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Serum and Blister Fluid Anticomplementary Activity in Pemphigus and Bullous Pemphigoid. Sucrose Density Gradient Studies[1] (39267)

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Pemphigus and bullous pemphigoid are blistering diseases of the skin with specific immunopathologic findings. By indirect immunofluorescence (IF), autoantibodies reactive with skin intercellular substance (ICS) occur in sera of pemphigus patients (1, 2), while skin basement membrane zone (BMZ) autoantibodies occur in sera of patients with bullous pemphigoid (3, 4).

Complement activation has recently been implicated in both diseases. Using IF methods, C1q, C4, C3, and Factor B deposition have been noted in ICS areas of early pemphigus lesions in addition to IgG (5-7). BMZ deposition of properdin has been reported in bullous pemphigoid skin lesions in addition to C1q, C4, and C3, findings compatible with activation of both the classical and alternate complement pathways in that disease (8-10). In addition, low levels of total hemolytic complement and of individual components have been noted in both pemphigus and bullous pemphigoid blister fluids when compared to serum levels (11, 12). Anticomplementary activity, suggestive of immune complex formation, has also been noted in most pemphigus blister fluids (12). In order to determine whether the anticomplementary activity present in these fluids was consistent in size with that of an antigen antibody complex (i.e., significantly greater than 7 S), we undertook to further characterize this material in pemphigus blister fluid by sucrose density gradient ultracentrifugation and to determine if it was also present in pemphigus sera and in bullous pemphigoid sera and blister fluids.

Methods. Patients with pemphigus and bullous pemphigoid included in this study had active disease when blood and blister fluid samples were collected. Suction and cantharidin blisters, induced experimentally on normal volunteers, as well as blister fluid from diabetic bullous skin lesions, served as controls. Paired samples of venous blood and blister fluid from patients or volunteers were collected under sterile conditions, allowed to clot, separated, aliquoted, and stored at −70° until tested.

Pemphigus and bullous pemphigoid sera and blister fluids were tested for ICS and BMZ reactive antibodies using IF as described before (2, 3). Total complement levels and total proteins of paired pemphigus and bullous pemphigoid sera and blister fluids were also determined as previously outlined (11, 12).

Linear 5 to 30% sucrose gradients were prepared as outlined by Blondin and McDuffie (13). Samples of serum or blister fluid (0.4 ml) sterilized by Millipore filtration and aged for 7 days at 25° to allow decay of complement activity were layered on gradients and centrifuged at 40,000 rpm (270,000 *g* at the tip) for 16 hr at 4° in a SB 283 rotor in a B-60 International ultracentrifuge.[2] Final volume of each gradient was 11.4 ml. All samples were run in duplicate. Gradient tubes were punctured following centrifugation, and 18 fractions (approximately 0.65 ml each) were collected from the bottom.

The fractions collected were tested for complement fixing activity (12, 14) by a microtiter system. One drop (0.025 ml) of sample was added to 0.025 ml of buffer,[3]

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[2] International Equipment Co., Needham Heights, Massachusetts.

[3] Glucose gelatin veronal buffer containing 1.5 ×

and six doubling dilutions were made. One drop of an appropriate dilution of complement (normal human serum) was added and the mixture was incubated at 30° for 30 min. One drop of EA (1×10^8 sensitized sheep red blood cells/ml) and two drops of buffer were added, and the mixture was incubated at 37° for 1 hr. The microtiter plates were then centrifuged at 1500 rpm for 10 min and examined for hemolysis.

The positive gradient fractions were also assayed for their ability to activate C1 and C3 in normal human serum. Equal volumes of positive gradient fractions and normal human serum were incubated at 37° for 1 hr and assayed for inhibition of hemolytic C1 and C3 as previously described (12). Cell intermediates (EAC14 and EAC4) and buffers (GVB^{2+} and $GGVB^{2+}$) used in these assays were prepared according to Borsos and Rapp (15) as modified by Day *et al.* (16).

Location of IgG, IgM, and C3 in the gradients was determined by radial immunodiffusion (17) with antisera made by us.

Results. The sera of all 10 pemphigus patients and 3 of the 5 bullous pemphigoid patients included in the study contained circulating antibodies against ICS and BMZ, respectively, as demonstrated by indirect IF staining. The blister fluids studied from the one patient with pemphigus and one of five with pemphigoid also contained these same antibodies. With the exception of bullous pemphigoid patient No. 4, blister fluid levels of total complement were relatively low when compared to the serum levels (Table I), consistent with our previous observations (11, 12).

Significant complement fixing activity was present in the one pemphigus blister fluid and in three of five pemphigoid blister fluids tested following sucrose density gradient ultracentrifugation. This activity was found in the bottom density gradient fractions (Table II). The peak complement fixing titer obtained for the pemphigus blister fluid (fraction 2) was 1:32, whereas the highest peak titer for bullous pemphigoid blister fluid was 1:64 (fraction 1, patient 4, Table II). These activities sedimented somewhat faster than, but overlapped, IgM. IgM was usually found in fractions 2–5, whereas IgG and C3 were in fractions 8–12. None of the control blister fluids, including five cantharidin induced blisters, two suction blisters, and one blister fluid from a patient with bullous lesions and diabetes mellitus, contained such activity.

10^{-4} *M* Ca^{2+} and 5×10^{-4} *M* Mg^{2+} ($GGVB^{2+}$), pH 7.4, 0.075 ionic strength.

TABLE I. SERUM AND BLISTER FLUID HEMOLYTIC COMPLEMENT.[a]

Case	Serum (S)	Blister fluid (BF)	BF/S ratio (%)[b]
1. Pemphigus[c]	17.2	<1.0	<6
2. Bullous pemphigoid[d]	22.2	2.5	11
3. Bullous pemphigoid[d]	23.9	7.8	33
4. Bullous pemphigoid[d]	28.6	18.2	64
5. Bullous pemphigoid[d]	26.9	5.8	22
6. Bullous pemphigoid[d]	9.0	4.0	44

[a] Expressed in CH50 units per 10 mg of total protein.

[b] The amount of complement in blister fluid compared to serum. Normal > 50 based on Table IV, ref. 11.

[c] Serum and blister fluid contained ICS antibodies.

[d] Sera and blister fluids contained BMZ antibodies.

Table III shows that 6 of 10 pemphigus sera also contained similar complement fixing activity, which sedimented somewhat more slowly at about 12–19 S (fractions 3–7). The complement fixing titers of these sera, however, were lower than that of the pemphigus blister fluid. Two of five bullous pemphigoid sera were found to have complement fixing activity in the same size range as the corresponding blister fluid complexes, but in somewhat lower titer.

Figure 1 depicts the relationship of the serum and blister fluid complement fixing activity in one bullous pemphigoid patient (patient No. 4). Higher levels of activity were present in blister fluid. Activity in both serum and blister fluid sedimented at the bottom of the gradient but overlapped IgM.

To shed some light on which components of complement were being activated by the blister fluids, hemolytic C1 and C3 in normal human serum were measured following addition of complement fixing gradient fractions from pemphigus and bullous pemphigoid blister fluids. As Table IV shows, blister fluid gradient fractions of both pemphigus and bullous pemphigoid activated both C1 and C3 in normal human serum, findings consistent with activation of the classical

TABLE II. SUCROSE DENSITY GRADIENT STUDIES: BLISTER FLUIDS.

Sample	Anticomplementary activity	Positive fractions	Fraction titers[a]
1. Pemphigus[b]	Pos	2-5	1:32, 1:16, 1:8, 1:2
2. Bullous pemphigoid[b]	Pos	1-4	1:32, 1:4, 1:4, 1:2
3. Bullous pemphigoid[b]	Pos	1-4	1:8, 1:4, 1:2, 1:2
4. Bullous pemphigoid[b]	Pos	1-4	1:64, 1:8, 1:4, 1:2
5. Bullous pemphigoid[b]	Neg	–	–
6. Bullous pemphigoid[b]	Neg	–	–
Diabetic bullae	Neg	–	–
Suction blister	Neg	–	–
Suction blister	Neg	–	–
Cantharidin blister	Neg	–	–
Cantharidin blister	Neg	–	–
Cantharidin blister	Neg	–	–
Cantharidin blister	Neg	–	–
Cantharidin blister	Neg	–	–

[a] Dilutions showing inhibition of hemolysis.
[b] Samples listed in Table I.

TABLE III. SUCROSE DENSITY GRADIENT STUDIES: PEMPHIGUS AND BULLOUS PEMPHIGOID SERA.

Sample	Anticomplementary activity	Positive fractions	Fraction titers[a]
1. Pemphigus[b]	Pos	4-6	1:16, 1:2, 1:2
Pemphigus	Pos	1-4	1:8, 1:8, 1:8, 1:4
Pemphigus	Pos	4-6	1:8, 1:8, 1:8
Pemphigus	Pos	4-6	1:16, 1:8, 1:4
Pemphigus	Pos	3,4	1:16, 1:8
Pemphigus	Pos	4-6	1:8, 1:8, 1:4
Pemphigus	Neg	–	–
Pemphigus	Neg	–	–
Pemphigus	Neg	–	–
Pemphigus	Neg	–	–
2. Bullous pemphigoid[b]	Pos	1-3	1:2, 1:4, 1:8
3. Bullous pemphigoid[b]	Neg	–	–
4. Bullous pemphigoid[b]	Pos	1-5	1:8, 1:4, 1:8, 1:4, 1:2
5. Bullous pemphigoid[b]	Neg	–	–
6. Bullous pemphigoid[b]	Neg	–	–

[a] Dilutions showing inhibition of hemolysis.
[b] Samples listed in Table I.

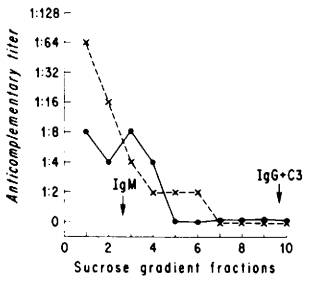

FIG. 1. Density gradient ultracentrifugation of complement fixing activity in serum (solid line) and blister fluid (broken line) of a patient (patient No. 4) with bullous pemphigoid. IgM, IgG, and C3 were used as molecular weight markers. Bottom tube to the left. Uppermost eight tubes (11-18) contained no activity and are not shown.

pathway. A noncomplement fixing fraction of each type of blister fluid did not result in similar activation of hemolytic C1 and C3.

Comment. Anticomplementary activity, thought to represent complement fixation by immune complexes, has recently been noted in pemphigus vulgaris blister fluids (12). The material responsible could activate complement both at 37° for 1 hr and at 4° for 8 hr and was not destroyed at 56° for 30 min. It could activate both early (C1 and C2) and later (C3 and C5) acting complement components and cause conversion of both C3 and Factor B in normal human serum.

In the present study this activity in pem-

TABLE IV. INHIBITION STUDIES OF HEMOLYTIC C1 AND C3 IN NORMAL HUMAN SERUM (NHS) WITH SELECTED POSITIVE BLISTER FLUID SUCROSE GRADIENT FRACTION.

	C1 (μ/ml)	Inhibition (%)	C3 (μ/ml)	Inhibition (%)[a]
NHS alone	310,000		1,260	
NHS + buffer[b]	140,000		650	
NHS + pemphigus[c]	112,000	20	480	26
NHS + pemphigoid[d]	64,000	54	390	40

[a] The amount of inhibition obtained for hemolytic C1 and C3 in NHS by the positive gradient fraction. (Inhibition was not seen with negative gradient fractions.)
[b] $GGVB^{2+}$.
[c] Positive gradient fraction of pemphigus vulgaris blister fluid.
[d] Positive gradient fraction of bullous pemphigoid blister fluid.

phigus blister fluid was found by sucrose density gradient ultracentrifugation to be 19 S and greater. The material appears to activate the classical pathway, a finding consistent with previous observations (12). Similar, slightly smaller (12–19 S) material was found in some pemphigus sera. The composition, mode of formation, and pathogenetic significance of this material is not known at the present time. Beutner *et al.* (18), using IF techniques, have also noted suspected immune complexes in the serum of one pemphigus patient in relapse. Their patient, however, had no circulating ICS antibodies, while all of our patients with anticomplementary material had such antibodies in their sera.

The finding of complement-fixing factors also in bullous pemphigoid blister fluids is not surprising since low complement levels and other immunoreactants have been previously identified (11). In the five cases studied, however, low blister fluid complement levels could not be correlated precisely with the amount of complement-fixing activity since patient No. 4 with a high level of anticomplementary activity had a normal level of blister fluid complement. The two patients with no anticomplementary activity in blister fluids, however, lacked circulating BMZ antibodies. As in pemphigus, the responsible material appears to activate the classical pathway and is also present in a few bullous pemphigoid sera.

Preliminary studies in our laboratory suggest that both pemphigus and bullous pemphigoid sera will also react with the C1q component of complement (19) as measured by a radioassay with ^{125}I-labeled C1q, as described recently by Nydegger *et al.* (20). These studies, presently being extended, are expected to shed further light on the properties of the material capable of activating complement present in these patients and its possible relationship to antigen–antibody complexes.

Summary. By sucrose density gradient ultracentrifugation and standard hemolytic complement assays, complement fixing activity of about 19 S and greater was found in blister fluids of one patient with pemphigus and three of five patients with bullous pemphigoid. Control blister fluids, including experimentally induced blisters, lacked such activity. Complement fixing activity was apparent, however, in 6 of 10 pemphigus sera and in two of five bullous pemphigoid sera tested. The material present in both pemphigus and bullous pemphigoid appears to activate the classical complement pathway.

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