrepancies between immunofluorescence and LA. These questions will require additional studies and perhaps new methods of immune complex analysis.

In conclusion, we feel these studies do provide functional evidence for the presence of C-activating immune complexes in the lesional skin of patients with BP. This finding suggests complexes are important in cutaneous inflammation and is consistent with, though certainly does not prove, an immune complex pathogenesis of the disease. In addition, the finding that LA is greater in lesional than normal BP skin and that LA activity in normal BP skin is not significantly different from normal human skin suggest immune complex function may be a reason why lesions develop at some sites and not others. It is anticipated that further studies using the LA method will be useful not only in defining the role of IC in the pathogenesis of BP, but in other suspected immune complex-mediated disease as well.

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