Complement Fixation by Pemphigus Antibody

V. Assembly of the Membrane Attack Complex on Cultured Human Keratinocytes

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Abstract

Previous studies have shown that pemphigus vulgaris (PV) IgG will fix early complement components (C1q, C4, and C3) to cultured murine epidermal cell surfaces and that PV IgG and complement alter epidermal cell membrane integrity. The present study was undertaken to determine if assembly of terminal complement components (C5, C6, C7, C8, and C9) and expression of C5b-9 neoantigens occur when PV IgG interacts with human keratinocyte (HuK) cell surface antigens in the presence of a source of complement. Monoclonal antibodies specific for C5, C6, C7, C8, C9, and C5b-9 neoantigens were screened for reactivity to the individual complement components in an assembled complex of human C5b-9 on rabbit red blood cell ghosts. Monoclonal antibodies (tissue culture supernatants) that bound to antigenic determinants accessible in the C5b-9 complex were selected for this study using immunofluorescence methods. HuK treated with PV IgG fixed C5, C6, C7, C8, C9, and C5b-9 neoantigens in a characteristic speckled pattern, while normal IgG did not. Heat inactivation or EDTA treatment of the complement source, or substitution of C2-depleted serum abolished C5, C6, C7, C8, C9, and C5b-9 neoantigen staining. PV IgG and complement also resulted in significant cytotoxicity to cell membranes as assessed using an ethidium bromide-fluorescein diacetate assay. These results suggest that PV IgG will activate the membrane attack complex of the complement system on HuK cell surfaces, resulting in cytotoxicity to cell membranes, further implicating complement in the pathogenesis of pemphigus.

Introduction

Pemphigus vulgaris (PV),[1] an autoimmune disease of the skin, is characterized clinically by flaccid weeping bullous lesions and histopathologically by intraepidermal blister formation and loss of cohesion of epidermal cells or acantholysis (1). Immunopathologically, this disease is characterized by the presence of serum autoantibodies that react with the intercellular substance areas of skin (2) or the surfaces of epidermal cells grown in tissue culture (3).

The mechanism by which epidermal cells detach in pemphigus (acantholysis) has received considerable investigative attention over the past several years. Some investigators have suggested that proteases such as activated plasmin might be the cause of this pathologic mechanism (4, 5). Our own investigations have focused on the role of complement in this process, since by direct immunofluorescence (IF) staining complement deposits are routinely found in early acantholytic lesions (6), and since evidence of complement activation is apparent in PV blister fluids (7).

An initial attempt by Jordon and co-workers (8) to demonstrate that pemphigus antibodies fix complement was unsuccessful. Later, Nishikawa et al. (9) and Hashimoto et al. (10), using in vitro complement IF staining, demonstrated that some pemphigus antibodies would fix C3 to skin intercellular substance areas. Kawana et al. (11) have confirmed and extended the above findings by showing that pemphigus antibodies will fix C1q, C4, and C3 to primate organ culture skin explants in vitro, and to tissue-cultured mouse keratinocytes. They further demonstrated that complement enhances pemphigus antibody-mediated murine epidermal cell detachment (12) and that pemphigus antibody and complement alter murine epidermal cell membrane integrity (13). Since PV antibodies fix early complement components, and since in the presence of complement they alter cell membranes, the present study was undertaken to determine if the terminal complement sequence, the "membrane attack complex," might be involved in this process. Carefully characterized monoclonal antibodies to C5, C6, C7, C8, C9, and C5b-9 neoantigens were used.

Methods

Tissue culture. Cultures contained human keratinocytes (HuK) that were obtained from a single normal newborn foreskin (stored at 4°C for < 24 h). Each foreskin was washed, trimmed of fat, cut into small pieces, and soaked in MCDB 153 medium (Irvine Scientific, Santa Ana, CA) containing 2.5 mg collagenase/ml (CooperBiomedical, Malvern, PA) for 90 min at 37°C. The epidermis was gently pulled free of the dermis and treated with 0.025% trypsin containing 0.01% EDTA to obtain a single-cell suspension. After 5 min 10% FCS was added to stop the digestion. The cell suspension was centrifuged at 1,000 rpm for 10 min and the cells were resuspended in MCDB 153 and counted. HuK were cultured in 25-cm flasks (Corning Glass Works, Corning, NY) in 5% CO_2 at 37°C. After 14 d the cells were removed with 0.025% trypsin containing 0.01% EDTA and plated on collagen-coated (Vitrogen 100; Flow Laboratories, Inc., McLean, VA) eight-well plastic tissue culture chamber slides (Miles Laboratories, Inc., Elkhart, IN) at 20,000 cells/

This paper was presented in part at the Annual Meeting of the Society for Investigative Dermatology, San Diego, CA, 4 May 1987.

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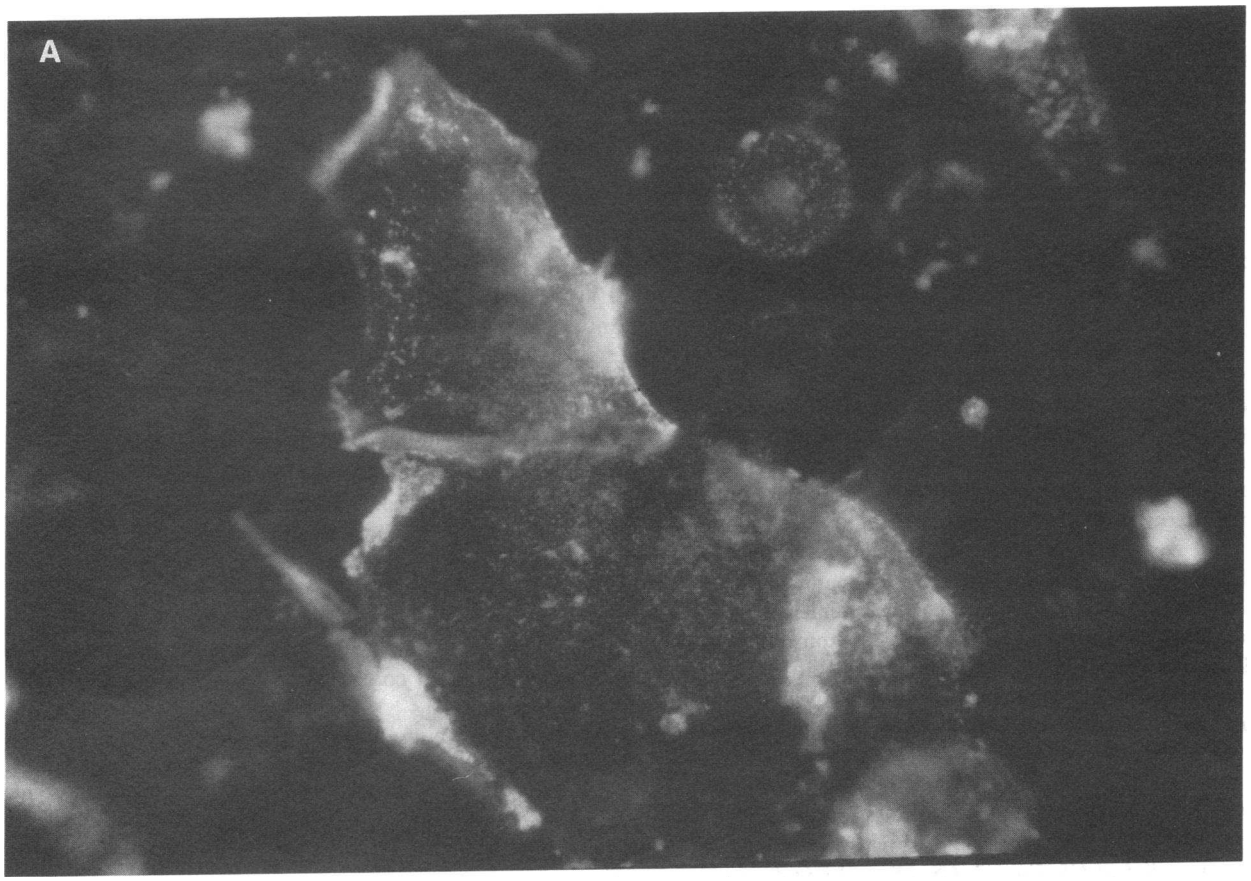
Received for publication 14 October 1987 and in revised form 20 May 1988.

1. *Abbreviations used in this paper:* EB, ethidium bromide; EIA, enzyme immunoassay; FDA, fluorescein diacetate; HuK, human keratinocytes; IF, immunofluorescence; NHS, normal human serum; PV, pemphigus vulgaris; RBC, red blood cell(s).

J. Clin. Invest.

0021-9738/88/12/1939/09 $2.00
Volume 82, December 1988, 1939–1947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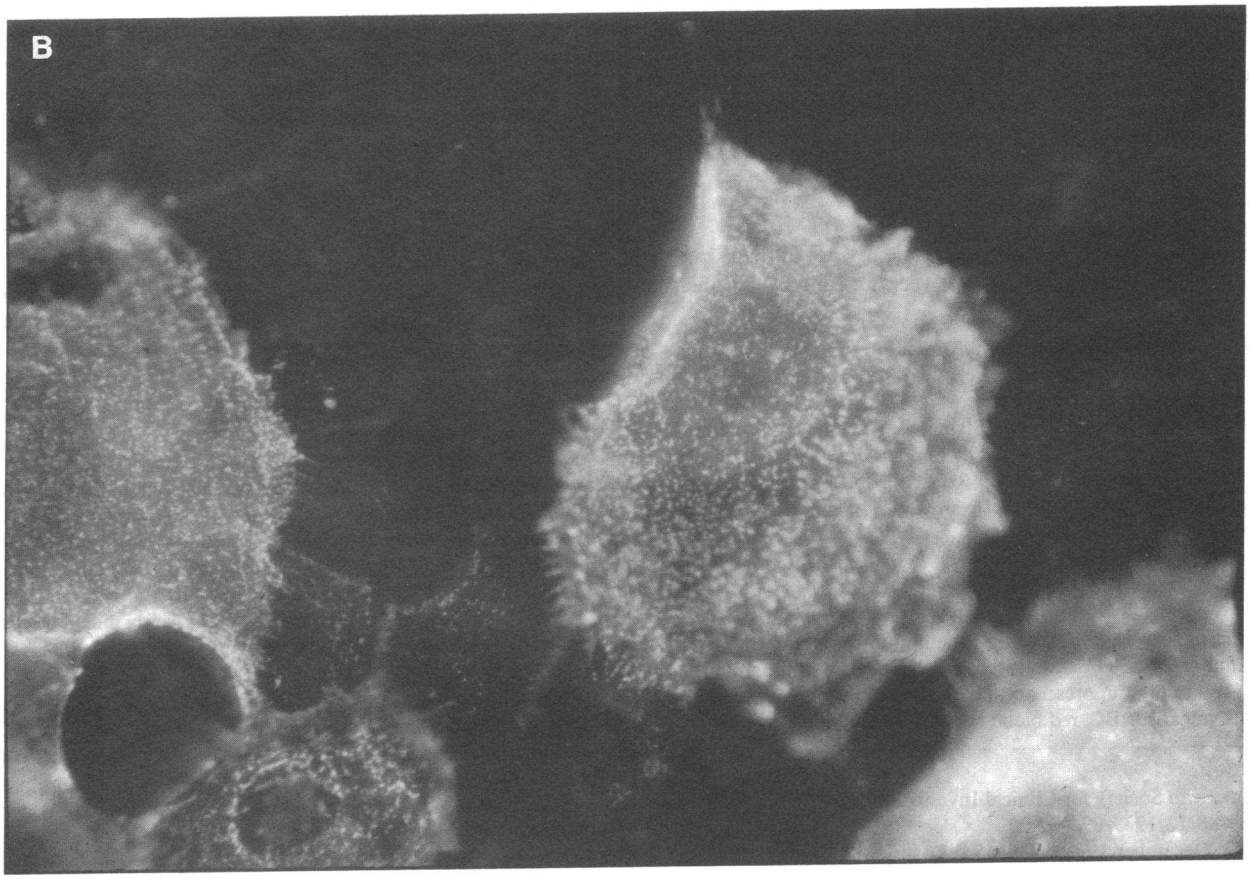
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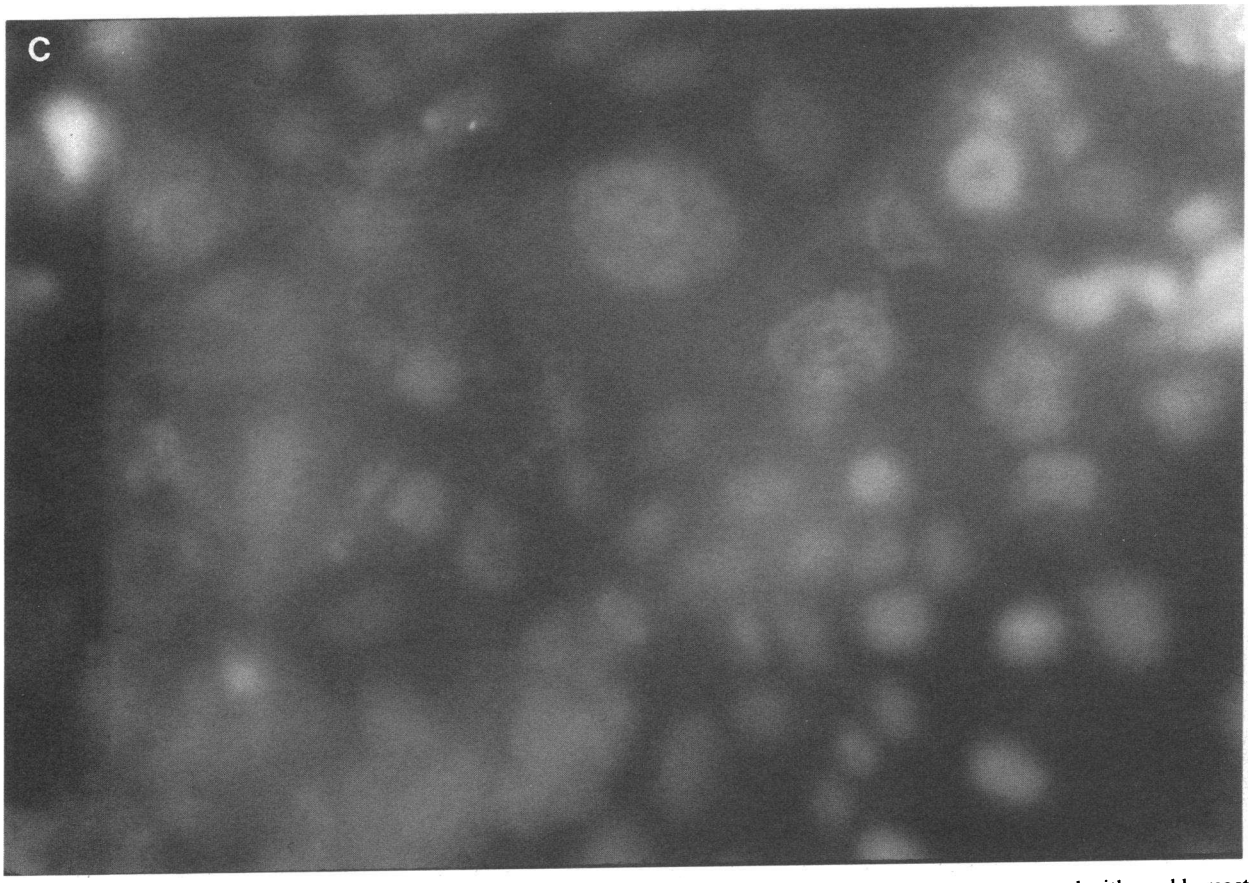


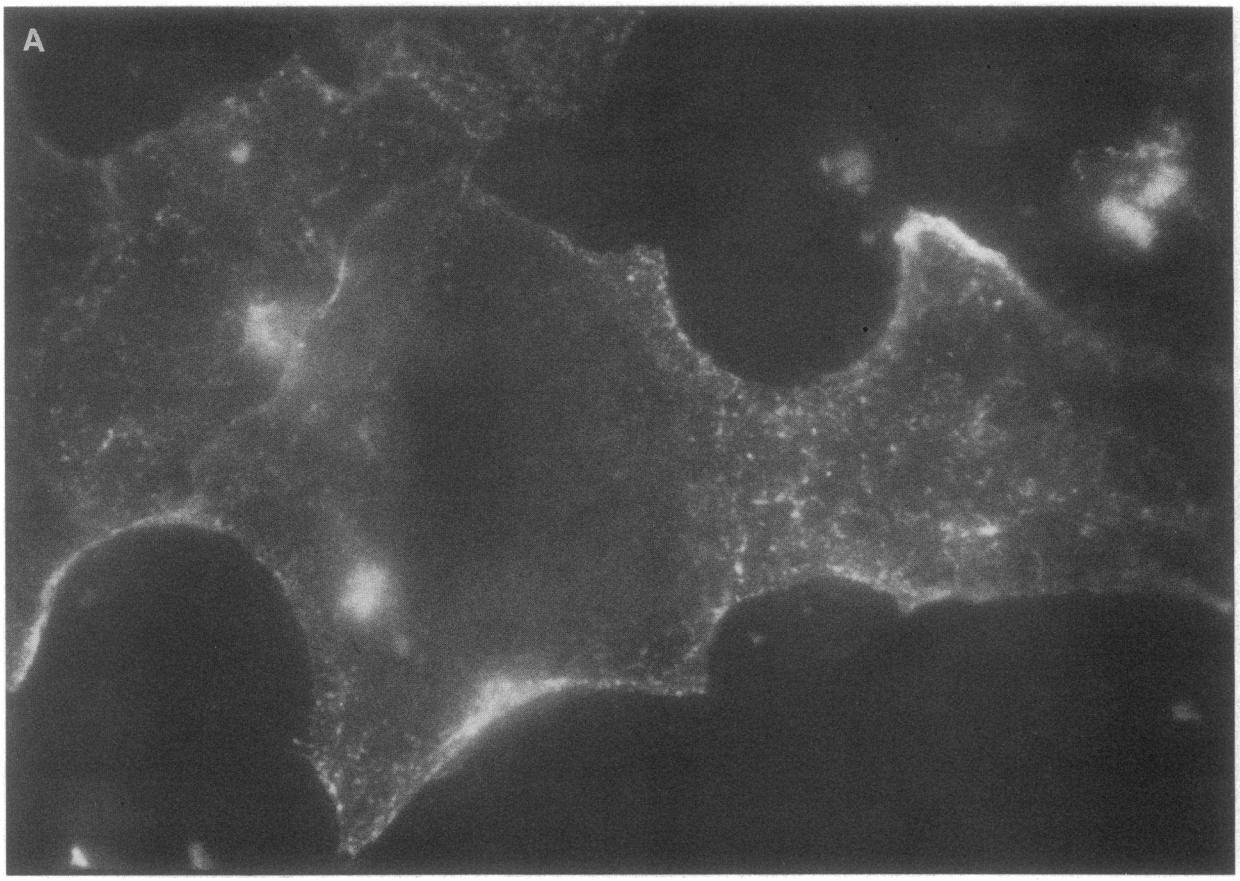
Figure 1. (*A*) Binding of FITC-labeled goat anti-human IgG-Fc to PV IgG-treated cells. Note weak or nonexistent staining pattern on smaller cells in the lower layer compared with the larger, flatter cells in the second layer. (*B*) Binding of FITC-labeled goat anti-mouse IgG-Fc to keratinocytes incubated in PV IgG plus complement treated with monoclonal anti-human C3c. Note heavily stained large keratinocyte in the upper layer compared with weakly reacting smaller cells in the lower layer. (*C*) Keratinocytes incubated in NHS IgG plus complement followed by monoclonal anti-human C3c and FITC-labeled goat anti-mouse IgG-Fc. Note general lack of staining of cell membranes. × 500.

well. The final vol/well was 0.5 ml. After 5 d the cells were fed with MCDB 153 containing 1.2 mM Ca^{++}. After being cultured in medium containing 1.2 mM Ca^{++} for 48 h, pemphigus antigen was expressed (14) and the HuK were used for experimentation.

Ig purification. Sera from three patients with PV were obtained by plasmapheresis. Two of the patients had serum pemphigus antibody titers of 1:640 while the other had titers of 1:160 as determined using a standard indirect IF staining assay (2). IgG was purified from normal and patient sera by precipitation at 50% saturation with ammonium sulphate followed by chromatography on DEAE (DE 52; Whatman Laboratory Products, Inc., Clifton, NJ) equilibrated with 0.01 M PBS, pH 8.0 (15). The unbound fraction consisting of IgG was dialyzed against 0.02 M PBS containing 0.25 M NaCl, pH 7.6, and then passed repeatedly over lysine-Sepharose 4B equilibrated with the same buffer to remove plasmin and plasminogen as previously described (15). The IgG preparations were tested for the presence of plasmin/plasminogen using a modification (15) of the caseinolytic assay described by Twinning (16). The IgG was then dialyzed against 0.01% ammonium bicarbonate, lyophilized, and stored at −20°C.

Complement sources. Fresh normal human serum (NHS), blood type AB, was used as a source of complement as previously described (11). This serum was absorbed with 3–4 × 10^6 HuK at 37°C for 3 h and overnight at 4°C with gentle rocking to remove natural antibodies to these cells. The AB serum was then centrifuged, sterile filtered, and stored at −70°C until used as previously described (11). C2-depleted normal serum (Cytotech, Inc., San Diego, CA) absorbed as described for NHS, NHS containing 10 mM EDTA, and heat-inactivated NHS at 56°C for 30 min were used as controls.

Antibodies. Murine monoclonal antibodies (tissue culture supernatants) to human complement components C5, C6, C7, C8, C9, and C5b-9 neoantigen were donated by Cytotech, Inc. These antibodies were derived from splenocytes removed from a BALB/c mouse immunized with highly purified human SC5b-9 (17, 18). The hybridoma supernatants were screened for their reactivity with immobilized SC5b-9 (19) using a modified enzyme immunoassay (EIA) (18). The positive supernatants were then screened by the same procedure for their reactivity to the individual purified components in their unassembled form. C5, C6, C7, C8, C9, and S protein were purified according to published procedures (20–25). We further screened all of the monoclonals for their ability to bind to individual complement components in the assembled human C5b-9 complex on rabbit RBC ghosts after a recommended procedure (Tamerius, J., personal communication). Briefly, rabbit RBC washed free of serum were incubated on poly-L-lysine–coated (48,000 mol wt; Sigma Chemical Co., St. Louis, MO) glass slides for 10 min at room temperature. After rinsing in 0.01 M PBS, 0.09% NaCl, pH 7.3, a drop of NHS (1:4 diluted with PBS) was placed on the slide and incubated for 8–10 min at 37°C (for controls, EDTA was used). This procedure resulted in the lysis of the rabbit RBC by the alternative pathway of human complement. The resulting rabbit RBC ghosts containing C5b-9 complexes remained bound to the poly-L-lysine-coated slides for use in IF staining. Each monoclonal was then incubated with the RBC ghosts. Reactive monoclonals were detected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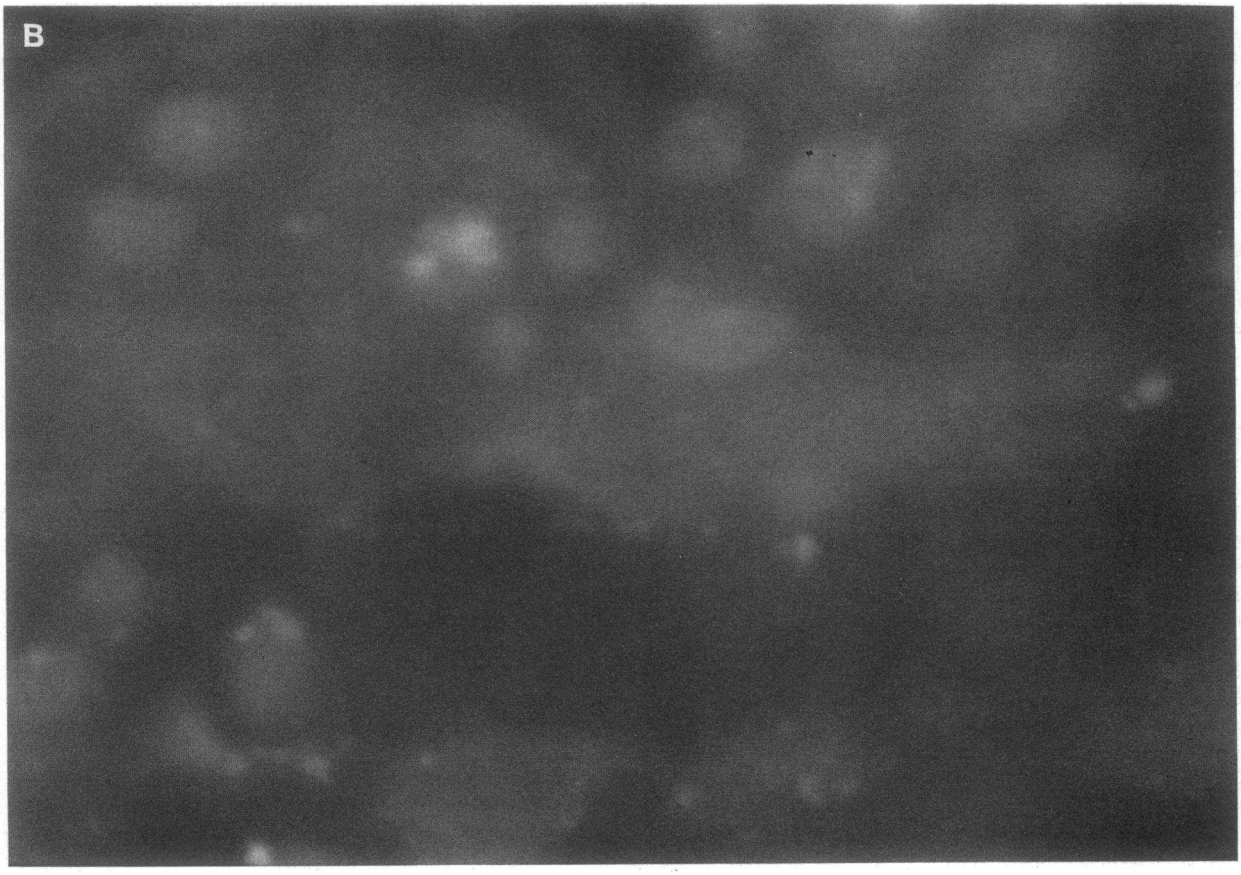
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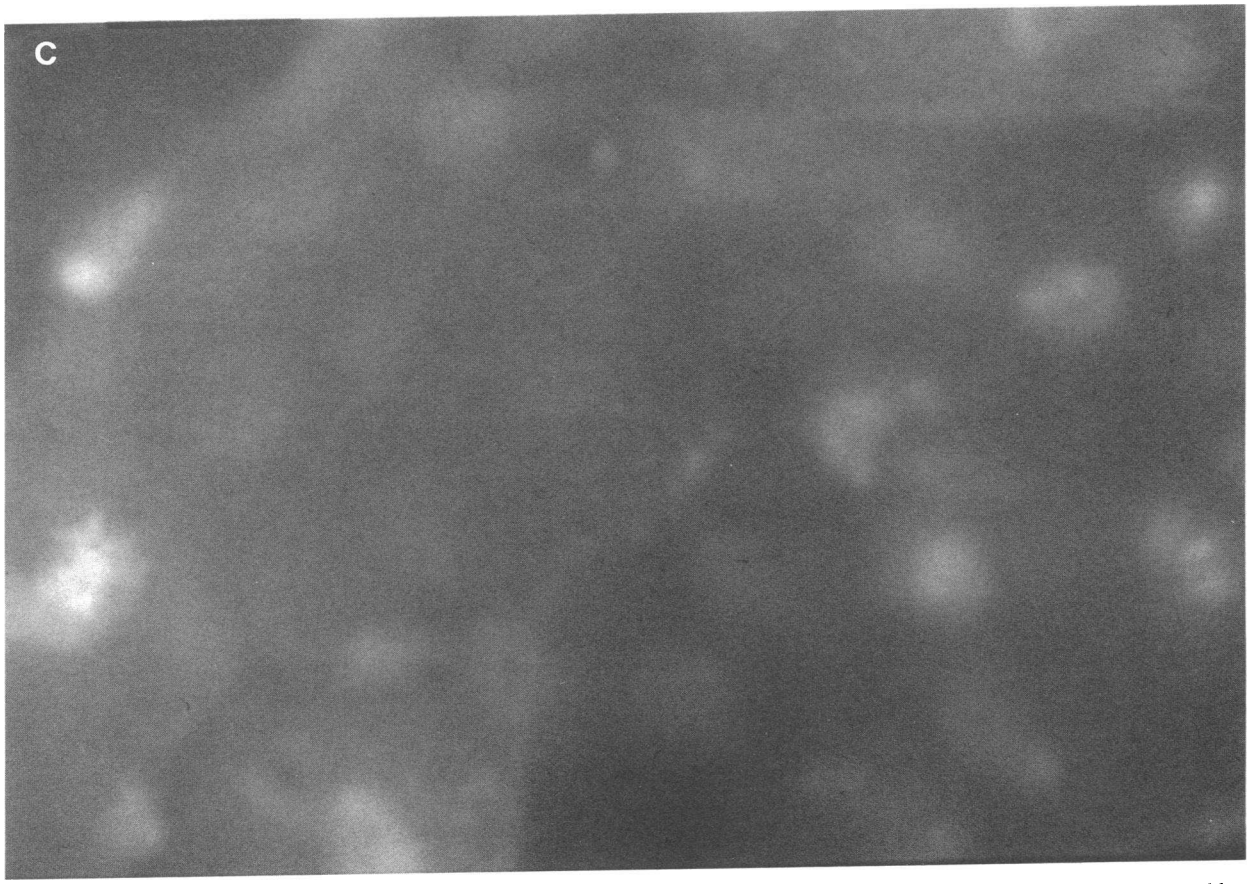


Figure 2. IF staining of complement C5 fixation by PV IgG. (*A*) PV IgG plus complement followed by monoclonal anti-human C5 and FITC-labeled goat anti-mouse IgG-Fc. Note weak fluorescence on smaller cells in lower layer and bright fluorescent pattern on larger, flatter cells. (*B*) PV IgG plus C2-depleted NHS followed by monoclonal anti-human C5 and FITC-labeled goat anti-mouse IgG-Fc. (*C*) PV IgG plus heat-inactivated AB NHS followed by monoclonal anti-human C5 and FITC-labeled goat anti-mouse IgG-Fc. × 500

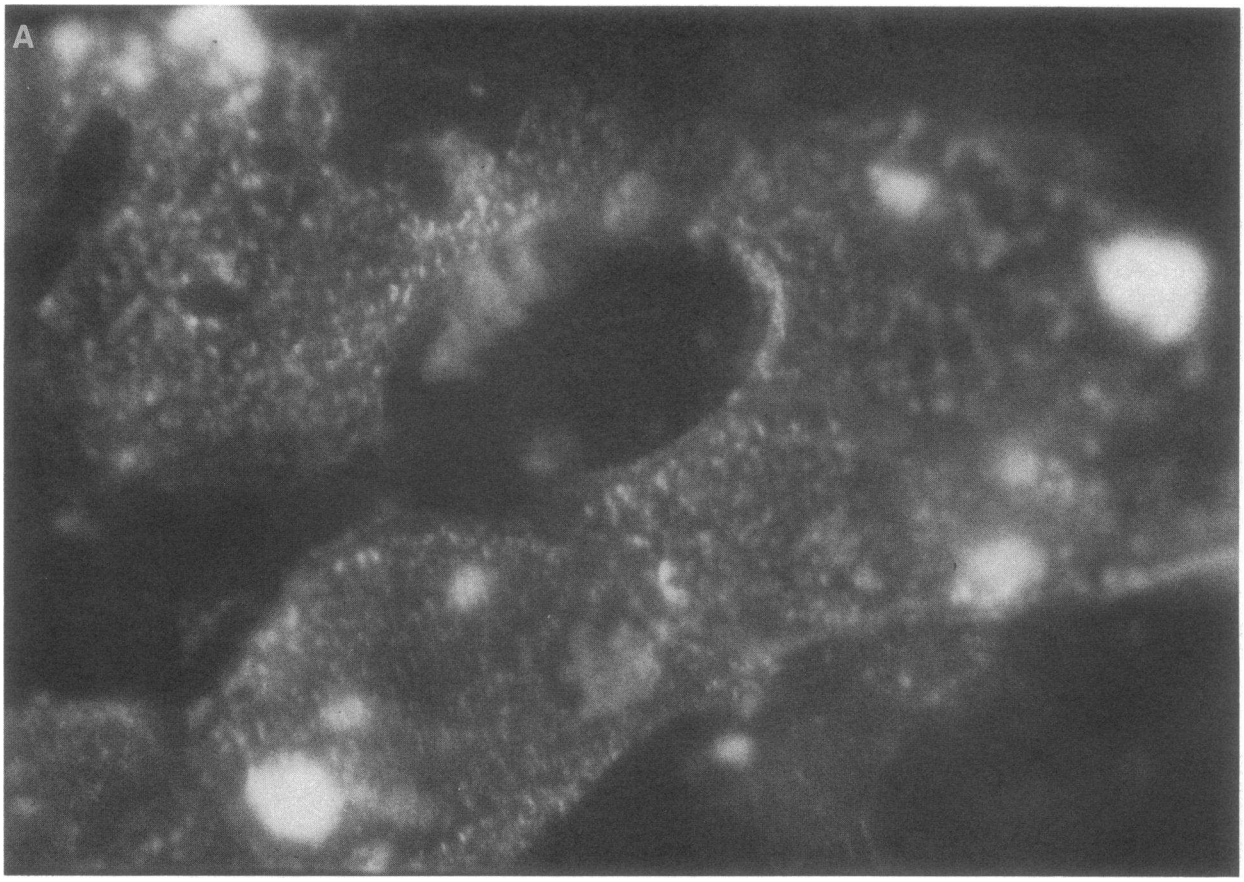
by using FITC-labeled goat anti-mouse IgG-Fc (Jackson Immuno Research Laboratories, Inc., Avondale, PA). Only monoclonals that bound to the assembled C5b-9 complex were used further. One monoclonal antibody, anti-C5b-9 neoantigen, bound only to the SC5b-9 complex used in the original selection of supernatants and to the C5b-9 complex assembled on the RBC membrane; it did not bind to any of the individual complement components, including the S protein. The remaining supernatants were specific for the individual complement components in the EIA and for exposed epitopes on the individual complement components in the assembled form on the rabbit RBC.

Immunofluorescence procedures. After 48 h of culture in 1.2 mM Ca^{++} medium, epidermal cells were incubated for 30 min in PBS containing either 3 mg PV IgG/ml or 3 mg normal IgG/ml that had been centrifuged at 100,000 rpm for 10 min in an ultracentrifuge (model TL100; Beckman Instruments, Inc., Palo Alto, CA) to remove IgG aggregates. After a brief washing with 0.01 M PBS the cells were incubated in NHS diluted with PBS containing 0.5 mM $MgCl_2$ and $CaCl_2$ (PBS^{++}). After washing with PBS^{++} the cells were incubated with monoclonal antibodies to C5, C6, C7, C8, C9, and C5b-9 neoantigens. Complement binding was detected with affinity-purified FITC-labeled goat anti-mouse IgG Fc (Jackson Immuno Research Laboratories, Inc.). Controls included heat-inactivated AB NHS, 10 mM EDTA-treated AB NHS, and C2-depleted AB NHS in place of the AB NHS complement source. Murine monoclonal anti-human C3c (Cytotech, Inc.) was used as a positive control in place of the monoclonal antibodies (tissue culture supernates) to the terminal complement components. Human IgG binding to HuK was detected by the use of goat anti-human IgG Fc (Jackson Immuno Research Laboratories, Inc.).

Cytotoxicity studies. Keratinocyte cytotoxicity (membrane integrity) studies were carried out as previously described (13) using the three PV IgG fractions. Fluorescein diacetate (FDA; Nutritional Biochemicals, Irvine, CA) and ethidium bromide (EB; Sigma Chemicals Co.) were purchased. 2 μl FDA stock solution (5 mg/ml in acetone) and/or 0.1 ml EB stock solution (200 μg/ml in HBSS) were added to 4.9 ml of HBSS just before use (26). The epidermal cells were gently rinsed with PBS and incubated in FDA/EB or EB to determine the integrity of cell membranes. After 5 min the solution was removed and the cells were immediately examined by fluorescence microscopy.

Results

In low Ca^{++} (0.1 mM) epidermal cells grow as a monolayer, becoming confluent but not stratified. The monolayers of human epidermal cells in low Ca^{++} formed multilayers within 48 h after being switched to high Ca^{++} (1.2 mM). The cells in the bottom layer are small and round; the cells in the upper layer are larger and flattened. The binding of PV IgG on the smaller round cells generally appeared to be less intense than binding on the larger flattened cells (Fig. 1 *A*). The expression of pemphigus antigen on cultured HuK may be related to keratinocyte differentiation, as pemphigus antigen was especially prominent on the large flattened cells found superficially in the stratified colonies. The binding of anti-C3c also appeared to follow the same pattern of IF intensity (Fig. 1 *B*).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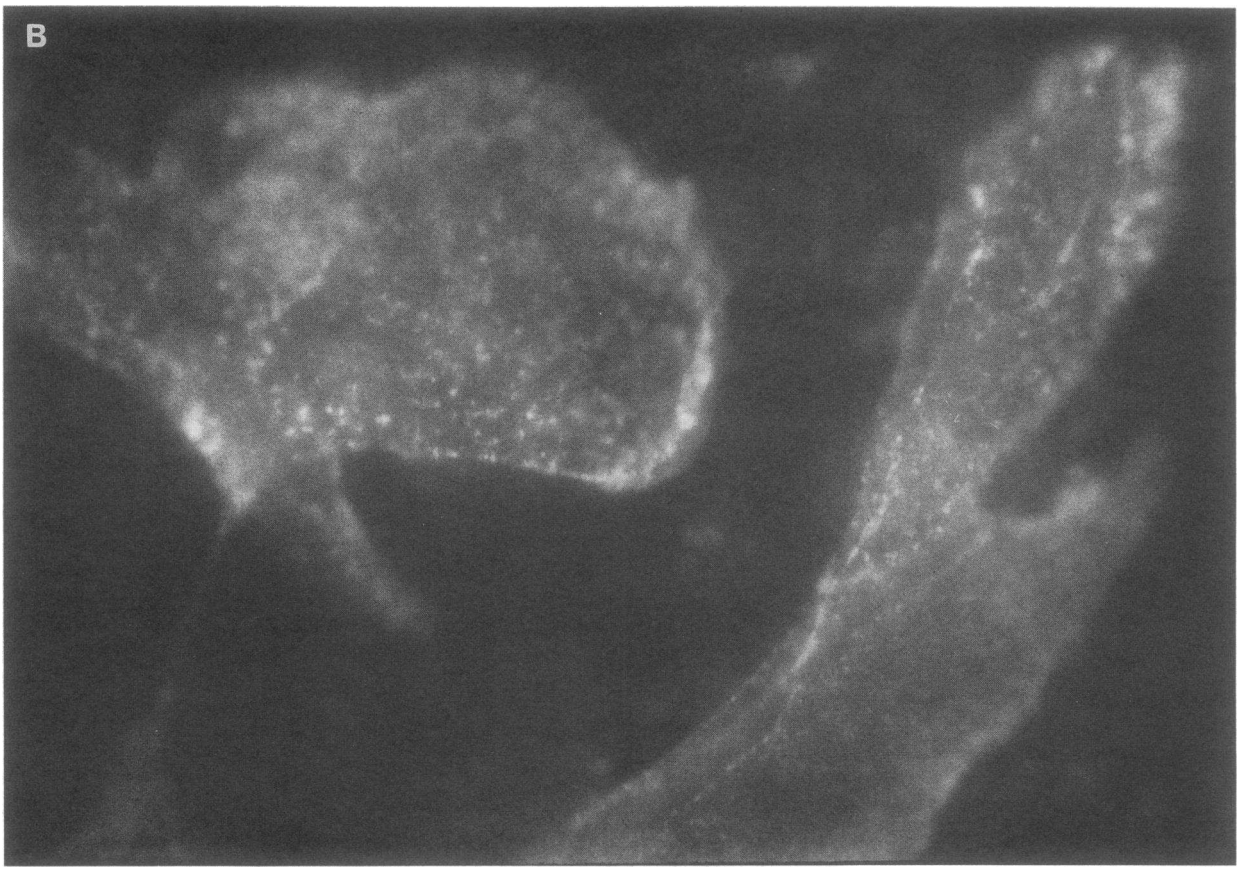
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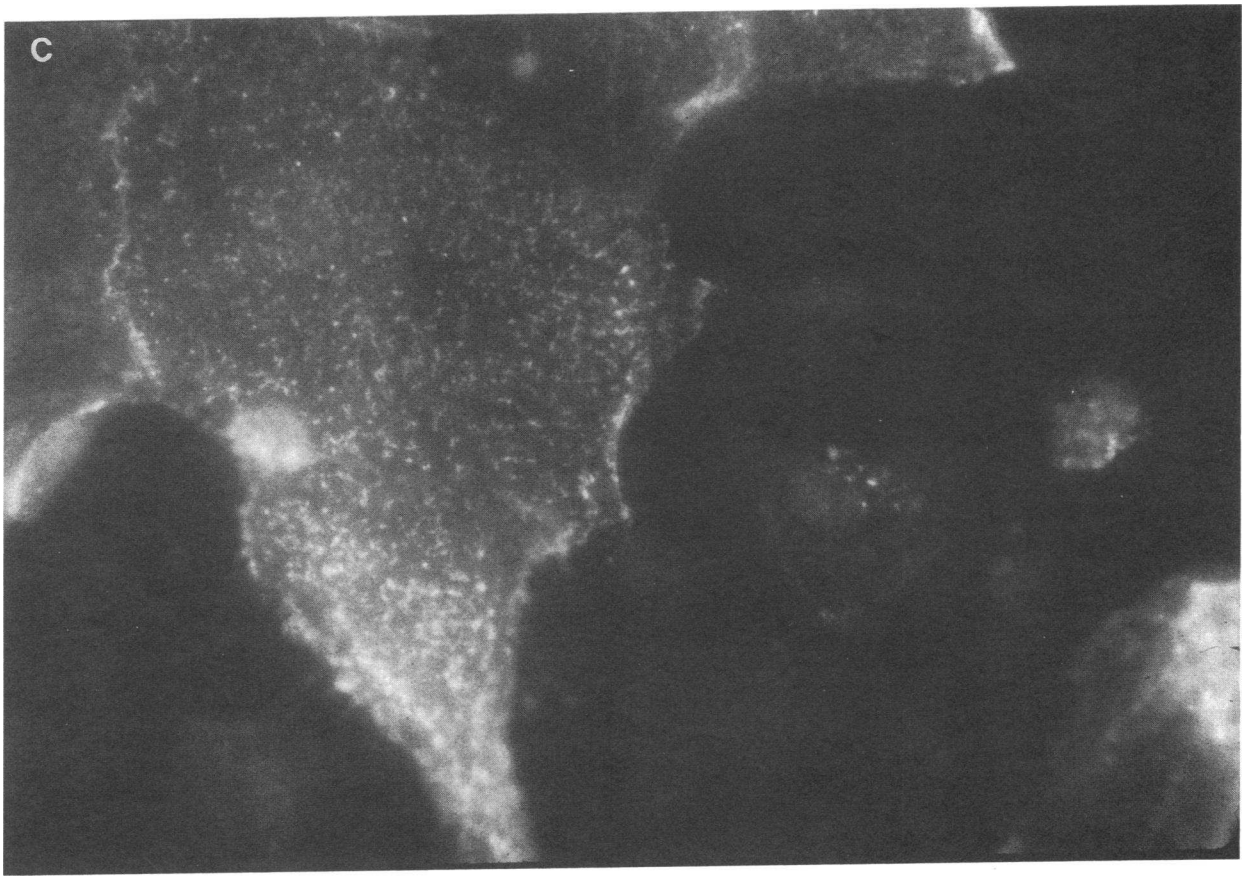

Figure 3. IF staining of complement. (*A*) PV IgG plus complement followed by monoclonal anti-human C6 and FITC-labeled goat anti-mouse IgG-Fc. (*B*) Same as *A*, except monoclonal anti-human C8 was used. (*C*) Same as *A*, except monoclonal anti-human C5b-9 neoantigen was used. × 500

HuK incubated with all three PV IgG fractions followed by fresh AB NHS (complement source) and then by monoclonal antibody to one of the complement components (C5, C6, C7, C8, C9, or C5b-9 neoantigens) bound FITC-labeled goat anti-mouse IgG-Fc with a characteristic speckled pattern. These results are similar to those using polyclonal antisera to C1q, C4, and C3 which we reported previously (11). IF staining was most intense on the exposed large flat cells located in the upper layers (Figs. 2 *A*, and 3, *A–C*) and was less intense on the small round cells attached to the collagen substrate on the slides. In controls, cells incubated with PV IgG and heat-inactivated AB NHS, EDTA AB NHS, or C2-depleted serum did not exhibit this characteristic speckled staining pattern (Fig. 2 *B, C;* Table I). IF staining was not observed on keratinocytes when normal IgG plus AB NHS was used in place of PV IgG plus AB NHS (Fig. 1 *C*).

FDA/EB staining was used to determine whether complement affected cell membrane integrity. We found that before incubation with PV IgG and AB NHS > 90–95% of the cells were FDA positive (green fluorescent cytoplasm). Only a small number of the cells were EB positive (orange nuclei) and FDA negative, consistent with our previous observations (13). After incubation with PV IgG and AB NHS almost all of the epidermal cells were EB positive and FDA negative, consistent with our previous observations (13). PV IgG in the absence of AB NHS had no effect and NH IgG in either the absence or presence of AB NHS also had no effect.

These studies were expanded to examine the effect of antibody titer on cytotoxicity. The effect of the quantity (titer) of PV IgG plus complement on the integrity of the cell membrane was determined using the EB test (13) (Table II). Three

Table I. Fixation of Terminal Complement Components (Membrane Attack Complex) by PV IgG

	Monoclonal antibodies						
Complement source	C3c	C5	C6	C7	C8	C9	C5b-9 neoantigen
PV IgG*							
C‡	+	+	+	+	+	+	+
C (56°C 30 min§)	−	−	−	−	−	−	−
C-EDTA‖	−	−	−	−	−	−	−
C2-depleted C¶	−	−	−	−	−	−	−
No C	−	−	−	−	−	−	−
Normal human IgG							
C	−	−	−	−	−	−	−

* PV IgG from three different patients was used.
‡ 10% NHS AB type, absorbed with human epidermal cells.
§ 10% NHS heat-inactivated at 56°C for 30 min.
‖ 10% NHS with 10 mM EDTA.
¶ C2-depleted NHS.
+, Fluorescence present (the degree of fluorescence is not indicated since this varied with each cell and with each monoclonal); −, no fluorescence.

Table II. EB Test of HuK Membrane Integrity

	mg IgG/ml*					
	3	0.3	0.03	0.015	0.0075	*n*
PV IgG + C 1	+	+	+	+	−	3
PV IgG + C 2	+	+	+	−	−	3
PV IgG + C 3	+	−	−	−	−	3
NHS IgG + C	−	ND	ND	ND	ND	3
PV IgG 1, 2, 3, No C	−	ND	ND	ND	ND	3

* The titer for each PV IgG was determined starting at a concentration of 2 mg IgG/ml. PV IgG binding to frozen sections of human foreskin was detected by indirect IF: Patient 1, 1:320; patient 2, 1:320; patient 3, 1:40. ND, not done.

PV IgG and NH IgG were compared. At 3 mg/ml all three PV IgG samples plus complement resulted in cytotoxicity as judged by EB staining of the nuclei, while the nuclei of keratinocytes in controls, NH IgG plus complement, were EB negative. As the quantity of PV IgG was reduced, the degree of EB staining appeared to decrease gradually until the nuclei were EB negative (Table II). Nuclear staining could be detected at greater dilutions for those patients having higher antibody titers (patients 1 and 2) than for the patient with the lowest titer (patient 3). In the absence of complement the nuclei of PV IgG-treated keratinocytes were negative at the highest PV IgG concentrations.

Discussion

The present investigation confirms and extends previous observations that PV IgG bound to the surface of keratinocytes is capable of activating the complement system (11). Our previous studies have demonstrated this phenomenon on murine and nonhuman primate tissues; this report extends these results to cultured HuK for the first time.

In our previous studies complement binding to murine cell membranes was determined by IF using polyclonal goat anticomplement antisera or rabbit IgG anticomplement reagents. These reagents repeatedly proved to be inadequate for use on cultured HuK; binding of the FITC-labeled antibody occurred even in the absence of complement. As neither the goat antisera nor the rabbit IgG were affinity purified, the positive binding observed in the controls was assumed to be the result of the binding of antibodies to other substances on the surface of the HuK. In an attempt to achieve maximum specificity, monoclonal antibodies produced in tissue culture were selected for these studies.

More than 100 different monoclonals specific for the individual complement components C5–C9 were tested for their ability to bind to C5b-9 assembled on RBC membranes. Although all of the monoclonals had been selected for their ability to bind SC5b-9 and/or the S protein, C5, C6, C7, C8, and C9 in solid phase EIA, most of them failed to bind to the membrane of rabbit RBC ghosts containing the C5b-9 complex. Only those monoclonals that bound to the terminal complement components in the assembled form on RBC membranes were used in these investigations. In addition, a murine monoclonal antibody purified from ascites fluid, anti-C3c, was selected as a positive control. Positive binding of this antibody to HuK was obtained only when PV IgG and complement were each present. Negative controls (no AB NHS) suggested that either the ascites fluid lacked antibodies to noncomplement proteins on the HuK or that noncomplement antibodies were not present in sufficient quantity to yield a positive result.

Our previous studies showed that PV IgG fixes C1q, C4, and C3 to Macaca organ culture skin explants and to monolayers of murine keratinocytes in culture (11), and that PV IgG and complement enhanced the detachment of cultured murine keratinocytes (12). More recently it was demonstrated that the activation of human complement in the absence of human plasmin/plasminogen was sufficient to generate detachment of murine keratinocytes in vitro (15). In addition, cell detachment requiring the presence of complement was demonstrated in preliminary experiments using HuK cultured in a defined medium totally lacking serum (15). Furthermore, we have demonstrated that PV IgG in the presence of complement results in limited cytotoxicity by significantly altering the permeability of murine epidermal cell membranes to EB (13).

The present investigations confirm and extend our previous observations by demonstrating that PV IgG will fix the late-acting complement components C5, C6, C7, C8, and C9 to HuK cell surfaces and result in expression of C5b-9 neoantigens. Formation of this latter complex (membrane attack complex) is thought necessary for cell damage to occur. Assembly of the membrane attack complex on RBC membranes after complement activation results in their lysis (27). The results of FDA/EB staining described by us previously (13) and in this study suggest that pemphigus antibody in the presence of complement alters the membrane integrity of HuK. Furthermore, this activity appears to parallel the antibody activity present. Thus, in all likelihood the assembly of the membrane attack complex on HuK surfaces as shown in the present study accounts for this altered membrane integrity. These findings, therefore, further implicate the complement system in the pathogenesis of pemphigus. Further studies, including identification of complement components responsible for cell detachment and altered cell membrane integrity, and identification of complement-mediated lesions on HuK surfaces by electron microscopy, are currently underway.

Acknowledgments

Dr. John Tamerius kindly provided monoclonal antibodies for use in these studies and reviewed this manuscript.

This work was supported in part by research grants AR-32974 and AR-35362.

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