Evidence for Complement Activation via the Alternate Pathway in Skin Diseases I

HERPES GESTATIONIS, SYSTEMIC LUPUS ERYTHEMATOSUS, AND BULLOUS PEMPHIGOID

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ABSTRACT A patient with herpes gestationis, 6 of 6 patients with bullous pemphigoid, and 5 of 25 patients with systemic lupus erythematosus were found to have properdin deposited along the skin basement membrane.

The patient with herpes gestationis demonstrated by immunofluorescence basement membrane deposition of C3 and C5 in the absence of C1q, immunoglobulins, and light chains. A second patient with herpes gestationis had C3 deposition with no demonstrable immunoglobulins or light chains. A thermolabile humoral factor(s) capable of depositing C3 (without C1q or C4) on normal skin basement membrane was found in the sera of both patients with herpes gestationis. No anti-basement membrane antibodies could be demonstrated in the sera of these patients.

The patients with systemic lupus erythematosus and bullous pemphigoid who manifested properdin deposition also showed skin basement membrane deposits of C1q, C4, C3, C5, and immunoglobulins. C3 proactivator (C3PA) was also found deposited along the skin basement membrane of three patients with systemic lupus erythematosus and all six bullous pemphigoid patients.

This study provides suggestive evidence that activation of complement is occurring via the alternate pathway in herpes gestationis. In systemic lupus erythematosus and bullous pemphigoid, both the classical (antibody) mediated activation of complement as well as the alternate pathway may be operative.

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INTRODUCTION

An alternate pathway for the activation of complement (the C3 shunt) has been described (1–3) which does not involve the earlier components of the complement sequence (i.e. C1, 4, 2). Activation of this pathway results in the consumption of the late complement components (C3, C5–C9) with little or no utilization of the factors preceeding C3 (C1, 4, 2). A variety of substances, including certain bacterial polysaccharides, $F(ab)_2$ fragments, and aggregated immunoglobulins such as IgA, IgE, and IgG_4, which are not capable of fixing complement through the classical route activate the C3 shunt (2–4). Although the detailed mechanisms involved in the activation of C3 are at present incompletely understood, evidence points to a relation to the previously described properdin system.

Properdin is a normal serum β-globulin of mol wt 220,000 which in combination with an activating substance such as yeast cell wall extract (zymosan) initiates the cleavage of C3 without the consumption of the earlier complement components (5, 6). This reaction requires Mg^{++} and two normal serum components; a hydrazine sensitive factor (factor A) and a heat-labile factor (factor B). Factor B has been reported to be functionally identical to C3 proactivator (C3PA)[1] (7). C3PA is a 6S β-globulin which in the presence of fresh serum and an activating substance such as zymosan is converted to an active enzyme (C3 activator) which cleaves C3. The activation of C3PA is accomplished by a serum enzyme

[1] *Abbreviations used in this paper:* C3PA, C3 proactivator; HG factor, herpes gestationis serum factor; PBS, phosphate-buffered saline.

which is a 3S α-globulin (C3Pase). This reaction also requires a hydrazine sensitive product of C3 which closely resembles C3b (8). Whether the latter component is identical with factor A of the properdin system is as yet unknown. The exact relationship of properdin itself to the C3 shunt has not been established although it seems likely that it is a component of the proenzyme system which activates C3PA.

Westberg, Naff, Boyer, and Michael (9) employing direct immunofluorescence have demonstrated deposition of C3 and properdin along the glomerular basement membrane and mesangium of patients with acute glomerulonephritis and chronic membranoproliferative glomerulonephritis (hypocomplementemic glomerulonephritis). In these patients, immunoglobulins and early components of complement (C1, C4) were either not observed or were present in small amounts compared with the heavy C3 staining. The latter was present in areas corresponding to the basement membrane and mesangial sites of properdin deposition.

In this report we describe two patients with herpes gestationis in which C3 was deposited along the dermal-epidermal basement membrane in the apparent absence of immunoglobulins. One of these patients also had C5 and properdin but not C1q staining along the basement membrane as determined by immunofluorescence. Subsequent screening of various inflammatory skin diseases revealed positive immunofluorescence for properdin in six patients with bullous pemphigoid and five patients with systemic lupus erythematosus. Three SLE patients were also found to have C3 proactivator (C3PA) deposition. All six bullous pemphigoid patients had C3PA deposited along their skin basement membrane.

METHODS

Patients. Two patients (F. L. and V. G.) with herpes gestationis were in their mid 30's and had previous normal pregnancies. A generalized pruritic bullous eruption developed at the end of the second or beginning of the third trimester. Skin biopsies demonstrated typical subepidermal bullae. Quantitation of immunoglobulins revealed a mild elevation in IgG, IgM, and IgA in both patients. C3 levels measured by radial immunodiffusion (Hyland Laboratories, Costa Mesa, Calif.), were normal on three occasions in patient F. L. 194, 205, 210 mg/100 ml, (normal 118–180 mg/100 ml). The C3 level in patient V. G. was 167 mg/100 ml. The total hemolytic complement (CH50) as determined by the method of Mayer (10) was 44, 50, and 54 in patient F. L. (normal >30). The CH50 level was not determined on the serum of patient V. G. The skin lesions improved rapidly on steroids although one patient (F. L.) had exacerbations at parturition and with several menses in the postpartum period. By 5 mo postpartum, both patients had no evidence of disease. The children were normal.

Immunofluorescent examination was performed on the skin biopsies of 25 patients with systemic lupus erythematosus. The patients were judged to have systemic lupus erythematosus by history, physical examination, and a variety of serological findings, including antinuclear antibodies, anti-DNA antibodies, and positive LE preparations. Only patients with classical disseminated lupus having positive serological findings were included. 20 of these patients had immunoglobulins and, in some cases, various components of complement deposited along the skin basement membrane of "normal" appearing skin. Serum complement levels varied. Three patients with properdin and C3PA on the basement membrane had low CH50(<20) and C3 (<100 mg/100 ml) complement levels. These patients had evidence of nephritis.

Six patients with classic bullous pemphigoid were studied. Diagnosis was established by history, physical examination, biopsy evidence of a subepidermal bullous disease and positive immunofluorescent staining of the basement membrane for complement (C3) and IgG. CH50 was 31, C3 122 mg/100 ml in one patient who was not on therapy. The five patients who were being treated with high doses of steroids had normal C3 and CH50 complement levels.

Experimental techniques. Immunofluorescence, utilizing both the direct and indirect techniques were performed on skin biopsies obtained with 4-mm punch biopsies. The biopsies were immediately "snap frozen" and stored at $-70°C$ until used. Placental tissue from F. L. was similarly treated.

Antisera to human immunoglobulins were prepared and rendered monospecific by appropriate absorptions as previously described (11, 12). The antiserum to rabbit globulin employed in the indirect immunofluorescence technique was raised in a goat and the antiserum to horse globulin in a rabbit. Dr. Jack Boyer kindly donated a properdin and C5 antisera. The preparation and characteristics of these antisera have been previously described (4). A rabbit anti-human C1q was a gift of Dr. Abeyounis (13). Subsequently human C1q was isolated and purified according to the technique of Yonemasu and Stroud and an antisera was raised in a rabbit (14). Fluorescein-conjugated rabbit anti-human C3 (BIC/BIA) and horse anti-human C4 were purchased from Hyland Laboratories.

Dr. Hans Müller-Eberhard kindly donated a rabbit anti-human C3 proactivator (C3PA) antisera. The preparation and characteristics of this antisera have been previously reported (3). This antisera employed in indirect immunofluorescence with fluorescein-conjugated goat anti-rabbit antisera failed to demonstrate C3PA deposition along the basement membrane in three normal skin biopsies or in the biopsies from patients with psoriasis, eczema, and cutaneous vasculitis.

All antisera were checked for monospecificity by gel double diffusion and immunoelectrophoresis. Each of the antisera were monospecific except for one of the C1q antisera which displayed a faint second system on gel diffusion analysis against normal serum. A 1:2 dilution of this antisera resulted in a disappearance of the unknown second system. This antisera was used at 1:12 dilution for immunofluorescent studies.

The antisera were conjugated with fluorescein isothiocyanate (FITC) according to the method of Clark and Sheppard (15). The F/P ratios of these antisera ranged between 2.8 and 5.2 as described by Beutner, Chorzelski, and Jordan (16). Controls consisted of normal skin removed at autopsy and biopsies from patients with various inflammatory skin diseases.

The sera of the two patients with herpes gestationis were subjected to salt fractionation with 50% ammonium sulphate. The precipitate was reconstituted with phosphate-buffered saline (PBS), pH 7.2, to the original volume

and dialyzed. The dialyzed supernatant was concentrated by positive pressure to the original serum volume. These fractions were then studied by the indirect immunofluorescent technique as follows: the fractions were layered on normal human skin, incubated at room temperature for 30 min. washed for 20 min with three changes of PBS, followed by the layering of fresh normal serum (as source of complement), incubated at room temperature a second time for 30 min, washed again with PBS, and stained with various conjugated anti-human immunoglobulin (IgA, IgG, IgM, and light chain) and complement antisera (C1q, C4, C3). The tissue was then stained with Evans blue dye at room temperature for 10 min. The Evans blue dye is used as a counter stain and is composed of (0.2 ml Evans blue, 0.4 ml of calf serum, and 0.1 ml PBS).

RESULTS

Tissues taken from active skin lesions and normal adjacent areas of both patients with herpes gestationis demonstrated linear deposition of C3 along the skin basement membrane (Fig. 1). Using monospecific antisera

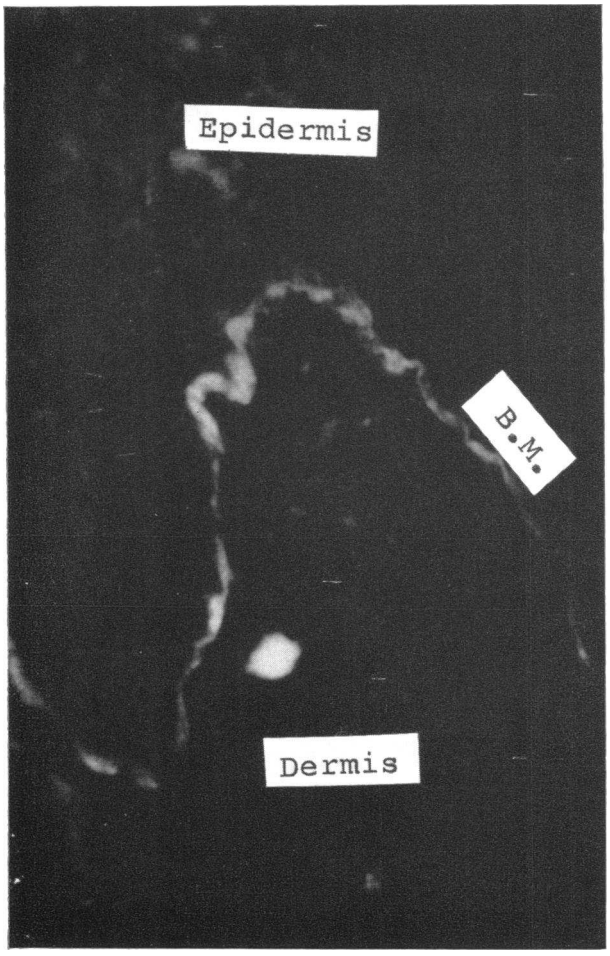

FIGURE 1 Patient F. L. with herpes gestationis. Direct staining of "normal" skin adjacent to bulla with conjugated anti-C3 demonstrating a "linear" staining pattern along the skin basement membrane (B.M.). × 40

TABLE I

Immunofluorescent Profile on Skin Biopsies of Patients with Herpes Gestationis (Basement Membrane Staining)

Name	C1q	C3	C5	Light chains	Properdin	Immunoglobulin
F. L.	Not tested	+	Not tested	−	Not tested	−
V. G.	−	+	+	−	+ Granular	−

to IgG, IgA, IgM, and a potent anti-light chain antisera having both kappa and lambda activity, no basement membrane fluorescence was detectable in either patient. Patient (V. G.) demonstrated linear fluorescence along the basement membrane of the skin with rabbit anti-human properdin antisera. Positive staining was also seen with anti-C5 but not with anti-C1q antisera (Table I). Unfortunately, skin from patient F. L. with herpes gestationis was not available for study with antisera to properdin, C5, and C1q. Anti-human C3PA and C4 antisera were obtained after all biopsy specimens on patients V. G. and F. L. had been utilized.

Fresh whole sera from patient F. L. placed on *normal* skin resulted in staining of the basement membrane with anti-C3 (Fig. 2) but not with anti-immunoglobulin or light chain antisera. The pattern of fluorescence was linear and very similar to that seen on direct examination of the patient's skin. Staining faded and disappeared with aging of the sera but could be reconstituted with the addition of fresh human serum as outlined in the indirect immunofluorescent procedure. Positive staining for C3 was also observed using the redissolved precipitate resulting from treatment of the serum with 50% ammonium sulfate. Although fresh serum was not available on patient V. G., her aged serum could be reconstituted with fresh human serum, as previously described. C3 deposition was consistently found using this technique employing either F. L. or V. G. sera but C1q, C4, C3PA, properdin, immunoglobulins, or light chains were not seen. Controls with five fresh normal sera alone or sera from patients with five other skin diseases (i.e., lichen planus, psoriasis, eczema, urticaria, and erythema multiforme) were negative. A partial characterization of the herpes gestationis serum factor (HG factor) is presented in Table II. The HG factor in both sera was present in a maximum titer of 1:16.

Direct and indirect immunofluorescence employing the patients' sera and conjugated antisera to IgG, IgA, IgM, and C3 was performed upon the placenta of F. L. No abnormal staining was found using either technique.

Using the indirect immunofluorescent technique, we have screened a limited number of biopsies from normal individuals as well as patients with various inflammatory

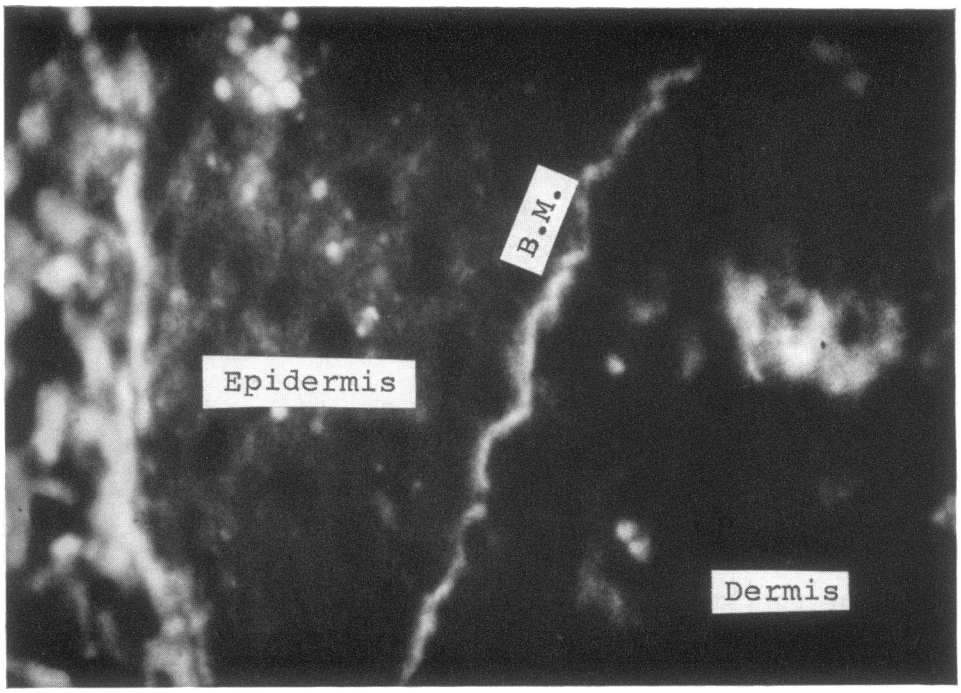

FIGURE 2 Patient F. L. with herpes gestationis. Fresh sera layered over normal human skin washed and stained with conjugated anti-C3. Note specific staining of basement membrane (B.M.). Nonspecific autofluorescence of reticulum fibers in the dermis is also seen below the basement membrane. × 40

skin diseases for properdin. The diseases which were studied and were negative for properdin are listed in Table III.

In addition to the patients with herpes gestationis positive staining for properdin was found on the skin basement membranes in 5 of 25 patients with systemic lupus erythematosus and 6 of 6 patients with bullous pemphigoid (Fig. 3). Deposition of properdin, immunoglobulins, and early as well as late components of complement were found in "normal" as well as involved skin of the patients with systemic lupus erythematosus (see Table IV). The biopsy of an active discoid plaque lesion in another patient with systemic lupus erythematosus displayed immunoglobulin and C3 deposition but no properdin. One patient, with discoid lupus of the face and no serologic evidence of systemic lupus erythematosus, had no properdin on the diseased skin basement membrane.

TABLE II

Characteristics of Serum Factor(s) in Herpes Gestationis Capable of Depositing C3 on Normal Skin Basement Membrane

1. Not an immunoglobulin (see text)
2. Present only during active disease
3. Nondializable
4. Pseudoglobulin
5. Thermolabile-destroyed at 56°C for 30 min
6. Precipitable by 50% ammonium sulphate
7. Not properdin (see text)

TABLE III

Skin Biopsies Showing Negative Properdin Staining at the Dermal-Epidermal Junction

Disease	Site of biopsy	No. of patients
Scleroderma	Active lesion	1
Eczema	"	2
Erythema multiforme	"	3
Urticaria	"	2
Psoriasis	"	2
Erythema elevation diutium	"	1
Necrotizing cutaneous vasculitis	"	4
Rheumatoid vasculitis	Normal skin	1
Vasculitis with cryoglobulinemia	Active lesion	1
Lichen planus	"	1
Discoid lupus erythematosus	Normal skin	1
Discoid lupus erythematosus	Involved skin	1
Systemic lupus erythematosus	Uninvolved skin	20
Systemic lupus erythematosus	Active discoid lesion	1
Pemphigus vulgaris	Active and normal skin	3
Normal skin		5
		49

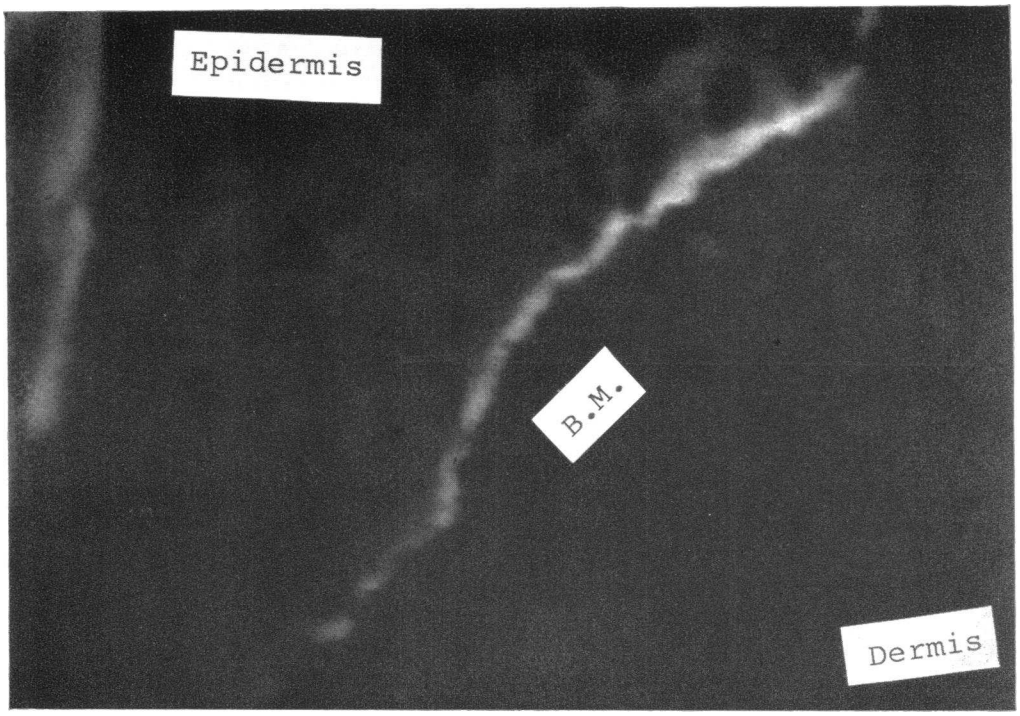

FIGURE 3 Patient C. C. with bullous pemphigoid. Positive staining of the skin basement membrane (B.M.) for properdin is seen at the edge of bullous lesion. × 95

The five patients with SLE who displayed properdin deposition lacked humoral factor(s) such as were seen in herpes gestationis which caused in vitro deposition of C3 on *normal* skin basement membrane. Three of these patients also had C3PA deposition along the skin basement membrane. Skin biopsy specimens from the other two patients are not available for study at this time.

All six of the bullous pemphigoid patients demonstrated IgG staining of the basement membrane together with C1, C4, C3, C5, and properdin (Table V). A linear staining pattern was seen in both clinically normal as well as active skin lesions. These six patients were also found to have C3PA staining of the basement membrane (Fig. 4).

DISCUSSION

Herpes gestationis is an uncommon bullous disease of pregnancy with an incidence of approximately 1 per 10,000 births (17). It is characterized by a rapidly progressive skin eruption heralded initially by severe gen-

TABLE IV

Immunofluorescent Profile on Skin Biopsies of Five Patients with Systemic Lupus Erythematosus (Dermal-Epidermal Junction Staining)*

Name	Clq	C4	C3	C5	Light chain	C3PA	Properdin	Immunoglobulins
P. P.	+	+	+	+	+	+ Granular	+ Linear	IgA, IgG, IgM, all +
P. W.	+	Not tested	−	+	+	Not tested	+ Granular	IgG +
M. F.	+	+	+	+	+	+ Granular	+ Granular	IgG, IgM +
V. S.	+	Not tested	+	+	+	Not tested	+ Linear	IgG, IgM +
A. M.	+	+	+	+	+	+ Granular	+ Granular	IgG, IgM +

* Five of 25 lupus patients having positive properdin staining.

TABLE V

*Immunofluorescent Profile of Skin Biopsies of Patients with Bullous Pemphigoid (Basement Membrane Staining)**

Name	Clq	C4	C3	C5	Light chains	C3PA	Properdin	Immuno-globulin
E. B.	−	+	+	+	+	+	+	IgG +
M. C.	+	+	+	+	+	+	+	IgG +
M. K.	+	+	+	+	+	+	+	IgG +
J. K.	+	+	+	+	+	+	+	IgG +
E. M.	+	Not tested	+	+	+	+	+	IgG +
H. E.	+	+	+	+	+	+	+	IgG +

* Staining pattern with these antisera is linear and homogeneous.

eralized pruritus. Erythematosus papules and urticarial-like plaques rapidly appear followed by a grouped, tense, vesicular bullous eruption. The disease most commonly occurs during the second and third trimesters and the skin eruption generally terminates 1–2 mo following delivery. Persistence long into the postpartum period as well as exacerbations with menses and the use of anovulatory drugs have occasionally been reported (18–22). Subsequent pregnancies may be accompanied by a more severe and earlier onset of the disease (23). There is no significant fetal wastage and prognosis for both mother and child is good. Steroids are a highly effective form of treatment. A variety of etiologies have been proposed including viral, toxic, psychogenic, Rh isoimmunization, and immunological mechanisms. However, none of these have been substantiated.

We are aware of at least two patients with herpes gestationis who have been previously evaluated employing direct and indirect immunofluorescent techniques (16, 24). No basement membrane immunoglobulin deposition was reported on direct immunofluorescence and autoantibodies could not be demonstrated by the indirect technique. Staining for C3 was performed on one patient's skin and was reported to be negative.

Our studies confirm the absence of immunoglobulin on the basement membrane of these patients. This study, however, demonstrates heavy deposition of C3 along the dermal epidermal basement membrane of both involved

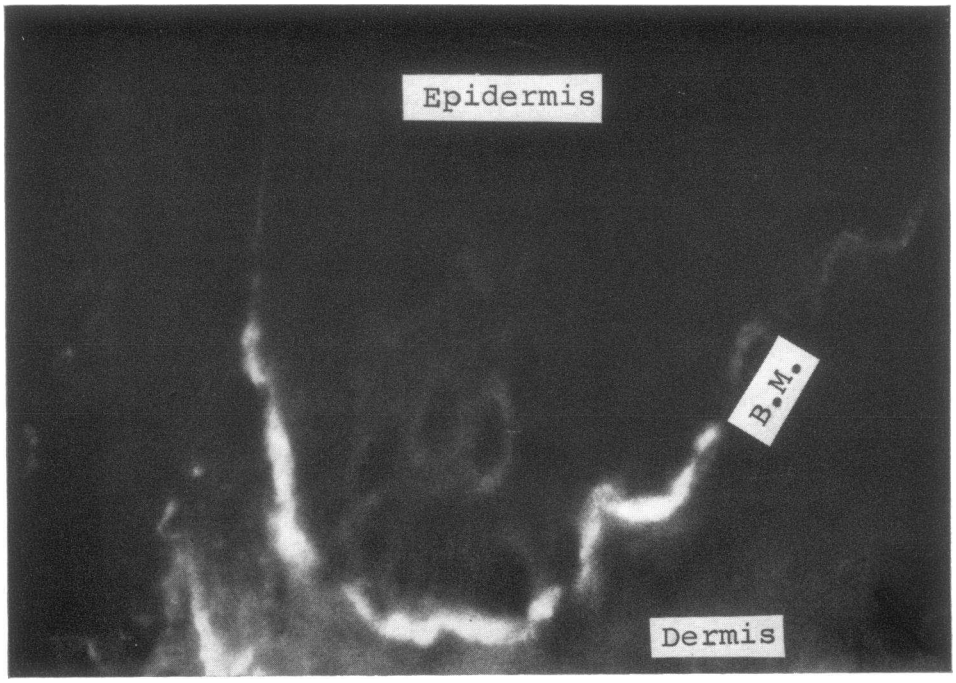

FIGURE 4 Patient G. B. with bullous pemphigoid. Positive staining of the skin basement membrane (B.M.) for C3 proactivator. × 54.

and uninvolved skin. The amount of C3 deposited decreased as the skin lesions improved. In one patient, C3 C5, and properdin but not C1q were found along the basement membrane. These findings taken together with the presence of the humoral factor discussed below suggest the complement activation in this disease may be occurring via the C3 shunt.

The possible activator of this system in our patients with herpes gestationis is unknown. Immunoglobulins and fragments of immunoglobulins containing light chains (such as $F(ab)_2$ fragments) could not be detected by fluorescence with specific anti heavy or light chain antisera. However, we cannot completely rule out the presence of small concentrations of immunoglobulins which are capable of initiating the shunt sequence but which are undetectable by immunofluorescence.

A humoral factor (HG factor) present in the sera of both patients which is capable of depositing C3 without C1q on *normal* skin basement membrane in vitro has been demonstrated. Although the participation of the HG factor in the pathogenesis of the skin lesion in vitro has not been established, this possibly is suggested by the disappearance of the HG factor from the serum concomitant with healing of the skin lesions following the termination of pregnancy. 11 serum specimens obtained from patient F. L. between the 7th mo of pregnancy and 2 mo postpartum demonstrated the presence of HG factor. Specimens taken 5 mo postpartum and 1 yr later failed to demonstrate this factor. That the HG factor may be influenced by female sex hormones is suggested by (*a*) the repetitive nature of the disease occurring only during and immediately following pregnancy, (*b*) exacerbation with parturition and menstrual cycles in the immediate post partum period, (*c*) the observations of reactivation of the disease with progestational hormones and subsidence of the disease with their discontinuance. At the present time we are unable to reconcile the direct fluorescent staining pattern on herpes gestationis skin in which properdin, C3, and C5 was demonstrated, with the fluorescent pattern obtained by layering the patient's serum over normal skin followed by a source of fresh serum. With the later procedure C3 deposition was found along the basement membrane but not properdin, C3PA, immunoglobulin light chains, C1q, or C4. Perhaps the simplest explanation is that the basement membrane is initially damaged by an unknown agent(s) including antibody undetectable by our techniques. The damaged basement membrane binds properdin and other factors including C3PA. C3 activator is generated with subsequent activation of the complement sequence beginning at C3. During this process a biologically active fragment (HG factor) is generated and released into the fluid phase. The HG factor has affinity for normal skin basement membrane together with the capability of activating the third component of complement. Once having generated the HG factor, properdin and C3PA may not be necessary for continued activation of this alternate pathway. Currently, we are investigating these possibilities and attempting to isolate and characterize the factor(s) responsible for the complement activation in this disease.

The finding of properdin staining on the skin basement membrane in systemic lupus erythematosus is not surprising in view of the report by Westberg et al. (9) of properdin deposition along the glomerular basement membrane and mesangium of 3 of 13 patients with systemic lupus erythematosus. They described the properdin staining as being faint (1+) while the C3 staining in the corresponding areas was heavy (3+). They also noted there was a deposition of properdin along the basement membrane in the absence of C3. The properdin staining in our lupus patients has so far been accompanied in all cases by heavy C3 staining. However, we have found a number of cases of C3 deposition in the absence of properdin.

Properdin and C3 staining of the glomerular mesangium was found in one of the five SLE patients with skin deposits. This was accompanied by glomerular basement membrane and mesangial deposition of IgG, C1q, and C5. Renal biopsies were not available on the other patients. Recently Rothfield, Ross, Menta, and Lepow (25) have also observed properdin staining of the skin basement membrane and glomeruli in three patients with systemic lupus erythematosus.

Since skin basement membrane deposition of properdin and C3PA in the lupus patients was always accompanied by the presence of immunoglobulins and early and late complement components, we suspect that both the classical activation mechanisms as well as the C3 shunt are operative in this disease.

We were unable to correlate the deposition of properdin and C3PA with any clinical feature of lupus erythematosus or with the effects of therapy. One patient, P. P., still displayed properdin and C3PA deposition after 3 mo of daily doses of 30–40 mg of prednisone.

The six patients with classic bullous pemphigoid had no unusual features of their disease. All six patients showed properdin and C3 proactivator deposition on the skin basement membrane. These findings were associated with positive fluorescent staining for IgG, C1q, and/or C4 plus C3 and C5. This combination of staining patterns suggest that both the classical as well as the alternate pathway may be operative in patients with bullous pemphigoid.

Sera obtained from these patients and stored at −70°C demonstrated on immunoelectrophoresis against anti-human C3PA, the β-migrating C3 proactivator. There was no evidence of the generation of the γ-migrating C3 activator (C3A). Fresh blister fluid from

three of these bullous pemphigoid patients, stored at −70° until used, however, demonstrated on immunoelectrophoresis the presence of the γ-migrating C3A as well as the β-migrating C3PA. Jordon, Day, Sams, and Good (26) have recently demonstrated low individual complement component levels in the blister fluid of bullous pemphigoid patients compared to normal serum complement values. In addition, immunoelectrophoresis of these blister fluids against anti-C3PA demonstrated the presence of the γ-migrating C3A. Controls consisting of blister fluids formed by the application of cantharidin and suction had normal complement component levels with no evidence of C3A generation. These results suggest that complement activation in bullous pemphigoid is occurring locally at the site of blister formation.

Patients with progressive membranoproliferative glomerulonephritis and acute poststreptococcal glomerulonephritis diseases in which the alternate pathway (C3 shunt) may be operative, have decreased total complement and C3 levels (27, 28). Patient F. L. with herpes gestationis had normal complement levels (CH50 and C3) before and during therapy. Patient V. G. had normal C3 levels while on steroid therapy and as the disease was remitting. We have thus far been unable to document a hypocomplementemic phase in either herpes gestationis or bullous pemphigoid. However, more frequent determinations during the height of the diseases and prior to therapy are necessary to rule out a hypocomplementemic phase and metabolic studies involving C3 synthetic and catabolic rates are required to exclude increased utilization of C3. Serum levels of properdin which are low in about one-half of the cases of hypocomplementemic nephritis were not measured in any of our patients (29).

A C3 nephritic factor has been described in progressive membranoproliferative glomerulonephritis (30, 31). This factor reacts with a normal serum component in the presence of magnesium to form a C3 lytic factor termed C3 lytic nephritic factor (C3 Ly Nef). This factor enzymatically cleaves C3 to C3a + C3c + C3d. The relationship of this C3 nephritic factor to the HG factor is unknown but this possible relationship is being investigated at the present time.

In another study to be published,[2] we have demonstrated the presence of IgA, C3, C3PA, and properdin along the skin basement membrane of some patients with dermatitis herpetiformis.

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