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Pemphigoid Antibody Mediated Attachment of Peripheral Blood Leukocytes at the Dermal-Epidermal Junction of Human Skin

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It has been proposed that cutaneous inflammation and blister formation in bullous pemphigoid is caused by antibodies to the cutaneous basement membrane zone which activate complement, thereby, attracting leukocytes to the dermal-epidermal junction. There is, however, no functional evidence which supports a role for pemphigoid antibodies in complement activation or leukocyte activity in skin. This study describes the *in vitro* attachment of human peripheral blood leukocytes to the dermal-epidermal junction of cryostat skin sections treated with 9/13 pemphigoid sera containing antibodies to the cutaneous basement membrane zone. A requirement for complement in the reaction was supported by the findings that only complement-fixing pemphigoid sera mediated the leukocyte response, a strong correlation existed between complement-fixation titers and leukocyte attachment titers and only leukocytes suspended in fresh serum but not buffer or heat inactivated serum attached at the junction. A requirement for antibody was supported by the observation that IgG fractions of 4 pemphigoid sera were as effective as whole sera in mediating leukocyte attachment. The leukocyte response was shown to be specific for complement-fixing pemphigoid sera since it was not observed with noncomplement-fixing sera or sera from 15 normal human and 22 non-pemphigoid disease controls. This study offers functional evidence for an interaction between pemphigoid antibody, complement and leukocytes in the immunopathogenesis of bullous pemphigoid and demonstrates that complement-fixing antibasement membrane zone antibodies may be important in initiating the cellular inflammatory events observed near the dermal-epidermal junction *in vivo*.

Bullous pemphigoid is a chronic acquired skin disease characterized clinically by tense blisters and bullae which develop on normal, erythematous, or urticarial skin and histologically by dermal-epidermal separation associated with variable degrees of dermal inflammation [1]. While the cause of pemphigoid is unknown, a number of observations suggest that inflammatory cells are important in pathogenesis and support the concept that leukocyte activity contributes to separation of the dermal-epidermal junction. Histologic studies show a progressive accumulation of leukocytes in the upper dermis and junctional zone prior to blister formation and degranulation of leukocytes at the junction appears to accompany damage to basement membrane structures [2]. In bullous lesions, the blister cavity contains numerous eosinophils, neutrophils, and mononuclear cells and these cells may be seen infiltrating the dermal-epidermal junction at the edges of blisters [3].

Since the original report that sera of most pemphigoid patients contain antibodies directed against the basement membrane zone (pemphigoid antibodies), it has been postulated that the cellular inflammatory events seen in the disease may have an antibody-mediated autoimmune pathogenesis [4,5]. The theory has been reinforced by the observation that all patients with active disease have complement (C3) and most have immunoglobulin (IgG) deposited in a linear pattern at the dermal-epidermal junction *in vivo* [6]. Most pemphigoid sera contain anti-basement membrane zone antibodies capable of depositing and presumably activating complement at the dermal-epidermal junction *in vitro* [7,8]. Supporting complement activation *in vivo* is the report that heat stable complement derived chemotactic activity is present in pemphigoid blister fluids [9].

The similarity of the histologic and immunologic observations in pemphigoid to features of experimental antiglomerular basement membrane disease suggest that cutaneous inflammation may be caused by complement fixing pemphigoid antibodies binding to antigen and causing activation of the complement system at the dermal-epidermal junction [10]. Complement activation may result in the formation of soluble mediators which directly and/or indirectly (via mast cells) cause the attraction and activation of leukocytes at the basement membrane zone. Complement-derived mediators and immunoglobulin may be responsible for triggering the release of lysosomal enzymes from leukocytes which may contribute to structural damage and separation of the dermal-epidermal junction analogous to immune-mediated injury in nephrotoxic nephritis [11,12].

While the preceeding hypothesis is consistent with the reported histologic and immunologic data it is unsupported by studies demonstrating functional activity for complement and antibody deposited at the dermal-epidermal junction and by experiments showing a relationship between antibody binding and leukocyte responses. This study describes a functional interaction between pemphigoid antibody, complement and leukocytes *in vitro* that provides additional evidence for a role of pemphigoid antibody in cutaneous inflammation and blister formation in bullous pemphigoid.

MATERIALS AND METHODS

Skin

Skins were obtained immediately after routine circumcision of healthy human blood group O, Rh type negative neonates. Excess fat and subcutaneous tissue was removed and the skin washed gently for 10 min at 25°C in 2 changes of sterile 0.15 M NaCl buffered with .01 M Na_2HO_4, pH 7.2 (PBS), dried briefly on filter paper and cut into 5 × 15 mm strips. Strips were quick frozen in liquid nitrogen and mounted for vertical sectioning in Ames O.C.T. compound (Ames Co., Elkhart, IN). All tissue blocks were frozen at −70°C until needed for indirect im-

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Abbreviations:
BSA: bovine serum albumin
GBSS: Gey's Balanced Salt Solution
PBL: peripheral blood leukocytes

munofluorescence, indirect complement fixation and leukocyte attachment studies.

Pemphigoid Sera

Serum samples containing antibodies to the basement membrane zone of skin were collected from 13 patients, ages 52–84, with active bullous pemphigoid diagnosed by previously described criteria [13]. Pemphigoid antibody titers were determined by indirect immunofluorescence using a previously described method [14]. Titers were found to range from 1:10 to greater than 1:1280. All serum samples were stored at −70°C until needed. Prior to use samples were thawed and heat inactivated for 30 min in a 56°C water bath.

IgG Fractions of Pemphigoid Sera

IgG fractions were prepared according to established methods from 10.0 ml of each of 4 pemphigoid sera containing complement-fixing pemphigoid antibodies [15]. Briefly, crude immunoglobulin fractions were prepared by salt precipitation of serum with 50% $(NH_4)_2$ SO_4. Precipitates were washed, ×2 in 25% cold $(NH_4)_2SO_4$, dissolved in 10.0 ml PBS dialyzed against 3 changes of cold PBS for 24 hr followed by dialysis against 3 changes of cold 0.01 M sodium phosphate pH 8.0 for another 24 hr. Samples were adjusted to 5.0 ml and layered over a diethylaminoethyl DE52 anion exchange column (Whatman, Ltd., Kent, England) previously equilibrated with 0.01 M sodium phosphate, pH 8.0. IgG was eluted with equilibration buffer and first peak fractions concentrated on an Amicon ultrafiltration cell, model 52 (Amicon, Corp., Lexington, MA.). Concentrate were dialyzed overnight against 3 changes of cold PBS and the protein concentrations were adjusted to 10.0 and 1.0 mg/ml. Purity of samples was determined by immunodiffusion and immunoelectrophoresis against goat anti-human IgG (Hyland Labs, Costa Mesa, CA) and goat anti-whole human serum (Gelman Inst. Co., Ann Arbor, MI) using an established method [16]. Samples, tested undiluted at both protein concentrations were found to contain complement fixing anti-basement membrane zone antibodies.

Control Sera

Control sera were obtained from 15 healthy adults (normal human controls) and 20 patients with a variety of active inflammatory skin diseases other than bullous pemphigoid (disease controls). Disease controls included patients with papular dermatitis of pregnancy, psoriasis, chronic muco-cutaneous candidiasis, systemic lupus erythematosus, Mucha-Habermann disease, disseminated granuloma annulare, toxic epidermal necrolysis, hyper IgE syndrome, atopic dermatitis, nummular eczema, aphthous stomatitis, pyoderma gangrenosum, lichen planus and urticarial vasculitis. All sera were stored at − 70°C and heat inactivated (56°C × 30 min) prior to use.

Complement-sufficient Serum

Fresh-frozen, platelet-poor serum from blood group AB Rh type positive donors was used as a source of complement in indirect complement fixation tests and as a suspension medium for peripheral blood leukocytes (PBL) in the luekocyte attachment method. Platelet poor serum was prepared according to a published method [17]. Briefly, blood was drawn into sterile, chilled, plastic centrifuge tubes and spun at 3500 rpm in a Sorvall refrigerated centrifuge at 4°C for 10 min to remove cells and platelets. Plasma was transferred to sterile glass tubes and allowed to clot at 25°C for 30 min. The clot was removed and serum aliquoted in 1 ml volumes and stored at −70°C until needed. Serum was thawed just prior to use, kept on ice and diluted to 20% with sterile Gey's Balanced Salt Solution (GBSS) (Flow Labs, McLean, VA), containing 2% bovine serum albumin (BSA).

Peripheral Blood Leukocytes (PBL)

PBL were prepared by dextran sedimentation of heparinized whole blood according to an established procedure [18] from healthy adult donors on no medications for at least 1 week prior to blood drawing. Twenty ml of blood was drawn and immediately anti-coagulated with 200 units of beef lung heparin (Upjohn Co., Kalamazoo, MI). Heparinized blood was mixed thoroughly with an equal volume of 3% dextran (MW 250,000, Pharmacia Co., Uppsala Sweden) and allowed to sediment at 37°C for 30 min at 1 ×g. The luekocyte rich supernate was removed and centrifuged at 250 ×g for 10 min at 25°C. Residual RBCs in the pellet were lysed by adding 10.0 ml of 0.2% aqueous NaCl and gently mixing for 5 seconds. Isotonicity was re-established by adding 10.0 ml of 1.6% NaCl. Leukocytes were resedimented at 250 ×g, suspended in 2.0 ml of GBSS + 2% BSA and counted on a hemocytometer. Cells were adjusted to a concentration of 16×10^6/ml with GBSS + 2% BSA. Cell viability as assessed by trypan blue exclusion was always greater than 98%. Before adding cells to skin sections, an equal volume of complement sufficient serum was added to the cell suspension giving a final concentration of 8×10^6 cells/ml.

Leukocyte Attachment Method

The method uses blood type O, Rh type negative human skin as a source of basement membrane antigen; heat-inactivated pemphigoid serum, undiluted or diluted with GBSS + 2% BSA as a source of anti-basement membrane zone antibody; PBL from normal donors and fresh frozen serum from blood type AB, Rh positive donors as a source of complement. Tissue slides were prepared by placing 2–3 freshly cut 8 μ cryostat sections of human skin side-by-side in the center of a sterile microscope slide. Sections were allowed to air dry for 15 min at 25°C and the slides rinsed for 10 min in cold PBS to remove embedding medium and tissue fluids. Excess moisture was wiped away and tissue sections were treated with 1 or 2 drops of heat inactivated neat or dilute pemphigoid or control serum. Sections were incubated with serum for 30 min at 4°C in a humidity chamber and washed in 3 changes of cold PBS for 15 min. Sections were then processed using a chamber constructed according to the method of Yamamoto et al as follows: [19]. Excess PBS was removed from the slides and strips of vinyl electrical tape (Fisher Scientific Co., Pittsburgh, PA) placed around each end. Tape strips were also placed around the ends of another microscope slide (covering slide) and the covering slide and tissue slide were taped together. This created a chamber between the 2 slides in which serum-treated skin sections were adherent to the bottom slide with a free air space above the tissue and below the covering slide (Fig 1). The chamber measured .2 mm in thickness with a volume of approximately 0.4 ml. PBL were diluted to 8×10^6 cells/ml with 20% complement sufficient serum in GBSS + 2% BSA and injected with a tuberculin syringe into the chamber taking care to completely fill the chamber and avoid air bubbles. Chambers were then incubated for 45 min at 37°C in a humified air atmosphere using a tissue culture incubator. After incubation, chambers were disassembled by cutting tape strips and nonadherent cells and excess serum were removed by gently washing the slides in cold PBS for 10 min. Slides were then allowed to dry for 10–15 min at 25°C, fixed in 95% ethanol and stained with hematoxylin and eosin. Leukocyte attachment at the dermal-epidermal junction was determined by examining stained sections at 250 × with a Zeiss microscope. A positive reaction was recorded if cells were seen nonrandomly distributed side by side at the dermal-epidermal junction over more than 50% of the junctional length in 2 skin sections. In all studies, both positive and negative controls were performed simultaneously, difficulties in interpretation of reactions were not encountered (Fig 2 and 3 are representative of positive and negative leukocyte attachment reactions).

Indirect Complement Fixation

Modifications of a previously described method were used to detect and titer complement-fixing pemphigoid antibodies in sera [7]. Thirteen pemphigoid sera known to contain pemphigoid antibodies were tested undiluted and at doubling dilutions from 1:10–1:1280. Freshly cut 8 μ cryostat sections of blood type O, Rh negative neonatal foreskin were air-dried on glass slides and incubated with pemphigoid serum diluted with GBSS + 2% BSA at 4°C in a humidity chamber for 30 min. Slides

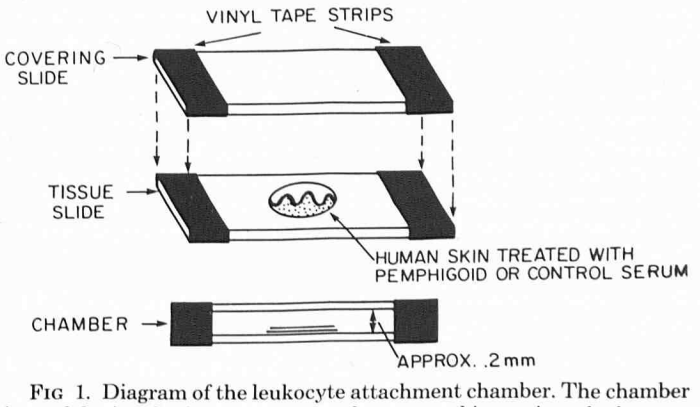

FIG 1. Diagram of the leukocyte attachment chamber. The chamber is used for incubating serum-treated cryostat skin sections, leukocytes and complement-sufficient serum (Yamamoto et al, reference 19).

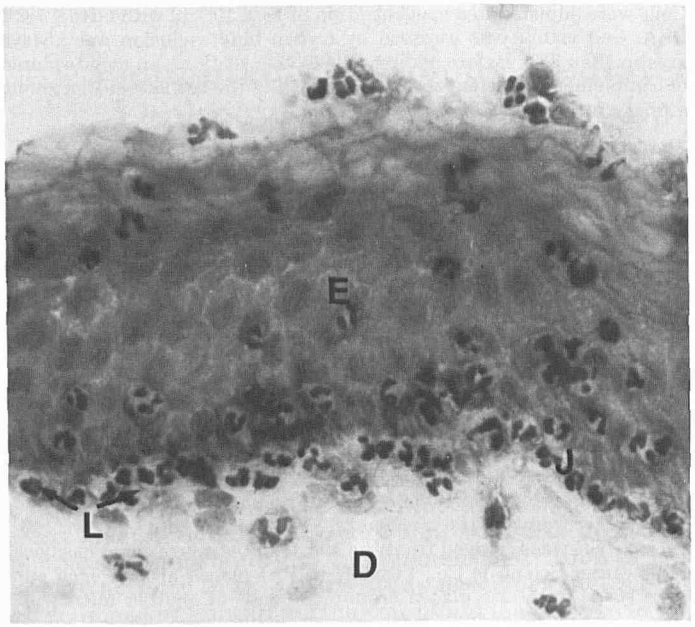

FIG 2. A positive leukocyte attachment reaction. Leukocytes (*L*), predominantly neutrophils, can be seen side-by-side in a linear nonrandom distribution at the dermal-epidermal junction (*J*) and an 8 μ cryostat section of skin treated with pemphigoid serum containing complement-fixing antibasement membrane zone antibodies, complement-sufficient serum and leukocytes. Randomly distributed leukocytes can be seen on the epidermis (*E*) and dermis (*D*) (reduced from × 400).

were then washed for 15 min in 3 changes of PBS at 25°C. Excess moisture was wiped from the slides and tissues incubated for 45 min with 1 or 2 drops of 20% complement-sufficient serum diluted with GBSS + 2% BSA. Slides were then rinsed, excess moisture removed and treated with one drop of a 1:10 dilution of fluorescein-conjugated goat anti-human C3 (Hyland Labs, Costa Mesa, CA) (specific Ab protein conc. 150 μg/ml, F.P. 3.2:1). Slides were incubated an additional 30 min, rinsed and immunofluorescence read on a Zeiss fluorescent microscope. Complement (C3) deposition was determined by the presence of a linear band of C3 fluorescence at the dermal-epidermal junction.

Interpretation of Data

Statistical analysis of data was performed using Fisher's Exact Test, (p) Chi-square Test (χ^2) and Correlation Coefficient Analysis[17].

RESULTS

Attachment of PBL at the Dermal-Epidermal Junction of Skin Treated with Pemphigoid Serum

Sera containing antibodies to the cutaneous basement membrane zone were selected from 13 patients with active bullous pemphigoid. All sera were shown by indirect immunofluorescence to have anti-basement membrane zone antibodies ranging in titer from 1:10 to greater than 1:1280. In these experiments, sera were tested for leukocyte attachment activity undiluted and at a dilution of 1:10. Table I lists the serum antibody titers and results of the leukocyte attachment reaction at both concentrations. Negative sera were tested at least twice and were found to be consistently negative. These results show that most, but not all, pemphigoid sera containing anti-basement membrane zone antibodies can mediate the attachment of PBL at the dermal-epidermal junction in the presence of complement-sufficient serum.

Microscopic Observations of the Attachment Pattern of PBL at the Dermal-Epidermal Junction

In all skin sections incubated with pemphigoid or control sera, a moderate number of leukocytes were randomly distributed over the glass slide and tissue sections. Of special interest was the observation that 9/13 pemphigoid sera caused the attachment of leukocytes (predominantly neutrophils but also eosinophils and mononuclear cells) in a nonrandom linear distribution along the dermal-epidermal junction. This pattern of cell attachment was apparent to observers who knew nothing of the study, the procedures or the results.

In some sections treated with pemphigoid serum, a single row of cells was aligned side by side in the upper dermis in contact with the junction (Fig 2). In others, larger numbers of leukocytes were crowded into the junction overlying one another and obscuring visualization of the upper dermis, junctional zone and basal layer of epidermal cells. Skin sections treated with 4 of the 13 pemphigoid sera did not cause leukocyte attachment as shown in a negative leukocyte attachment response (Fig 3). Leukocyte attachment to the junction was similar to the localization of leukocytes at the dermal-epidermal junction as seen in some biopsies of lesional pemphigoid skin (Fig 4).

Specificity of the Attachment Reaction

To determine if the reaction was specific for pemphigoid sera, sera from 15 normal donors and 22 nonpemphigoid disease controls were examined undiluted and diluted 1:10. In no case

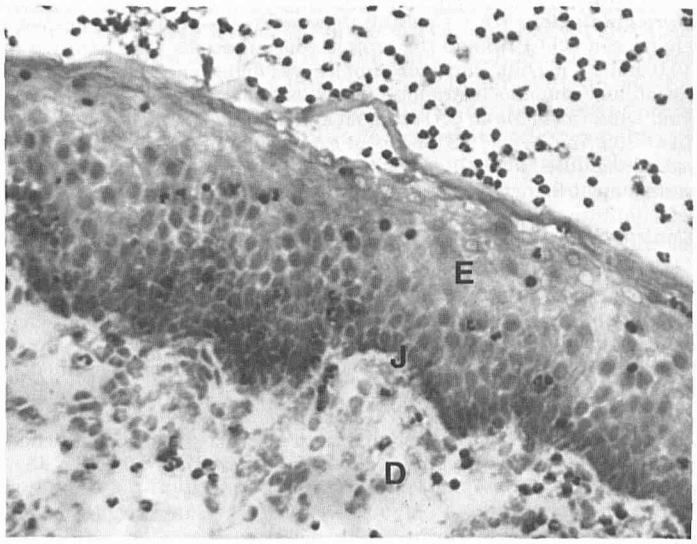

FIG 3. A negative (control) leukocyte attachment reaction on skin treated with normal human, nonpemphigoid disease control or noncomplement-fixing pemphigoid sera. Leukocytes are randomly distributed on the slide, epidermis (*E*) and dermis (*D*). Only an occasional leukocyte can be seen at the dermal-epidermal junction (*J*). (Compare to Fig 2) (reduced from × 250).

TABLE I. *Leukocyte attachment activity in 13 pemphigoid sera*

Serum	Pemphigoid antibody[a] titer	Leukocyte attachment[b]	
		undiluted	diluted 1:10
1	1/20	N	N
2	1/640	P	P
3	1/640	P	P
4	1/1280	P	P
5	1/1280	P	P
6	1/160	P	P
7	1/10	N	N
8	1/640	P	P
9	1/1280	P	P
10	1/80	N	N
11	1/1280	P	P
12	1/1280	P	P
13	1/10	N	N

N = negative and P = positive.

[a] Antibody titers were determined by indirect immunofluorescence.

[b] Attachment activity of the sera was tested neat and diluted 1:10 with GBSS + 2% BSA.

was a positive response observed with either normal or disease controls (Table II). To insure that negative results were not a result of the method, a pemphigoid serum (#12) previously proven to mediate leukocyte attachment was used as a positive control with each days experiments and consistently showed a positive response. Analysis of the data (by Fisher's Exact Test) showed that the attachment response is specific for pemphigoid sera when compared to normal human ($p < .001$) or nonpemphigoid disease control sera ($p < .001$).

Correlation Between Complement Fixation and Leukocyte Attachment Titers In Bullous Pemphigoid Sera Containing Anti-basement Membrane Zone Antibodies

The 13 pemphigoid sera known to contain anti-basement membrane zone antibodies were assayed simultaneously for complement-fixation and leukocyte attachment activity undiluted and diluted 1:10. The tests were performed by 2 separate investigators (WRG & DML) without knowledge of the other's results until all data were recorded and compared. As can be seen (Fig 5) only sera containing complement fixing antibasement membrane antibodies were positive in the leukocyte attachment test. These results were significant (p=.0014).

To further establish a correlation between complement fixation and leukocyte attachment, the 9 sera positive in both tests were serially diluted from 1:10 to 1:1280 and the dilutions tested by separate investigators (WRG & DML) for complement-fixation and leukocyte attachment activity. The results of this study are shown in Fig 5. Figure 6 graphically demonstrates the high correlation between the maximum positive titers of each serum in both tests. $r = 0.91$.

Evidence Supporting a Role for Complement in the Leukocyte Attachment Response

The results of the above studies strongly suggested a role for complement in the reaction. To test the requirement for com-

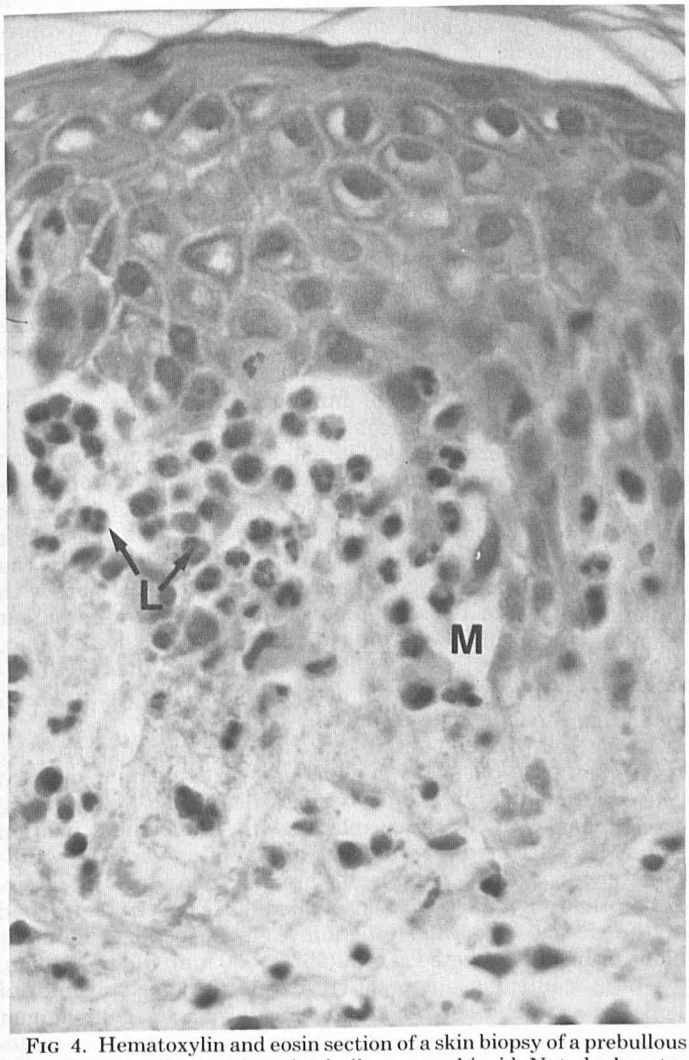

FIG 4. Hematoxylin and eosin section of a skin biopsy of a prebullous lesion from a patient with active bullous pemphigoid. Note leukocytes (*L*) infiltrating the dermal-epidermal junction and compare to leukocyte attachment reaction (Fig 2) *M*=microvesicle. (reduced from × 400).

TABLE II. *Comparison of bullous pemphigoid, normal human and disease control sera in mediating leukocyte attachment*

Serum source	Number tested	Results	
		Positive	Negative
Bullous pemphigoid	13	9	4
Normal human	15	0	15
Nonpemphigoid disease controls	22	0	22

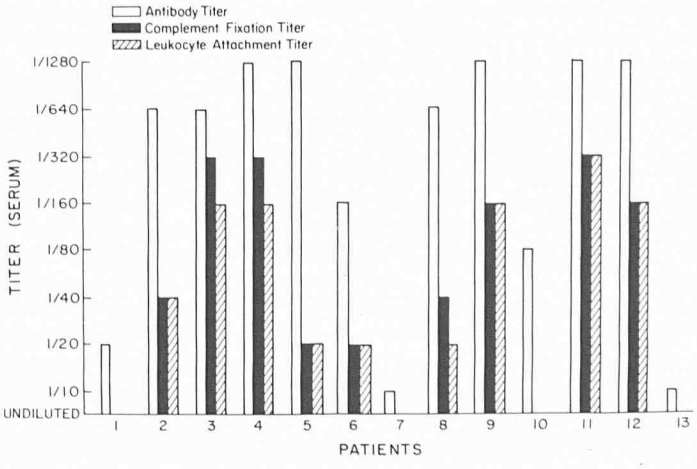

FIG 5. Comparison of pemphigoid antibody, complement fixation and leukocyte attachment titers in 13 pemphigoid sera. Four patient's sera (1,7,10 and 13) had only antibody activity. The remaining 9 patients had similar titers of complement fixation and leukocyte attachment activity.

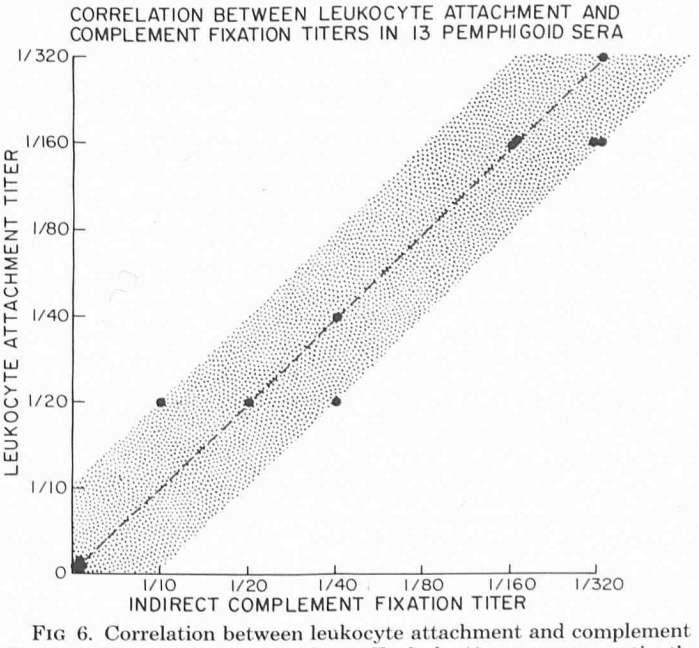

FIG 6. Correlation between leukocyte attachment and complement fixation. Titers in 13 pemphigoid sera. Each *dot* (.) represents a patient's serum. The correlation co-efficient for the 13 sera was 0.91.

plement further, skin sections were treated with a pemphigoid serum (diluted 1:10) previously shown to mediate leukocyte attachment. Skin sections were subsequently exposed to washed PBL suspended in (a) 1:10 dilution of fresh serum (b) 1:10 dilution of heat-inactivated (56°C × 30 min) serum or (c) buffer. All tests were performed in duplicate. The results showed that only sections treated with PBL suspended in fresh serum attached at the dermal-epidermal junction.

Evidence for Pemphigoid Antibody in Mediating Leukocyte Attachment

To support the hypothesis that antibodies were responsible for the leukocyte attachment activity in pemphigoid sera, immunochemically pure IgG fractions from 4 sera known to promote the binding of PBL to the dermal-epidermal junction were obtained and tested at protein concentrations of 10 and 1 mg/ml. All 4 fractions were positive at both concentrations.

Evidence Supporting A Requirement for Viable Leukocytes in the Attachment Response

To test cell viability as a requirement for attachment, the following experiment was performed: PBL were obtained and divided into 3 groups of 24×10^6 cells suspended in 1 ml of GBSS + 2% BSA. To group 1 was added 2 ml of GBSS + 2% BSA. Group 2 received 2 ml of GBSS + 2% BSA containing 2% sodium azide. Group 3 received 2 ml of GBSS + 2% BSA containing 1% sodium merthiolate. Cells were incubated at 37°C for 1 hr, washed in GBSS + 2% BSA and tested for viability by trypan blue exclusion. Cell viability was greater than 95% for buffer-treated cells but less than 30% for cells treated with either merthiolate or azide. Cells were then diluted in 20% complement sufficient serum and tested for leukocyte attachment. While buffer treated cells effectively attached to the dermal-epidermal junction, azide and merthiolate treated cells did not.

DISCUSSION

Clinical observations of skin lesions in patients with bullous pemphigoid suggest an important role for inflammation in the pathogenesis of the disease. Types of skin lesions include erythematous macules and plaques and blisters which usually arise on clinically inflamed skin. In some patients, one can observe a transition of lesions from erythematous macules to papules to blisters [21].

The histology of evolving skin lesions suggests an important role for cellular inflammation in their pathogenesis and evolution. Studies on the temporal sequence of histological events preceeding blister formation report a progressive increase in the number of inflammatory leukocytes infiltrating the upper dermis [21]. Schamburg-Lever, et al reported a "pronounced" infiltrate of leukocytes in apposition to the dermal-epidermal junction in prebullous lesions and observed contact between cells and basement membrane structures prior to dermal-epidermal separation [2]. Other investigators have reported the presence of eosinophils, neutrophils and histiocytes in close proximity to the junction bordering blisters and papillary microabscesses in prebullous and bullous lesions [22]. Evidence supporting functional activity for leukocytes is reinforced by the finding that degranulated cells have been observed near the junction [2].

While the histologic evidence is good that leukocytes are important in the pathogenesis of the disease, the factors which initiate and regulate leukocyte responses remain obscure. A number of observations, however, suggest that anti-basement membrane antibody and complement are important in initiating cellular inflammation. Particularly important is the observation that most patients have serum antibodies reactive with a glycoprotein antigen located in the lamina lucida between the basal cell plasma membrane and the basal lamina [4,23,24]. Most of these sera deposit complement in vitro at the dermal-epidermal junction in a pattern which corresponds exactly to that of antibody deposition [7,25]. Even in patients who lack serum antibodies, complement and usually immunoglobulins can be detected in the lamina lucida, which is the ultrastructural site of dermal-epidermal separation [26].

Additional evidence supporting a role for complement in inflammation is the finding that multiple components of the classical and amplification pathways as well as complement regulating proteins are deposited at the dermal-epidermal junction *in vivo* [13,27,28,29]. *In vitro* studies show that complement-fixing pemphigoid antibody can deposit complement at the dermal-epidermal junction by the classical and amplification pathways [30–32]. Evidence for functional complement activity in the disease is provided by the finding that pemphigoid blister fluids contain reduced levels of total hemolytic complement and individual component levels and increased amounts of complement-derived chemotactic factor activity [9,33]. Lacking thus far has been direct *in vivo* or *in vitro* evidence that pemphigoid antibody can interact with complement to influence cell functions which might be important in initiating inflammation *in vivo*.

The purpose of this study was to develop a method to demonstrate a functional relationship between leukocyte activity and the binding of pemphigoid antibody at the dermal-epidermal junction. To reduce nonspecific antigen-antibody interactions and represent as closely as possible events which might occur *in vivo* only human tissues with minimal blood group antigenic incompatibilities were used. Peripheral blood leukocytes were employed rather than enriched suspensions of a particular cell type since mononuclear cells and neutrophils as well as eosinophils have been shown to participate in the inflammatory response seen in lesions.

When cryostat sections of human skin were treated with complement fixing pemphigoid sera, washed and subsequently incubated with peripheral blood leukocytes suspended in fresh serum, cells including neutrophils, eosinophils and mononuclear cells attached along the dermal-epidermal junction in a specific and reproducible pattern. Evidence supporting anti-basement membrane antibody in mediating the reaction was based on the findings that normal human sera and sera from patients with inflammatory skin diseases unassociated with anti-basement membrane zone antibodies did not cause leukocyte attachment. Additional support was provided by the finding that IgG fractions of pemphigoid sera were just as effective as whole sera in mediating the leukocyte response.

A role for complement in the reaction was suggested by several observations. Only complement fixing pemphigoid sera caused attachment of leukocytes and the titers of sera causing attachment were closely correlated with complement fixation titers demonstrated by indirect immunofluorescence. Additional evidence for a complement requirement was the observation that only leukocytes suspended in fresh serum attached at the dermal-epidermal junction. Cells suspended in buffer alone or heat inactivated serum (56°C × 30 min) did not attach.

These studies do not precisely define the mechanism(s) of leukocyte attachment but do support a role for complement fixing antibasement membrane zone antibodies, serum complement and viable leukocytes. Any explanation of the attachment response would have to account for these factors. Complement-fixing pemphigoid antibodies which are predominantly IgG could function in leukocyte attachment by either 1 or both of 2 mechanisms based on knowledge of immune complex-leukocyte interactions. Antibodies may play a direct role by opsonizing the basement membrane zone. Leukocytes could adhere to the Fc portion of antibody via membrane receptors for IgG Fc present on granulocytes [34–36] and macrophages [37,38]. Secondly, the requirement for serum complement suggests an additional function of antibody, deposition and activation of the complement system at the dermal-epidermal junction. Pemphigoid antibodies which have been shown in this and other studies to deposit complement at the dermal-epidermal junc-

tion could cause the formation of both soluble and membrane-fixed complement intermediates which could attract and mediate attachment of leukocytes at the junction.

The soluble complement intermediates C3a and particularly C5a formed at the dermal-epidermal junction could diffuse and attract leukocytes to the junction by chemotaxis. Both intermediates have been shown to be chemotactic for granulocytes and macrophages [39–41]. While chemotaxis has not been proven in this study, a role for leukocyte mobility can be inferred from the nonrandom distribution of cells at the junction and the requirement for viable cells in the attachment response.

An additional complement dependent mechanism could result from the formation of fixed complement intermediates at the junction to which leukocytes could bind. Again, granulocytes and macrophages have cell membrane receptors for one or more complement intermediates which might be present at the junction [34]. Cells could, therefore, attach by complement-dependent as well as antibody-dependent immune adherence.

A causative role for pemphigoid antibodies capable of depositing and activating complement and mediating cellular inflammatory events *in vivo* can be inferred from the results of these studies on pemphigoid sera. However, as shown by these and other studies not all pemphigoid sera contain functionally active antibodies. The absence of complement-fixation and leukocyte attachment activity in some sera may be due to the fact that all functionally-active antibodies have been absorbed and bound to the dermal-epidermal junction *in vivo*. It has been shown that pemphigoid antibodies are functionally heterogeneous and belong to several different IgG subclasses which vary from active to inactive in complement-fixation [8]. Furthermore, complement fixing antibodies tend to have a higher affinity for antigen. These antibodies may be preferentially absorbed *in vivo* leaving only low affinity noncomplement fixing antibodies in the circulation. The finding that all patients do have complement deposits at the dermal-epidermal junction *in vivo* would support this contention.

The specific mechanism of leukocyte attachment and their relevance to events occurring *in vivo* remain to be established. These studies do demonstrate, however, that pemphigoid antibody, probably through complement activation can mediate a specific leukocyte response *in vitro*. The similarity of the *in vitro* response to the histology of inflammatory prebullous lesion *in vivo* can be readily appreciated. These studies add functional support to the histologic, immunologic and clinical observations that antibody and complement are important in the inflammatory responses observed in bullous pemphigoid and provide additional evidence that leukocytes may mediate dermal-epidermal separation in the disease.

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