The Immunopathology of Herpes Gestationis

IMMUNOFLUORESCENCE STUDIES AND CHARACTERIZATION OF "HG FACTOR"

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ABSTRACT Nine skin biopsies from seven herpes gestationis patients were studied by immunofluorescence (IF) techniques. Basement membrane zone (BMZ) deposition of C3 and properdin was present in all nine skin specimens, while IgG deposition was apparent in only one. With in vitro C3 IF staining, positive BMZ staining (HG factor activity) was noted with all seven of our patients' serum samples tested. By standard indirect IF staining, however, only one of these serum samples contained BMZ antibodies of the IgG type. Two cord serum samples, tested by these same methods, yielded positive in vitro C3 staining (HG factor activity) but negative indirect IF staining (IgG). HG factor activity was found to be stable at 56°C for 30 min and in two of three specimens at 56°C for 1 h. Treatment of the complement source (normal human serum) used in the in vitro C3 staining assay with Mg_2-EGTA or use of C2-deficient serum as the complement source inhibited HG factor activity. HG factor blocked the specific staining of the BMZ of normal human skin by labeled bullous pemphigoid antibodies. By sucrose density gradient ultracentrifugation and gel chromatography (Sephadex G-200), HG factor activity eluted with IgG-containing fractions. The highly purified IgG fraction of two herpes gestationis sera was also positive for HG factor activity. Our studies suggest that HG factor is an IgG antibody that may not be demonstrable by conventional IF methods, but which activates the classical complement pathway.

INTRODUCTION

Herpes gestationis is an interesting and uncommon vesiculobullous dermatosis of pregnancy and the postpartum period. Characterized histopathologically by subepidermal bulla formation, this disease has long been confused with other subepidermal blistering diseases, including dermatitis herpetiformis, erythema multiforme, and bullous pemphigoid. The etiology of herpes gestationis remains unknown.

Using immunofluorescence (IF)[1] techniques, Provost and Tomasi (1) first delineated immunopathologic findings in two cases of herpes gestationis. Basement membrane zone (BMZ) deposition of C3 and properdin was noted in a skin lesion of one of these patients, but in the absence of immunoglobulins (IgG, IgA, IgM, and IgE) and early complement components (Clq and C4). In addition, a heat-labile factor, called "HG factor," was identified in one herpes gestationis serum. This factor would bind C3 to normal skin BMZ but without demonstrable binding of immunoglobulins. They concluded that the alternative or properdin pathway might be involved in such a deposition of C3 (1).

A patient with herpes gestationis who had immunologic findings similar to bullous pemphigoid, however, has recently been reported (2). This patient had circulating antibodies to the BMZ (IgG type) and deposi-

tion of IgG and C1q in skin lesions, in addition to properdin and C3. The authors further suggested that herpes gestationis might be more closely related, immunopathogenetically, to bullous pemphigoid than to other subepidermal blistering diseases (2). Other herpes gestationis patients who demonstrate IgG deposition in skin lesions but who lack circulating BMZ antibodies, like some bullous pemphigoid patients, have also recently been described (3, 4).

Using various IF techniques, we wish to report our observations in six cases of herpes gestationis in addition to our previously reported case (2). IF studies of cord serum samples from two of our patients and our attempts to further characterize the so-called HG factor as IgG, are also presented.

METHODS

Specimens. Blood samples were collected under sterile conditions from patients with active disease, separated, aliquoted, and stored at − 70°C until used. All of our patients presented with active blistering lesions characterized histopathologically by subepidermal bulla formation consistent with herpes gestationis. Two cord blood samples, obtained at the time of delivery, were handled similarly. Skin lesions, obtained by punch biopsy, were quick-frozen in liquid nitrogen and were stored at − 70°C until used. Normal human skin, obtained from surgical specimens, served as an antigen source for both indirect and in vitro complement IF staining assays. All sections, both lesions and normal human skin, were cut in a cryostat at − 30°C and were used unfixed in the various IF staining procedures.

Electrophoresis and immunodiffusion. Immunoelectrophoresis (IEP) was performed according to Scheidegger (5) in 25 mM Veronal buffer in 2% agarose at 10 V/cm for 2–3 h. IEP for components of the complement system were run similarly but with the addition of 10 mM EDTA to the buffer. Slides were developed at 37°C and read after 24 and 48 h.

Polyacrylamide gel disc electrophoresis was performed according to Ornstein (6) and Davis (7) in 5% acrylamide gels, pH 9.5. Gels were run in duplicate, and approximately 100 μg protein, as determined by the Hartree modification (8) of the Lowry et al. method (9) against solutions of known concentrations of human gamma globulin as standards, were applied to each gel. After electrophoresis, one of the gels was fixed in 12% trichloroacetic acid and stained with Coomassie blue, the other sliced longitudinally and analyzed by immunodiffusion in 1% agarose.

Single radial immunodiffusion was carried out according to Mancini et al. (10) in Veronal-buffered saline: 1.5% agarose, 10 mM Veronal, 100 mM NaCl, pH 7.5. Plates were developed at 37°C and read after 48 h.

Double radial immunodiffusion (Ouchterlony) was carried out in 2% agarose in Veronal-buffered saline, pH 7.5. Plates were developed at 37°C and read after 24 and 48 h.

Antisera. Antisera to human IgG,[3] C1q,[3] C4,[3] and to normal human serum[2] and fluorescein isothiocyanate (FITC)-labeled antisera to human IgM,[2] IgA,[2] and C3,[2] as well

as FITC-labeled goat anti-rabbit[3] and rabbit anti-goat[3] IgG antisera were purchased.

Antiserum to human IgG was prepared, assayed, and conjugated with FITC by methods previously reviewed in detail (11, 12). FITC-labeled antiserum to human IgE was a gift from Dr. Gerald Gleich of the Mayo Clinic.

Rabbit antiserum to Factor B was prepared and assayed by the method of Götze and Müller-Eberhard (13). When this antiserum was tested by IEP, addition of inulin to normal human serum produced a gamma-migrating arc in addition to the single beta-migrating protein arc seen in normal human serum alone. Antiserum to human properdin was made in a goat with properdin isolated and assayed by the method of Pensky et al. (14). This antiserum demonstrated a single cathodally migrating precipitin arc against purified properdin with IEP, similar to the pattern described by McLean and Michael (15) for aged serum. RP reagent also was prepared by the method of Pensky et al. (14), but was absorbed twice more with zymosan[4] so that the RP reagent was completely free of properdin. Antiproperdin antiserum was absorbed with RP to ensure its specificity. Details of the preparation and use of the anti-Factor B and antiproperdin have been published previously in detail (16).

All antisera were tested for specificity and activity by double immunodiffusion (Ouchterlony) and IEP. Units of antiserum, antibody protein assays, fluorescein-protein ratios, and dilutions at which these conjugated antisera were used conformed to our previous standards (11, 12).

The globulin fraction of one bullous pemphigoid serum with an anti-BMZ antibody titer of 1: 1280 by indirect IF staining was obtained by precipitation in 33% saturated $(NH_4)_2SO_4$ and labeled with FITC to a molar fluorescein-protein ratio of 2.96 according to Beutner et al. (11).

Staining procedures. Direct IF staining of herpes gestationis skin lesions with antisera to IgG, IgA, IgM, IgE, and C3 was carried out by established methods (11, 12). A modified indirect IF method recently outlined (1, 16) was used to test these same lesions for the presence of C1q, Factor B, and properdin. In this procedure, skin sections treated initially with rabbit anti-C1q (diluted 1: 100) and rabbit anti-Factor B (diluted 1: 80) were followed by labeled goat anti-rabbit IgG. Similarly, when goat anti-properdin (diluted 1: 10) was used, labeled rabbit anti-goat IgG was used in the second step of the procedure. Specificity controls for this modified indirect IF test have also recently been reported (16).

Standard indirect IF staining, used to test herpes gestationis serum samples and cord serum samples for the presence of BMZ antibodies, was performed as outlined before (11, 12). Labeled antisera to IgA, IgM, and IgE, however, were employed in addition to IgG.

In vitro complement IF staining, used to test these same samples for the presence of complement-fixing activity (HG factor), was carried out by previously summarized methods (17, 18). For in vitro C3 staining, normal skin sections were first treated with dilutions of sera or cord sera (1:2, 1:4, 1:8, etc.) plus human complement (fresh normal human serum), followed by labeled anti-C3. In vitro staining for C1q and C4 was performed by a three-step procedure instead of the two-step procedure used for C3 staining. Briefly, normal human skin sections were treated initially with serum or cord serum dilutions with normal human comple-

ment added. The tissues were then treated with unlabeled rabbit anti-C1q (diluted 1:100) or unlabeled rabbit anti-C4 (diluted 1:80). As the third and final step of this procedure, labeled goat anti-rabbit IgG was used. Experimental controls for in vitro C1q, C4, and C3 IF staining were identical to those outlined previously in detail (17, 18).

Two additional controls, however, the substitution of C2-deficient serum for the normal human serum used as the source of complement and the addition of 15 mM Mg_2-ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (Mg_2-EGTA) [5] to the complement source, were added. The C2-deficient serum, a gift from Dr. John Leddy, Rochester, N. Y., was tested alone and after the addition of inulin by IEP with antisera to C3 and Factor B. Conversion of C3 and Factor B was apparent only after the addition of inulin. No hemolytic C2 activity was apparent in this serum.

Blocking of BMZ staining with a FITC-labeled bullous pemphigoid serum was done as follows: Labeled bullous pemphigoid serum was titered on normal human skin and gave a titer of 1:50. It was then used at this dilution. Normal human skin was incubated three times for 30 min with the IgG fraction from herpes gestationis sera, washed between each incubation for 10 min in phosphate-buffered saline, pH 7.2, and then stained for 30 min with labeled bullous pemphigoid serum. Phosphate-buffered saline, normal human serum, and unlabeled bullous pemphigoid serum were substituted for the herpes gestationis serum fraction as controls.

Sucrose density gradient studies. The globulin fraction of one herpes gestationis serum (case 4, Table II) was also prepared by $(NH_4)_2SO_4$ precipitation, layered on a 5–30% sucrose gradient, and centrifuged at 40,000 rpm (270,-000 g at the tip) for 16 h at 4°C in a SB 283 rotor in a B-60 International ultracentrifuge.[6] The sample was run in duplicate. The gradient tubes were punctured after centrifugation, and 18 fractions (approximately 0.65 ml each) were collected. Each fraction was assayed for IgM and IgG activity by radial immunodiffusion (Mancini) and HG factor activity by the in vitro C3-staining method.

Column chromatography. The same herpes gestationis globulin fraction was also passed over a column of superfine Sephadex G-200 [7] (1.5 × 50 cm, downward flow, phosphate-buffered saline, pH 7.5) and again assayed as above. 2 ml of whole serum from two patients with herpes gestationis were dialyzed against 0.01 M TRIS-HCl buffer, pH 8, and passed over a DEAE-Sephadex A50 [7] column (1.5 × 30 cm), equilibrated with the same buffer. Effluents of all columns were monitored for protein by measuring their OD_{280}. The first peak emerging from the DEAE column containing highly purified IgG was pooled and concentrated to half the original serum volume by ultrafiltration with a PM 30 membrane.[8] Purity of the IgG obtained was checked by IEP and polyacrylamide gel disc electrophoresis. This fraction was again tested for IgG, IgM, and in vitro C3-staining activity, as described above.

RESULTS

Skin lesions. Direct IF and modified indirect IF studies of herpes gestationis skin lesions are summarized

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[5] Sigma Chemical Co., St. Louis, Mo.
[6] Damon/IEC Div., Damon Corp., Needham, Mass.
[7] Pharmacia Fine Chemicals, Piscataway, N. J.
[8] Amicon Corp., Scientific Sys. Div., Lexington, Mass.

in Table I. A total of nine skin biopsies was studied from our seven patients. In only one of these skin lesions (the second skin biopsy from case 1) was BMZ deposition of IgG noted, findings reported previously (2). No immunoglobulins (IgG, IgM, IgA or IgE) were noted to be bound to the BMZ in any of the other skin lesions tested. Only cases 5 through 7 were tested with labeled antiserum to IgE, however.

C3 and properdin deposition, on the other hand, were demonstrable in all nine skin lesion specimens (Fig. 1), but as noted in Table I and as seen in Fig. 1, properdin staining was always less intense than C3 staining. These observations, therefore, are similar to those reported previously by Provost and Tomasi (1). Factor B deposition, on the other hand, was not detectable in any of our lesional specimens. C1q deposition was noted in three of the immunoglobulin negative lesions in addition to case 1.

Sera. Table II summarizes our studies of serum samples of herpes gestationis patients by indirect IF and in vitro complement IF staining methods. Only one patient (case 1) had circulating BMZ antibodies (IgG type) similar to those observed in patients with bullous pemphigoid (11, 12). As reported previously (2), the titer of these antibodies was 320. No other herpes gestationis sera tested, however, have demonstrated similar BMZ antibodies.

All of the patients' serum samples were tested by indirect IF staining with labeled antisera to IgA, IgM, and IgE, in addition to IgG. None of the sera tested demonstrated positive BMZ staining reactions with labeled antisera to these other immunoglobulins (Table II).

By in vitro C3 IF staining, however, very intense BMZ staining reactions were noted with all of the serum samples tested. But positive reactions were detectable only at low dilutions of patients' sera (1:2, 1:4, etc.) and, except for case 1, were not detectable beyond a 1:20 dilution. The strongest C3 staining reactions occurred at 1:2 or 1:4 dilutions.

Again, as in true bullous pemphigoid (18), positive in vitro C1q and C4 BMZ staining reactions were apparent when serum from case 1, with circulating BMZ antibodies, was used. Similar C1q staining of the BMZ was not noted when serum samples of our other patients were similarly tested. In addition to case 1, a positive C4 staining reaction was noted with the serum of one additional case (case 4), but only at a 1:2 dilution. The intensity of this C4 staining reaction, however, was much less than its C3 staining reaction. None of the other serum samples tested yielded similar positive C4 staining reactions (Table II).

Cord sera. Cord serum samples from two of our patients were also tested by the various indirect IF and

TABLE I
Summary of (IF) Studies of Skin Lesions from Herpes Gestationis Patients (BMZ Staining)

Case no.	Specific antisera							
	IgG	IgA	IgM	IgE	C1q*	C3	Factor B*	Properdin*
1‡ (a)	−	−	−	NT§	−	+ + +	−	+ +
(b)	+	−	−	NT	+	+ + +	−	+
2	−	−	−	NT	−	+ + +	−	+
3	−	−	−	NT	+	+ +	−	+
4 (a)	−	−	−	NT	+	+ + +	−	+ +
(b)	−	−	−	NT	−	+ + +	−	+ +
5	−	−	−	−	−	+ +	−	+
6	−	−	−	−	+	+ +	−	+
7	−	−	−	−	−	+ +	−	+

* Modified indirect IF staining used.
‡ This case was reported previously (2).
§ Not tested.

in vitro complement IF staining methods (Table III). Both cord serum samples yielded negative indirect IF reactions with labeled antisera to IgG, IgA, IgM, and IgE. Low dilution (1:2 and 1:4) C3 staining activity, however, was apparent with both samples, as with serum samples. Negative C1q and C4 staining reactions were also apparent with both of the samples tested.

Nature of HG factor. To check the specificity of the observed C3 staining reactions further, selected control tests, including blocking experiments with EDTA, Mg₂-EGTA, heat inactivation (56°C for 30 min and 56°C for 1 h), and substitution of C2-deficient serum for fresh normal human serum as a complement source, were performed (Table IV). As in previous studies with bullous
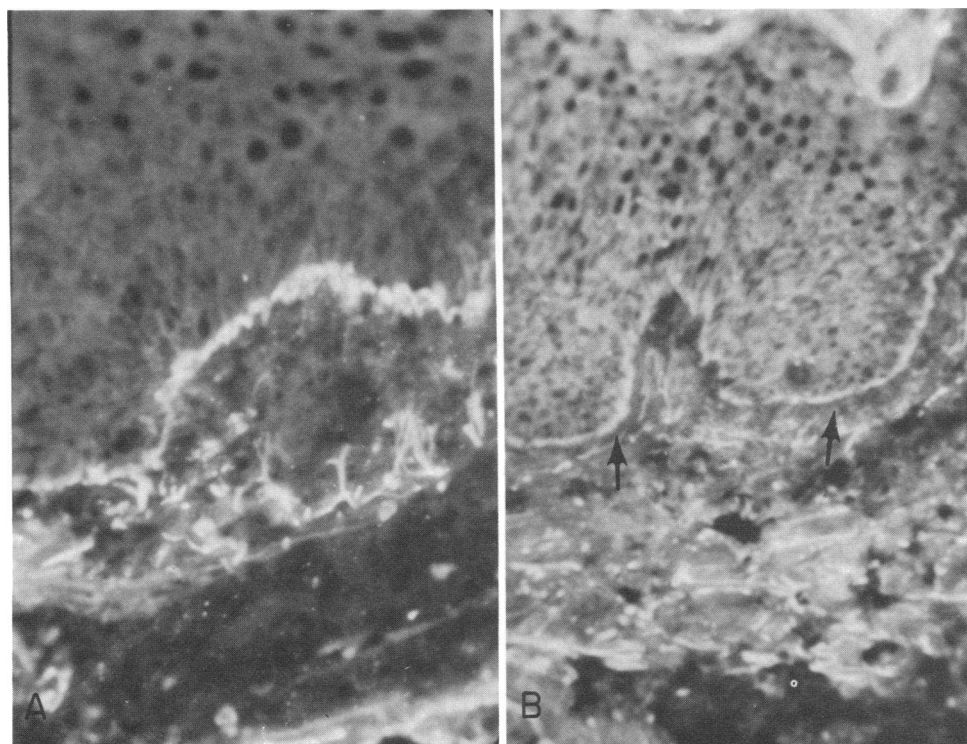

FIGURE 1 IF staining of skin lesions of patients with herpes gestationis, demonstrating BMZ deposition. A. C3 deposition (× 250). B. Properdin (arrows) deposition (× 250).

TABLE II

Summary of Indirect and In Vitro Complement Studies of Herpes Gestationis Sera (Basement Membrane Staining)

Case no.	Specific antisera						
	IgG	IgA	IgM	IgE	C3	C1q	C4
1*	+ +	−	−	−	+ + +	+	+ + +
2	−	−	−	−	+ + +	−	−
3	−	−	−	−	+ +	−	−
4	−	−	−	−	+ + +	−	+
5	−	−	−	−	+ +	−	−
6	−	−	−	−	+ + +	−	−
7	−	−	−	−	+ +	−	−

* This case was reported previously (2).

pemphigoid serum samples (17, 18), all routine controls (antisera alone, complement plus antiserum, and normal human serum plus complement plus antiserum) yielded negative staining reactions. Bullous pemphigoid serum, used as a positive control, yielded positive BMZ staining reactions with complement and labeled anti-C3.

Controls performed with three herpes gestationis sera to characterize HG factor activity further are listed in Table IV. As expected, all three sera yielded positive BMZ reactions (at 1:2 dilutions) with complement and labeled anti-C3. When the HG sera were heated at 56°C for 30 min and fresh complement was added, the staining was not abolished but the intensity was reduced. Further heating at 56°C for 1 h abolished the staining in serum from case 6, but not cases 1 and 4. As expected, heat inactivation (56°C for 30 min) of both HG sera and the complement source resulted in inhibition of C3 staining.

Addition of EDTA to the HG sera, however, again resulted in blocking of BMZ staining in cases 4 and 6, whereas only the intensity of staining was reduced in case 1. Addition of EDTA to the complement source, with or without adding EDTA to the HG sera, also resulted in inhibition of BMZ staining. Addition of Mg-EGTA to the complement source also inhibited the positive staining.

Finally, if C2-deficient serum is used as the complement source instead of fresh normal human serum, complete inhibition of the C3 staining was apparent with all three serum samples (Fig. 2B). This finding, along with the Mg-EGTA studies, suggests that HG factor must be activating complement via the classical pathway (i.e., via C1, C4, and C2).

In an attempt to delineate the site of deposition of HG factor in the skin, studies with labeled bullous pemphigoid antibodies were performed. Repeated treatment of human skin sections with HG factor almost completely inhibited the staining of the BMZ of skin with labeled bullous pemphigoid antibodies. Similar

treatment of human skin sections with normal human serum did not result in inhibition of BMZ staining with this reagent.

Sucrose density gradient and chromatographic studies. The globulin fraction of serum from case 4 (Table II) was subjected to sucrose density-gradient ultracentrifugation and to gel chromatography on Sephadex G-200. By the Mancini technique, IgM was located in early sucrose gradient fractions (fractions 2 and 3), while IgG eluted in later fractions (8 through 12). Hg factor activity eluted in these same later fractions (fractions 9–11). This suggests a molecular weight for HG factor similar to that of IgG.

Comparable results were also obtained with this serum on gel filtration. Hg factor activity was found again in the fractions containing IgG after separation on Sephadex G-200. IgG was obtained in highly purified form from the sera of cases 4 and 7 by chromatography on DEAE-Sephadex. By IEP and polyacrylamide gel disc electrophoresis, this IgG, the first peak emerging from the DEAE column, did not contain demonstrable amounts of other proteins. HG factor activity was present in the IgG fractions from these two sera (Fig. 2A). One of the positive DEAE-Sephadex fractions did yield a positive BMZ staining reaction with antiserum to IgG, after concentration to half its original volume. But this finding was not consistently reproducible.

DISCUSSION

By IF criteria alone, our previously reported case (case 1 in this report) would appear to have bullous pemphigoid rather than herpes gestationis. BMZ antibodies were present in her serum and IgG and C1q deposition was apparent in her skin lesion, in addition to C3 and properdin. These findings, therefore, could be compatible with a diagnosis of bullous pemphigoid (1, 12, 16). In addition, her circulating BMZ antibodies will fix C1q and C4 (classical pathway) in addition to C3, as do the BMZ antibodies present in most bullous pemphigoid sera (18).

Although all of the skin lesions studied had both C3 and properdin deposition, IgG deposition was conspicu-

TABLE III

Summary of Indirect and In Vitro Complement Studies of Herpes Gestationis Cord Sera (BMZ Staining)

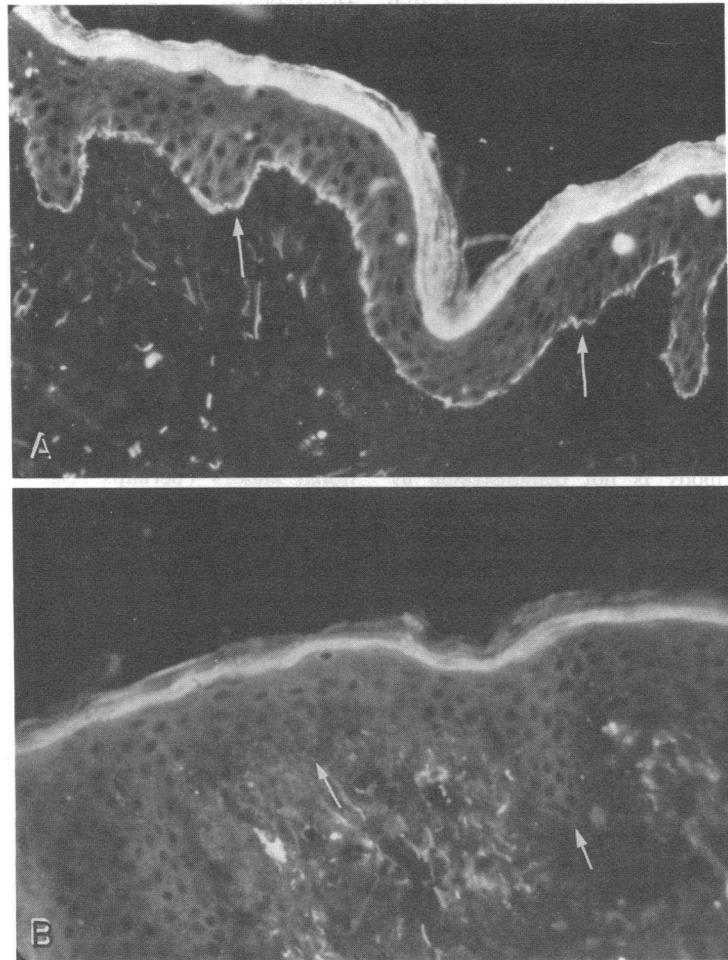| Case no.*	Specific antisera						
	IgG	IgA	IgM	IgE	C3	C1q	C4
4	−	−	−	−	+ +	−	−
6	−	−	−	−	+	−	−

* Case numbers refer to those listed in Tables I and II.

FIGURE 2 In vitro complement (C3) staining of normal human skin with herpes gestationis serum. A. DEAE-Sephadex-purified HG factor (× 250). Positive BMZ staining (arrows) is evident. B. Substitution of C2-deficient serum for normal human serum as the complement source has inhibited positive in vitro C3 staining (arrows) with HG factor (× 250).

TABLE IV

In Vitro C3 Staining Reactions with Herpes Gestationis Sera: Inhibition Studies Demonstrating the Nature of Action of HG Factor (BMZ Staining)

Serum	C treatment*	Case no.‡		
		1	4	6
1:2	C	+ + +	+ + +	+ + +
1:2 56°C for 30 min	C	+ +	+ +	+
1:2 56°C for 1 h	C	+ +	+	−
1:2 56°C for 30 min	C 56°C for 30 min	−	−	−
1:2 56°C for 30 min	C plus EDTA	−	−	−
1:2 56°C for 30 min	C plus Mg₂-EGTA	−	−	−
1:2 56°C for 30 min	C2-deficient serum	−	−	−

* C, complement (fresh normal human serum or C2-deficient serum).
‡ Case numbers refer to those listed in Tables I and II.

ously absent in the remaining six cases. C1q deposition was apparent in three specimens, in spite of the lack of IgG or IgM. Serum samples of these six cases, however, did not contain circulating BMZ antibodies to account for their C3-binding properties. In addition, most of these sera did not bind amounts of C1q and C4 sufficient to be demonstrable by in vitro complement IF staining methods. From our standard IF findings, then, one might conclude that the C3 binding in most herpes gestationis sera is independent of IgG, the early complement components (C1q and C4), and thus the classical complement pathway, as suggested originally by Provost and Tomasi (1).

Our more recent studies, however, suggest that HG factor may be an IgG type of BMZ antibody similar to those found in bullous pemphigoid. But, for as yet unknown reasons, this antibody is not demonstrable by conventional indirect IF staining.

The factor is more heat-stable than previously reported (1), especially at 56°C for 30 min. It crosses the placenta, as does IgG. We have demonstrated that HG factor activity is recovered in IgG-containing fractions after sucrose density gradient ultracentrifugation and Sephadex G-200 separation of HG serum. Further purification of IgG from HG serum on DEAE Sephadex also supports our contention that HG factor is an IgG antibody. In addition, purified HG factor will block specific BMZ staining of normal human skin sections by fluorescein-labeled bullous pemphigoid antibodies, suggesting at least a close anatomic relationship of the binding sites.

Preliminary studies recently presented by Yaoita et al. (19) support our contention that HG factor is IgG. By various absorption techniques, including affinity chromatography, removal of the IgG from their patient's serum resulted in inhibition of the positive C3 staining. They have also reported that HG factor is heat-stable. Like our six additional patients, their patient's serum does not contain IgG antibodies demonstrable by conventional IF techniques.

It also appears that HG factor activates the classical pathway of the complement system, rather than the alternative pathway as suggested earlier (1). Substitution of C2-deficient serum for fresh normal human serum as the complement source, or addition of Mg_2-EGTA to the complement source, resulted in complete inhibition of the positive in vitro C3-staining reactions. The early complement components, therefore, must be required for fixation of C3 by HG factor. In light of our IF studies, however, the amounts of C1q and C4 fixed to the BMZ by HG factor must again be below the limits of resolution of the IF techniques used.

If the above observations are correct and HG factor activates the classical complement pathway rather than the alternative pathway, the consistent finding of properdin in skin lesions must be clarified. Recent reports by Fearon and Austen (20) and Schreiber et al. (21), however, may help explain this finding. They have reported that C3b, generated by C3 activation, will bind properdin. Conceivably, then, with activation of C3 to C3b by HG factor via the classical pathway, properdin is binding to the BMZ because of the presence of C3b on the complex. This possibility, however, must be investigated further.

Although Hg factor appears to be an IgG antibody, but not demonstrable by IF techniques, its further characterization seems warranted. This antibody, however, may be present in serum but in amounts too low to detect. Perhaps the activity resides in one particular IgG subclass that fixes complement avidly. Possibly additional factors (perhaps related to pregnancy) may be required by HG factor to allow the enhancement of activation of C3. Such factors could be interfering with the C3b inactivator, which could explain the amplification of C3 activation. These and other possibilities will be the subject of future investigations. In any event, our studies would suggest that herpes gestationis is a form of bullous disease in pregnancy mediated by BMZ deposition of complement. Whether herpes gestationis is related to classical bullous pemphigoid, however, also remains to be determined.

ACKNOWLEDGMENTS

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