The Complement System in Bullous Pemphigoid

I. COMPLEMENT AND COMPONENT LEVELS IN SERA AND BLISTER FLUIDS

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A B S T R A C T Compared with other serum and blister
fluid proteins. total hemolytic complement was reduced
in the blister fluid of six serologically positive bullous
pemphigoid patients while four serologically negative
cases had blister fluid complement levels closely ap-
proaching the serum levels. Except for pemphigus vul-
garis blisters, blister fluids from most patients with other
bullous diseases and experimentally induced blisters had
blister fluid complement levels more closely approaching
the serum levels. With the exception of the two terminal
components. C8 and C9, individual components of the
complement sequence were also reduced in the blister
fluids of the six bullous pemphigoid patients with cir-
culating basement membrane zone antibodies. On the
other hand, transferrin and IgG levels of these same six
serologically positive blister fluids closely approached
the corresponding serum levels. Conversion of C3 proac-
tivator was also demonstrable in the serologically posi-
tive bullous pemphigoid blister fluids, but not in the cor-
responding sera. Our studies. therefore, are suggestive
of local activation of the complement sequence, by both
the classical and alternate pathways, in blisters of sero-
logically positive bullous pemphigoid patients.

INTRODUCTION

Bullous pemphigoid, a disease characterized clinically by
the presence of large, tense blisters and histopathologi-
cally by subepidermal bulla formation affects primarily
elderly individuals. The etiology of this disease is still
unresolved.

Indirect immunofluorescent (IF)[1] procedures have
firmly established that antiepithelial autoantibodies spe-
cific for the basement membrane zone occur in the sera
of most patients with bullous pemphigoid (1-5). The
staining pattern observed is disease specific and when
present is of considerable diagnostic importance. Inter-
testingly, these antibodies, also referred to as pemphigoid
antibodies, react rather precisely with the corresponding
histopathologic site, the basement membrane area, but
their role, if any, in disease production must still be
determined.

Past immunopathologic investigations of bullous pem-
phigoid lesions, using direct IF staining, have revealed
IgG and complement (C3 and C4) in virtually all skin
specimens examined to date (6-9). This includes, in ad-
dition, bullous pemphigoid patients with active disease
but without demonstrable circulating antibodies (7-9).
The staining pattern observed is identical with the
indirect IF pattern.

With an in vitro complement-staining method, we
have also demonstrated that most serologically positive
bullous pemphigoid sera fix complement again with a
pattern identical with indirect IF staining (10). Blister

fluids from these patients, tested by both indirect IF staining and complement IF staining, also contain complement-fixing pemphigoid antibodies (10).

Since the above IF studies suggest that bullous pemphigoid might represent an immunologic disease mediated by the complement system, the present studies were undertaken to determine if abnormalities in the complement system occur in sera and blister fluids of these patients, and how such abnormalities might relate to the IF findings.

METHODS

Patient population. All patients included in this study had active disease when blood and blister fluid samples were collected. Included were 10 cases of bullous pemphigold, 4 cases of pemphigus vulgaris, 3 cases of diabetes mellitus and large spontaneous bullae of the lower legs and feet, 2 cases of erythema multiforme bullosum, 1 case of a bullous id reaction, 1 case of epidermolysis bullosa, and 1 case of bullous contact dermatitis. Except for the pemphigus vulgaris blisters which were characterized histologically by acantholysis and intraepidermal bulla formation, all the other patients had subepidermal bullae with varying degrees of inflammation. Overlying epidermal necrosis was seen in the two erythema multiforme lesions, but not in the other subepidermal lesions examined. All subepidermal lesions did have cellular infiltrates, both perivascular and within the bullous cavity. A mixture of lymphocytes, neutrophils, and eosinophils was commonly seen.

Sera and blister fluids. Paired samples of venous blood and blister fluid collected from patients under sterile conditions were allowed to clot at 0°C for 1 h and were then aliquoted. In most instances, blister fluids were collected within 48 h after bulla formation. For IF staining, serum and blister fluid samples were stored at — 20°C while those for hemolytic complement studies were stored at — 70°C until used. Studies on blister fluids, however, were limited by the volumes available.

Experimentally induced blisters. Experimentally induced suction blisters were produced on normal volunteers according to the method of Küstala and Mustakallio (11). Suction blisters were chosen as one type of experimentally induced blister because they closely resemble bullous pemphigoid blisters. Both are characterized histologically by subepidermal bulla formation with minimal epidermal cell necrosis (11, 12). In addition, cantharidin [a]-induced blisters, characterized by intraepidermal bulla formation (12), were produced by painting a small area of skin of a volunteer with a 0.7% solution. The area was then covered, and the blister fluid was harvested 24 h later. Liquid nitrogen-induced blisters, characterized by subepidermal bulla formation with epidermal necrosis (13), were produced by freezing a small area of skin for about 60 s. These blisters were also harvested 24 h later. Paired samples of venous blood and blister fluid were then collected and handled as above.

IF studies. For both indirect IF staining and complement IF staining, guinea pig esophageal mucosa and normal human skin sections were cut in a cryostat at — 20°C and were used unfixed in both staining procedures. Antiserum to human IgG, labeled with fluorescein isothiocyanate, was prepared in goats and was standardized by methods described previously in detail (14). Conjugated antiserum to $\beta 1C/$

[a] Cantharone, Ingram Pharmaceutical Co., San Francisco, Calif.

$\beta 1A$ globulins or C3 (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), used for in vitro complement staining was also tested as previously described (9, 10). Units of antiserum, antibody protein assays, and molar fluorescein-to-protein ratios for each antiserum were determined by routine methodology (9, 10), and each was used at a strength of 1/4 U/ml. Indirect IF and complement IF staining procedures were performed in the standard fashion (6, 9, 10).

Reagents for complement assays. Gelatin Veronal buffers with and without Ca++ and Mg++ (GVB++, GVB--) and glucose gelatin Veronal buffer with Ca++ and Mg++ (GG-VB++) were prepared as previously outlined (15). EDTA reagent, used for cell intermediate preparations and in the hemolytic complement component assays, was prepared in the routine manner (16). Partially purified C2 of guinea pig serum (Texas Biological Laboratories, Inc., Fort Worth, Tex.) was prepared according to the method of Nelson, Jensen, Gigli, and Tamura (17). Cell intermediates (EAC1, EAC14, and EAC4) used in complement component assays were prepared according to the methods of Gewurz, Page, Pickering, and Good (18), and Boros and Rapp (19).

Total complement and total protein assays. Total hemolytic complement of sera and blister fluids measured by methodology described by Day, Pickering, Gewurz, and Good (20) were expressed in 50% hemolytic units (CH_{50}). Total protein concentrations for each of these fluid compartments were also determined using a Folin method (21), and the complement levels of both sera and blister fluids were then expressed as a function of the total protein of each, or as CH_{50} units per 10 mg of total protein.

Anticomplementary tests. All sera and blister fluids were tested for anticomplementary activity. This was accomplished by adding equal volumes of the test serum or blister fluid and normal human serum with a known complement level. This mixture was incubated at 37°C for 1 h and then tested for total complement in the routine manner (20). A serum or blister fluid was considered anticomplementary if it lowered the complement concentration of the normal human serum to a greater degree than did an equal volume of buffer (GVB++).

Individual complement component assays (C1–C9). Using the appropriate cell intermediates, hemolytic C1, C4, and C2 titers were determined by previously described methods (18). Functionally pure complement components (Cordis Laboratories, Miami, Fla.) were used for assays of components C3, C5, C6, and C7 and EAC1-7 human (Cordis Laboratories) were used for C8 and C9 assays according to methodology described by Nelson et al. (17). Experimental error for these component assays ranges between 5 and 10% (16). In this study the individual complement components are also expressed as a function of the total proteins, or as CH_{50} units per 10 mg of total protein.

Transferrin and IgG measurements. Transferrin and IgG were measured in both sera and blister fluids by the Mancini method (22). Immunodiffusion plates for measuring transferrin were the gift of Dr. Hans J. Müller-Eberhard, La Jolla, Calif. IgG measurements were kindly performed by Dr. Gary Litman, Department of Pathology, University of Minnesota.

C3 proactivator (C3PA) and C3PA conversion. C3PA was measured in both sera and blister fluids by the Mancini method (22). C3PA conversion was demonstrated by immunoelectrophoresis according to the method of Götze and Müller-Eberhard (23). Antiserum to C3PA was also prepared according to Götze and Müller-Eberhard (23).
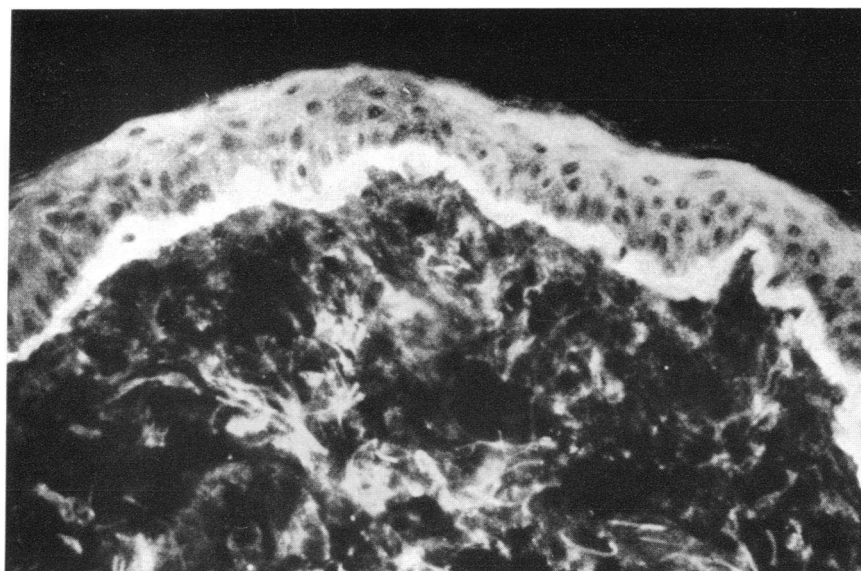
FIGURE 1 Indirect IF staining of normal human skin with the serum of a bullous pemphigoid patient (case 3 in Tables I and II) using labeled antiserum to IgG. Basement membrane zone staining is evident. × 400.

RESULTS

IF studies. Indirect IF staining was positive for both sera and blister fluids in 6 of the 10 cases (Table I). Four cases (cases 7, 8, 9, and 10 in Table I) did not have circulating basement membrane zone antibodies, despite the fact that IgG and C3 were bound to the basement membrane zone in their skin lesions. None of the control sera or blister fluids (other bullous diseases and experimentally induced blisters) yielded staining reactions with the basement membrane zone. Positive indirect IF staining, demonstrating pemphigoid antibodies in the serum of one of the serologically positive cases, appears in Fig. 1. In vitro complement staining was positive in both sera and blister fluids in five of the six serologically positive bullous pemphigoid cases (Table I).

Total complement and total proteins. A comparison of total complement levels in both sera and blister fluids revealed markedly reduced levels in the bister fluid compartments and relatively normal or elevated levels in the corresponding sera of the six serologically positive patients. Blister fluid protein levels, on the other hand, more closely approach their corresponding serum levels than do the complement levels.

Since previous studies of rheumatoid joint fluids (24, 25) suggest that complement activity in pathologic fluids is a function of the total protein content, we have expressed the serum and blister fluid complement values in CH₅₀ units per 10 mg of total protein (Table II). As Table II shows, the total complement levels of the

blister fluids in the six serologically positive bullous pemphigoid patients were lower than the corresponding serum levels. In one case (case 4), the total complement level of the blister fluid was too low to measure. The blister fluid-to-serum ratio, the amount of complement in blister fluid as compared with serum, expresses this difference. The four serologically negative cases, on the other hand, had levels more closely approaching their corresponding serum values.

TABLE I

IF Studies of Sera and Blister Fluids of 10 Cases of Bullous Pemphigoid for Basement Membrane Zone Staining

Case no.	Indirect IF staining*		In vitro complement staining‡	
	Serum	Blister fluid	Serum	Blister fluid
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	−	−
5	+	+	+	+
6	+	+	+	+
7	−	−	−	−
8	−	−	−	−
9	−	−	−	−
10	−	−	−	−

* Performed using monospecific antiserum to IgG.
‡ Performed using monospecific antiserum to β1C/β1A globulins (Hyland Div., Travenol Laboratories).

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Table III and IV summarize similar complement studies on 4 cases of pemphigus vulgaris, 8 cases of other blistering diseases, and 20 experimentally induced blisters. Except for the pemphigus vulgaris blisters, most of the control blisters more closely approach their correspond-

ing serum levels as do the four serologically negative bullous pemphigoid cases.

Two cases listed under "other bullous diseases" however, did exhibit lowered blister fluid complement levels. In this respect, one patient with epidermolysis bullosa and one patient with a bullous "id" reaction did have blister fluid-to-serum ratios of 42 and 40%, respectively. It becomes apparent from these two cases, and from the pemphigus vulgaris cases, that lowered complement blister fluid levels will not be confined solely to bullous pemphigoid blisters. The epidermolysis bullosa patient grew *Staphylococcus aureus* coagulase positive from the

blood and blister fluid which might account for the low value. The cause for the low value obtained in the id reaction blister fluid is not known. However, id reactions have been linked to antigen-antibody complex injury (26).

Suction blister fluid values were low for both total complement and total proteins, approximately ½ and ¼ of the serum values, respectively. This is true when the blister fluid and serum is collected immediately after blister formation. By expressing the complement values as a function of the total proteins, however, blister fluid

complement levels (except for case 8, Table IV) more closely approach the serum levels as seen in Table IV.

By harvesting the suction blister fluid 24 and 48 h after blister formation, both total complement and total protein content of the blister fluid rises; but by again expressing the complement in terms of the total protein, the complement levels of these older blisters are almost identical with those harvested immediately after formation. The total complement blister fluid-to-serum ratios, therefore, are essentially the same.

Cantharidin-induced blisters and liquid nitrogen-induced blisters yielded higher levels of both total complement and total proteins when compared with the suction blisters. When the complement values of these experimentally induced inflammatory blisters are expressed

TABLE V
Complement Component Profiles A. Bullous Pemphigoid
(Case 3, Tables I and II)

Components*	Serum‡	Blister fluid‡	Blister fluid-to-serum ratio§
			%
Total C	12.3	4.5	36
C1	38,630	9230	23
C4	26,300	6831	25
C2	401	111	27
C3	371	166	44
C5	575	314	54
C6	712	255	35
C7	474	162	34
C8	13,700	10,150	74
C9	17,810	11,080	62

* Normal values from this laboratory previously reported by Day et al. (16).
‡ Values expressed as CH_{50} units per 10 mg of total protein.
§ The amount of total complement or individual complement component in blister fluid compared with serum.

as a function of the total protein, their blister fluid-to-serum ratios are higher than those obtained for the suction blisters (Table IV). Further studies of complement activities in experimentally induced blisters are currently under way.

Anticomplementary tests. Except for three pemphigus vulgaris blister fluids, no serum or blister fluid demonstrated anticomplementary activity including the 10 bullous pemphigoid sera and blister fluids. One pemphigus vulgaris blister fluid, from a patient with widespread bullous lesions, exhibited no complement activity and completely inhibited complement activity in an equal volume of normal human serum. Two other pemphigus blister fluids reduced the complement level of an equal volume of normal human serum by better than one-half.

TABLE V
Complement Component Profiles B. Diabetic Bullae
(Case 7, Table III)

Components*	Serum‡	Blister fluid‡	Blister fluid-to-serum ratio§
			%
Total C	13.0	11.2	86
C1	44,600	14,600	33
C4	51,200	30,000	59
C2	175	153	87
C3	345	250	72
C5	560	404	72
C6	512	346	68
C7	1024	770	75
C8	12,900	10,000	78
C9	12,500	9800	78

* Normal values from this laboratory previously reported by Day et al. (16).
‡ Values expressed as CH_{50} units per 10 mg of total protein.
§ The amount of total complement or individual complement component in blister fluid compared with serum.

These blister fluids account for the low blister fluid-to-serum ratios for the pemphigus vulgaris patients seen in Table III. The nature of the anticomplementary activity in pemphigus vulgaris blister fluids is not known at this time but is currently under investigation.

Complement component assays. Individual complement components (C1-C9) were then measured in the serologically positive bullous pemphigoid patients using hemo-

TABLE V
Complement Component Profiles C. Suction Blister
(Case 2, Table IV)

Components*	Serum‡	Blister fluid‡	Blister fluid-to-serum ratio§
			%
Total C	12.5	8.4	67
C1	35,000	10,300	30
C4	4250	4420	104
C2	275	232	84
C3	156	116	74
C5	256	187	73
C6	169	112	66
C7	190	145	76
C8	47,500	17,370	37
C9	6625	7105	104

* Normal values from this laboratory previously reported by Day et al. (16).
‡ Values expressed as CH_{50} units per 10 mg of total protein.
§ The amount of total complement or individual complement component in blister fluid compared with serum.

Case no.	Serum		Blister fluid		Blister fluid-to-serum ratio‡	
	TF§	IgG	TF§	IgG	TF§	IgG
					%	%
1	451	1984	437	2192	96	101
2	509	2424	382	2436	75	101
3	445	2630	459	2385	103	91
4	554	2549	208	2154	37	85
5	394	1968	389	1970	98	100
6	292	2105	296	2293	101	109

* Values expressed in micrograms per 10 mg of total protein.
‡ The amount of transferrin or IgG in blister fluid compared
with serum.
§ TF, transferrin.

lytic assays (17, 18). Again, the serum levels of the individual components of the complement sequence are relatively normal. When serum levels are compared with blister fluid levels, however, reduced concentrations of individual complement components are noted in the blister fluid compartments, except for the two terminal components (C8 and C9) which more closely approximate the serum levels. A complement component profile from a representative case (case 3), appears in Table V*A*. As Table V*A* shows, the blister fluid-to-serum ratios for C1 through C7 are less than 50% except for C5 (54%). The C5 level still represents a significant reduction when compared with other blister fluid proteins. C8 and C9 levels, on the other hand, more closely approach the serum levels.

Complement component profiles were also determined on one patient with diabetic bullae (Table V*B*) and an experimentally induced suction blister (Table V*C*). Interestingly, the hemolytic C1 levels in both of these control blisters were low as in the bullous pemphigoid blisters. The C4 level of the diabetic bulla was also some-

FIGURE 2 Immunoelectrophoretic pattern of serum and blister fluid from a patient with bullous pemphigoid using 2% agar, barbital buffer, pH 8.5, ionic strength 0.05, containing 0.01 M EDTA and a potential gradient at 6 V/cm for 2½ h at 10°C. Middle trough contains antiserum to C3PA. Patient's serum (upper well) does not demonstrate C3PA conversion while the blister fluid (lower well) does.

what low (59% of serum level), but the remainder of the components (with the exception of C8 and C9) were higher than observed in the bullous pemphigoid blisters. In addition to a low C1 value (30% of the serum level), the C8 value of the experimentally induced suction blister was also low (37% of the serum value). The remainder of the components, however, more closely approached the corresponding serum levels in contrast to bullous pemphigoid blisters. C4 and C9 levels were slightly higher in the suction blister fluid compared with the serum.

Transferrin and IgG measurements. In an attempt to rule out protein size as a factor for the observed low complement and individual component blister fluid levels, we have measured two proteins, transferrin (mol wt 90,000) and IgG (mol wt 150,000), again in the sera and blister fluids of the six serologically positive bullous pemphigoid patients (Table VI). Except for transferrin levels in one patient (case 4), blister fluid levels of transferrin and IgG closely approached the corresponding serum levels. Again, the blister fluid-to-serum ratios express these differences. Although the IgG level of blister fluid closely approached the serum, case four exhibited a ratio of only 37% for transferrin. No total complement activity, however, was detected in this patient's blister fluid. In addition, the individual components of this blister fluid were all low, and in fact C4, C3, and C5 were not measurable at the lowest dilution used.

C3PA studies. Except for case 4 (Table VII), significantly reduced levels of C3PA using radial immunodiffusion were not detected in bullous pemphigoid blister fluids. This includes both the serologically positive group and the serologically negative group (Table VII). In fact, five blister fluids had higher levels of C3PA than did their corresponding sera.

Three blister fluids from serologically positive patients (cases 3, 5, and 6) were then tested by immunoelectrophoresis against antiserum to C3PA. Interestingly, all three blister fluids demonstrated C3PA conversion while the corresponding sera did not. Similar C3PA conversion was not demonstrable in diabetic bulla, suction, and cantharidin blister fluid. Fig. 2 illustrates C3PA conversion in one bullous pemphigoid blister fluid.

DISCUSSION

These studies show that hemolytic complement and individual components of the complement sequence are present in the blister fluids of bullous skin diseases of man and experimentally induced blisters. Similar findings have been reported for other pathologic fluids, such as synovial fluids in various types of joint diseases (25).

Katz, Inderbitzen, and Halprin (27) recently measured C3 levels in the serum of six bullous pemphigoid
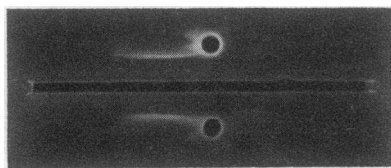
cases by radial immunodiffusion, and except for a few elevated levels no abnormalities were noted. Blister fluid levels were not included in their studies. These authors did not exclude involvement of complement in bullous pemphigoid, however, since consumption of C3 would have to reach drastic levels to be detected as a decrease in total serum complement. Our present studies, using hemolytic assays, corroborate their serum findings.

In our initial studies, we decided to look at bullous pemphigoid blister fluid complement levels using functional hemolytic assays to ascertain if they would more closely parallel the disease activity than do the serum levels. Our studies suggest that activation of the complement system occurs in blisters of patients who manifest circulating pemphigoid antibodies, especially of the complement-fixing type. Whether this relationship will be borne out in subsequent definitive analyses of complement activation must still be determined.

An analogous situation has been reported in patients with rheumatoid arthritis (24, 25); that is, with normal serum levels of complement and complement components, disproportionately low levels of complement are present in synovial fluids when compared with other joint fluid proteins and serum proteins (24, 25). In addition, the individual complement components (C1 through C3) are significantly lowered, and by-products of the complement sequence and active principals reflecting complement activation are found in synovial fluids (24). Leukotatic factors, consisting of C5-related products (28) and immune complexes (29, 30), have also been identified in rheumatoid synovial fluids. Rheumatoid arthritis, therefore, represents at least in part an important example of an immunologic disease in which local activation of the complement system plays a pathogenetic role.

The finding of relatively normal levels of the two terminal components (C8 and C9) in the blisters from the serologically positive bullous pemphigoid patients is not clear at this time but is being investigated further. It could, however, reflect the molecular relationships of the terminal components to the more proximal components in the cascade as has been clarified in a recent study by Kolbe, Haxby, Arroyave, and Müller-Eberhard (31). Our findings, however, are in contrast to similar studies of rheumatoid joint fluids where the two terminal components are lowered in addition to the early components (32).

The low C1 levels observed in the blister fluids studied thus far, including one diabetic bulla and one suction blister in addition to the pemphigoid blisters, is puzzling but might be related to the size of the C1 molecule. This finding is being investigated further. The low C8 level of one suction blister fluid also needs further clarification.

The abnormally elevated C3PA levels in some of the

TABLE VII
Bullous Pemphigoid Serum and Blister Fluid
C3PA Measurements*

Case no.	Indirect IF‡ results	Serum	Blister fluid	Blister fluid-to-serum ratio§
				%
1	+	30.5	31.9	104
2	+	33.1	29.6	89
3	+	34.9	28.3	82
4	+	16.7	‖	—
5	+	33.9	51.4	151
6	+	33.3	39.2	117
7	−	45.7	42.4	92
8	−	27.4	27.3	99
9	−	35.9	49.0	136
10	−	42.5	45.1	106

* Values expressed in micrograms per 10 mg of total protein.
‡ Indirect immunofluorescence for basement membrane zone staining.
§ The amount of C3PA in blister fluid compared with serum.
‖ Too low to measure.

bullous pemphigoid blisters is not fully understood at this time. This is possibly due to the conversion of C3PA which was apparent in three blister fluids tested thus far.

Although our hemolytic studies suggest that complement activation in bullous pemphigoid occurs by the "classical pathway," i.e. C1 through C9, our recent demonstration of C3PA conversion in blister fluid suggests recruitment of the alternate pathway (C3 through C9) as well. Recent studies by Provost and Tomasi,[3] who have demonstrated both properdin and C3PA bound to the basement membrane zone in bullous pemphigoid skin lesions by direct IF staining, tend to support this contention. C1q and C4 (9),[3] however, have also been demonstrated in bullous pemphigoid skin lesions, findings which support our contention of involvement of the classical pathway in addition to the alternate pathway.

Our studies of the complement system in bullous pemphigoid, although in the initial stages, are suggestive of local activation and utilization of complement, i.e., in the blister fluid. Other studies now in progress, including complement-mediated functions and in particular chemotactic activity, should further implicate complement in the pathogenesis of this disease.

ACKNOWLEDGMENTS

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