Cutaneous IgA Deposits in Bullous Diseases Function as Ligands to Mediate Adherence of Activated Neutrophils

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Linear IgA bullous dermatosis and dermatitis herpetiformis are inflammatory subepidermal blistering diseases characterized by IgA deposits at the cutaneous epithelial basement membrane and in dermal papillae, respectively. Inflammation in both disorders localizes to sites of IgA deposition and is characterized by a predominance of neutrophils. From these observations we postulate that IgA deposits in both diseases may contribute to the recruitment and/or localization of neutrophils. In this study we examined the ability of *in vitro* and *in vivo* bound IgA anti-basement membrane autoantibodies from patients with linear IgA bullous dermatosis and *in vivo* bound IgA deposits in dermal papillae from patients with dermatitis herpetiformis to mediate adherence of neutrophils stimulated by granulocyte macrophage colony-stimulating factor. The study showed that stimulated neutrophils adhered to basement membranes and dermal papillae containing IgA deposits. Adherence was IgA anti-basement membrane antibody concentration dependent and correlated with the immunofluorescence staining intensity of IgA deposits in dermal papillae. Adherence to IgA deposits but not IgG deposits could be inhibited by purified exogenous secretory IgA but not IgG and adherence to IgG deposits could be inhibited by purified exogenous IgG but not secretory IgA. These results provide direct experimental evidence that cutaneous IgA deposits in linear IgA bullous dermatosis and dermatitis herpetiformis can function as ligands for neutrophil adherence and have a role in the localization of inflammation in these disorders. *J Invest Dermatol 94: 667–672, 1990*

Dermatitis herpetiformis (DH) and linear IgA bullous dermatosis (LABD) are subepidermal blistering diseases characterized by neutrophil-predominant inflammation and cutaneous IgA deposits [1,2]. The IgA deposits in LABD are located in a linear pattern along the basement membrane (BM) of stratified squamous epithelia and those in DH are mainly located in the upper dermis in the tips of dermal papillae. The IgA deposits in LABD are anti-BM autoantibodies (ABM) [3]. The specificity of the deposits in DH is unknown [3]. In both diseases, neutrophils are abundant at sites of IgA deposition, suggesting that the deposits may contribute to recruitment and/or localization of neutrophils.

A mechanism by which IgA deposits could contribute to localization of neutrophils is immune adherence. In vitro studies have shown that neutrophils adhere to particle-bound or aggregated IgA, and evidence has been presented that adherence is mediated via specific membrane receptors for the Fc portion of IgA [4–7]. However, there is no experimental evidence that tissue IgA deposits in any disease can mediate the adherence of neutrophils.

In this study we examined the ability of cutaneous IgA deposits in DH and LABD to mediate adherence of neutrophils. The results show that tissue IgA deposits in both disorders can function as specific ligands to mediate adherence of stimulated neutrophils.

MATERIALS AND METHODS

Human Skin and Sera Neonatal normal human foreskin was obtained immediately following elective circumcision. Punch biopsies (3–4 mm) were obtained under local anesthesia from perilesional skin of three patients with LABD and five patients with DH. All tissues were immediately frozen in liquid nitrogen, mounted in OCT compound (Miles Scientific, Naperville IL), and stored frozen at −80°C. Patient biopsies were assayed by direct immunofluorescence for IgG, IgA, IgM, and C3 deposits as previously described and the deposits characterized according to type, location, and immunofluorescence staining intensity [8].

Platelet-poor normal human serum (NHS) was prepared from normal donors as previously described [9]. Sera were obtained from three patients with LABD and assayed by indirect immunofluorescence for IgG, IgA, IgM, and C3-binding ABM as previously described [8]. The sera contained IgA ABM at titers of 1:40–1:80. No IgG, IgM, or C3-binding ABM were detected. All sera were heat-inactivated (56°C × 30 min), aliquoted, and stored frozen at −80°C.

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Abbreviations:

ABM: anti-basement membrane autoantibody
BM: epithelial basement membrane
C: complement
DH: dermatitis herpetiformis
IgA: immunoglobulin A
IgG: immunoglobulin G
Il-3: interleukin 3
LABD: linear IgA bullous dermatosis
NA: neutrophil adherence
PB: polymyxin B
PBS: phosphate-buffered saline
rGM-CSF: recombinant granulocyte macrophage colony-stimulation factor

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Neutrophils Neutrophils were purified from the blood of normal human donors as previously described and suspended in RPMI at 20 million cells/ml [10]. These preparations routinely contained > 95% viable neutrophils.

Other Reagents Purified recombinant human granulocyte-macrophage colony-stimulating factor (rGM-CSF, 100 ng/ml) and interleukin 3 (rIL3) (Genzyme Corp., Boston, MA) were aliquoted and stored frozen at −80°C. IgG fraction of rabbit antiserum to rGM-CSF was obtained from Genzyme Corp, aliquoted, and stored frozen at −80°C. Purified human secretory IgA and human IgG were obtained from Sigma Chemical Co., St. Louis, MO. Polymyxin B (PB) was obtained from Boehringer Mannheim, W. Germany.

Neutrophil Adherence (NA) Assay Neutrophil adherence to tissue IgA deposits was performed by a previously described technique [11]. Briefly, four sequential 8-μm-thick cryostat sections of fresh frozen DH, LABD, or normal human skin were placed on sterile gelatin-coated glass slides and briefly air dried. Normal human skin sections were overlaid with 25 μl/section of LABD serum or NHS diluted in sterile 0.1 M phosphate-buffered saline (PBS) and incubated for 30 min at room temperature in a humidity tray. Slides were washed for 15 min in three changes of sterile PBS and excess PBS removed by blotting. Tissue sections from patient biopsies were not pretreated. Wells corresponding to each tissue section were constructed on separate slides and loaded with 25.0 μl of RPMI medium containing 250,000 neutrophils, 12% heat-inactivated NHS, and indicated concentrations of cytokines, anti-cytokine antibodies, PB, or normal human secretory IgA or IgG. Skin sections were placed over the wells and inverted to allow neutrophils to settle over the sections. Wells were incubated 45 min at 37°C in a humidified air incubator and then sections were washed free of nonadherent cells, fixed in ethanol, and stained with hemotoxylin and eosin (H&E).

Quantitation of NA and Analysis of Results Neutrophil adherence to dermal papillae in DH skin and to the BM in LABD skin or normal skin treated with IgA ABM was determined with a light microscope equipped with rotating linear or grid ocular micrometers. Neutrophil adherence to dermal papillae was measured by counting the number of neutrophils in three 1 mm² fields in each of three dermal papillae/section and expressed as the mean (±SEM) neutrophils/mm² dermal papillae in quadruplicate sections. Neutrophil adherence to the BM was measured by counting the number of neutrophils adherent to three 1-mm-long segments of BM/section and expressed as the mean (±SEM) neutrophils/mm BM in quadruplicate sections. All experiments were performed 3–6 times with consistent results. Means were tested for variance within groups and no significant variance was found. Means between groups were then compared by the matched Student t test.

RESULTS

rGM-CSF Stimulates NA to Sites of IgA Deposition in Skin Sections In initial studies, we examined NA to the BM of skin sections with in vitro and in vivo bound IgA ABM and to dermal papillae of DH skin with in vivo bound IgA deposits. In vitro bound IgA ABM were prepared by treating normal human skin sections with 10% LABD sera (n = 3) containing 1:40–1:80 titers of IgA ABM or 10% NHS (n = 2) as a negative control. Skin sections with in vivo bound IgA ABM (1–2 + IgA staining) and IgA deposits in dermal papillae (2–3 + IgA staining) were obtained from the biopsies of three patients with LABD and five patients with DH, respectively. Normal skin (n = 2) was used as a negative control. The effect of rGM-CSF was tested by examining NA to all skin sections in the presence and absence of 67.0 ng/ml rGM-CSF. The results of studies on skin with in vitro and in vivo bound IgA ABM (Figs 1 and 2) showed significantly greater ($p < 0.001$) NA to the BM when sections were treated with rGM-CSF and neutrophils than when treated with neutrophils alone. Furthermore those studies showed that in the presence of rGM-CSF, NA to the BM was significantly

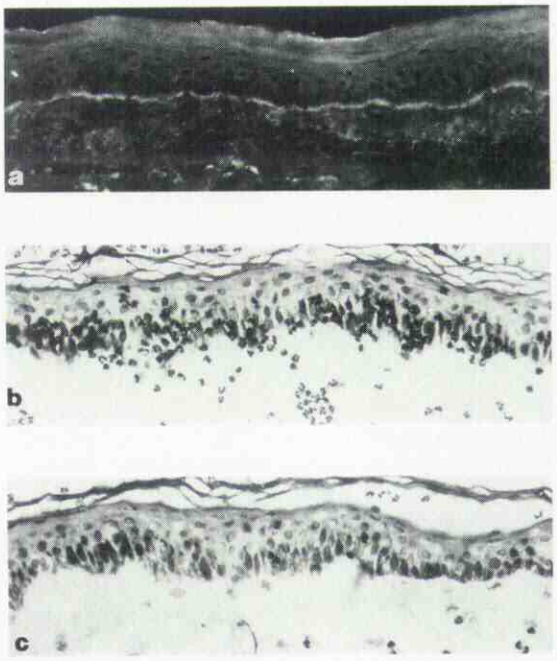

Figure 1. Adherence of rGM-CSF-stimulated neutrophils to the BM of skin from a patient with LABD (magnification ×250): *a*) photomicrograph showing direct IgA immunofluorescence staining of a section from a patient with LABD. Note linear IgA deposits at the BM. Similar results were obtained when normal human skin was incubated with LABD serum containing IgA ABM; *b*) photomicrograph of a sequential skin section from the same patient incubated with rGM-CSF stimulated neutrophils. Note neutrophils along the BM. Similar results were observed when stimulated neutrophils were incubated with skin pretreated with serum containing IgA ABM; *c*) photomicrograph of another sequential section from the same patient treated with neutrophils in the absence of rGM-CSF. Note relatively few neutrophils are adherent to the BM compared to *b*. Similar results were obtained with normal human skin pretreated withIgA ABM and subsequently incubated with neutrophils in the absence of rGM-CSF, or normal human skin pretreated with NHS and subsequently incubated with rGM-CSF-stimulated neutrophils.

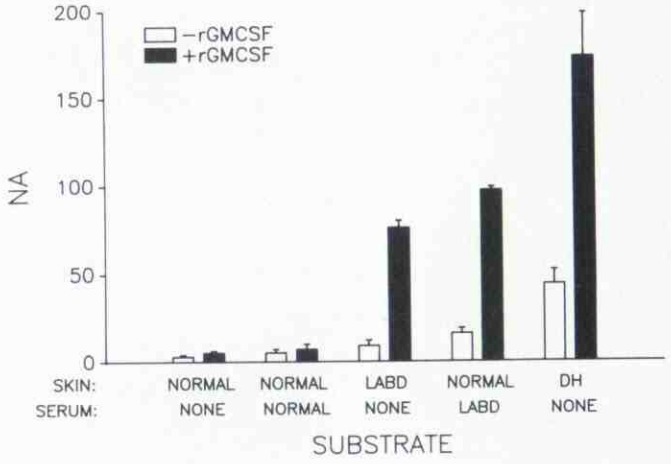

Figure 2. The effect of substrate and rGM-CSF on neutrophil adherence. *Open bars* represent the results of NA on skin sections treated with neutrophils in the absence of rGM-CSF and *solid bars* represent results on sections treated with neutrophils in the presence of 67.0 ng/ml rGM-CSF. NORMAL SKIN is normal human skin. NORMAL SERUM is 10% normal human serum (n = 2). LABD SKIN is skin from patients with LABD (n = 3) and in vivo bound IgA ABM. LABD SERUM is 10% LABD serum (n = 3) containing IgA ABM. DH SKIN is skin from patients with DH (n = 5) and granular deposits of IgA in dermal papillae. NA is expressed as neutrophils/mm BM for the first four substrates and neut/mm² dermal papillae for the DH SUBSTRATE. *Error bars* represent SEM.

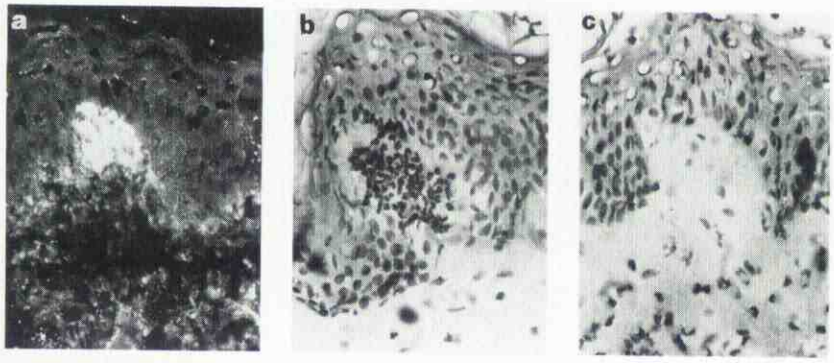

Figure 3. Adherence of rGM-CSF-stimulated neutrophils to dermal papillae of DH skin (magnification ×400). *a*) photomicrograph showing direct IgA immunofluorescence of a section of DH skin with 3+ deposits of IgA in dermal papillae; *b*) sequential section from the same patient incubated with rGM-CSF and neutrophils. Note neutrophils in the dermal papillae; *c*) sequential section from the same patient incubated with neutrophils in the absence of rGM-CSF. Note relatively few neutrophils in the dermal papillae compared to *b*.

greater ($p < 0.001$) on sections with IgA ABM than control sections without those ABM. Likewise, NA to dermal papillae of DH skin containing IgA deposits was significantly greater in the presence of rGM-CSF ($p < 0.001$) than in its absence. In the presence of rGM-CSF, NA to DH skin with IgA deposits was significantly greater ($p < 0.001$) than NA to normal human skin without IgA deposits (Figs 2 and 3). These results provided evidence that rGM-CSF could stimulate NA to cutaneous IgA deposits and that those deposits could function as ligands to mediate the adherence of rGM-CSF-stimulated neutrophils to the BM and dermal papillae.

rGM-CSF Stimulated NA to IgA Deposits at the BM and in Dermal Papillae is rGM-CSF Selective, Specific and Concentration Dependent In these studies we examined the specificity, selectivity, and concentration dependency of rGM-CSF-stimulated NA to BM and dermal papillae with IgA deposits. Specificity and concentration dependency were examined as follows: Skin sections with in vitro bound IgA ABM and in vivo bound dermal papillary IgA deposits were prepared as described above and incubated with neutrophils and increasing concentrations (0.067–67.0 ng/ml) of rGM-CSF alone or rGM-CSF plus: 1) 40 μg/ml PB; 2) 1.0 mg/ml normal (preimmune) rabbit serum; or 3) 1.0 mg/ml rabbit anti rGM-CSF. The results (Fig 4) showed a concentration-dependent increase in NA to the BM and to dermal papillae with IgA deposits in the presence of increasing concentrations of rGM-CSF. There was no significant effect ($p < 0.01$) of PB or normal preimmune rabbit serum on rGM-CSF-stimulated NA to those substrates. However, there was significantly less ($p < 0.01$) NA to all substrates and at all concentrations of rGM-CSF in the presence of anti-rGM-CSF. These results showed that rGM-CSF-mediated NA to BM and dermal papillae containing IgA deposits is concentration dependent, cytokine specific, and not due to endotoxin because it was not inhibited by the addition of PB (an inhibitor of endotoxin) but could be selectively inhibited by GM-CSF antiserum. In other studies (results not shown), we compared the ability of rGM-CSF and rIL-3 mediate NA to IgA deposits and found that rIL-3 was ineffective at concentrations up to 10.0 ng/ml. This result showed that stimulation of NA to BM and dermal papillae with IgA deposits is at least partially cytokine selective.

Adherence of rGM-CSF Stimulated Neutrophils to IgA Deposits is IgA ABM Concentration Dependent and Dependent on the Intensity of Dermal Papillary IgA Immunofluorescence Staining The observation that IgA was the only immunoglobulin class in circulating and tissue-bound ABM and in DH dermal papillae suggested that IgA was functioning as the ligand for adherence of stimulated neutrophils to sites of IgA deposition. To obtain additional support for that conclusion, we examined the relationship between adherence of rGM-CSF-stimulated neutrophils to the BM and serum IgA ABM concentration used to treat skin sections, and between adherence and the intensity of IgA immunofluorescence staining in DH dermal papillae. The results of studies with IgA ABM showed a serum IgA ABM concentration-dependent increase in adherence of rGM-CSF-stimulated neutrophils to the BM and a good correlation between the lowest serum concentration mediating NA and IgA binding to the BM (Fig 5). To examine the relationship between NA and immunofluorescence intensity of IgA deposits in dermal papillae, we assayed 12 biopsies from five DH patients by direct IgA immunofluorescence and rGM-CSF-stimulated NA. The results of immunofluorescence showed 2–3+ IgA deposits in five biopsies, 1–2+ deposits in four, and 0–1+ deposits in three (Fig 6). The results of NA (Fig 7) showed 173 ± 25 neutrophils/mm² in dermal papillae with 2–3+ IgA deposits; 71 ± 20 neutrophils/mm² in papillae with 1–2+ deposits, and 35 ± 8 neutrophils/mm² in papillae with 0–1+ deposits. As in previous experiments, we observed that optimal adherence to IgA ABM and dermal papillae required neutrophil suspensions containing rGM-CSF.

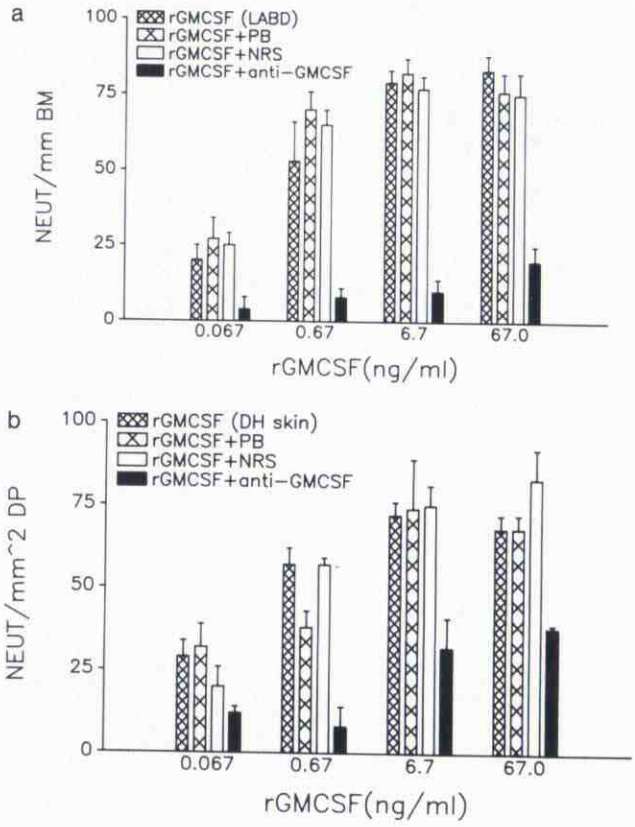

Figure 4. *a*) Normal human skin was preincubated with 10% serum containing IgA ABM and subsequently incubated with neutrophils in the presence of increasing concentrations (0.067–67.0 ng/ml) of rGM-CSF alone [rGMCSF (LABD)], rGMCSF plus 40 μg/ml PB, rGM-CSF plus 1.0 mg/ml normal (preimmune) rabbit serum (NRS), or rGM-CSF and 1.0 mg/ml antiserum (anti-GMCSF) to rGMCSF. *b*) Same conditions as in *a* using DH skin as substrate.

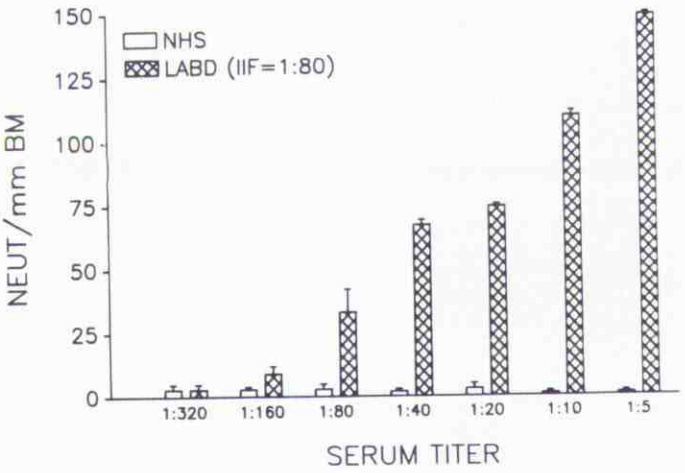

Figure 5. The results of treating skin with increasing concentrations of NHS *(open bars)* or LABD serum containing a 1:80 titer of IgA ABM *(cross-hatched bars)* and subsequently incubating sections with 67.0 ng/ml rGM-CSF and neutrophils. Note a serum concentration-dependent increase in NA on sections pretreated with IgA ABM but no effect of NHS. These results also show that significant NA begins at an IgA ABM serum titer of 1:160.

Adherence of rGM-CSF-Stimulated Neutrophils to IgA Deposits at the BM and in Dermal Papillae is Specifically Inhibited by Exogenous Purified Secretory IgA To obtain additional evidence that NA to BM and dermal papillae was mediated by IgA, we attempted to block adherence with exogenous purified secretory IgA. In these studies, NA was performed on skin sections treated with 10% LABD serum containing a 1:80 titer of IgA ABM, and on DH sections with 2–3+ deposits of IgA. NA was examined in the presence of 67.0 ng/ml rGM-CSF and increasing concentration of purified human secretory IgA. As controls, we examined the ability of purified human IgG to inhibit NA to skin sections with IgA deposits and the ability of purified secretory IgA to inhibit NA to sections with BM IgG deposits. IgG deposits at the BM were prepared by treating normal human skin sections with 10% serum containing IgG ABM (IgG ABM titer 1:160; IgA ABM titer 0) from a patient with epidermolysis bullosa acquisita. The results of those studies (Fig 8) showed that increasing concentrations of exogenous secretory IgA could inhibit NA to BM and dermal papillary IgA deposits by 70% to more than 90% in a dose-dependent manner while equivalent concentrations of exogenous IgG inhibited by only 5–20% with no evidence of an IgG concentration effect. Alternatively, exogenous IgG inhibited NA to BM IgG by nearly 100% and in a dose-dependent manner, while exogenous secretory IgA inhibited by less than 5% at equivalent concentrations (Fig 8).

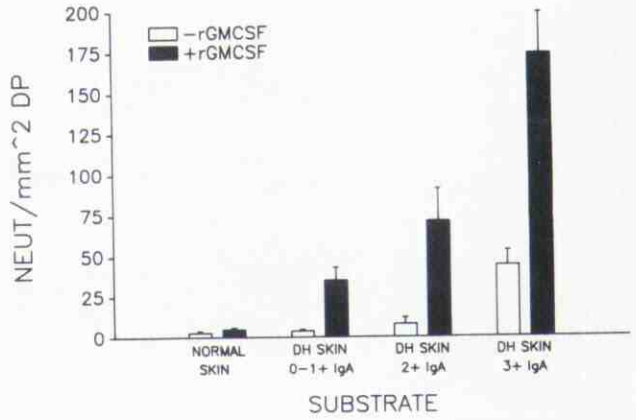

Figure 7. Results of NA on normal human skin (NORMAL SKIN) and DH skin with 0–1+, 2+, or 3+ IgA deposits in dermal papillae. *Open bars* represent adherence in the absence of rGM-CSF and *closed bars* represent adherence in the presence of rGM-CSF. *Error bars* represent SEM.

DISCUSSION

Previous in vitro studies have shown that IgA binds to neutrophils, and that neutrophils adhere to aggregated IgA, and IgA-coated particles [4–7,12]. Binding of IgA to neutrophils and neutrophil adherence to IgA-coated surfaces demonstrates saturation kinetics, and can be inhibited by IgA but not IgG suggesting that neutrophil-IgA interactions are mediated through specific membrane receptors for IgA [4,5,13]. Binding of neutrophils to aggregated IgA and IgA-coated substrates can stimulate release of lysosomal enzymes and enhance neutrophil phagocyosis [4,12,13,14]. Both the expression of IgA-dependent neutrophil functions and the affinity of IgA receptors can be enhanced by GM-CSF [13]. These observations suggest IgA deposits in tissues may act as specific ligands to mediate neutrophil inflammatory functions and host defense and that neutrophil-tissue IgA interactions may be enhanced by biologic response modifiers such as GM-CSF.

Several inflammatory diseases including LABD and DH are characterized by deposits of autoantibodies and immune complexes containing predominantly or exclusively IgA. The role of those deposits in the pathogenesis of inflammation is unclear. It has been suggested that they may activate the alternative complement pathway and generate C5-derived chemotactic factors that recruit leukocytes. However, the ability of IgA to generate chemotactically significant amounts of C5-derived peptides remains an open question. Some IgA molecules appear to activate the alternative complement pathway; however, that appears to be a relatively inefficient means of forming C5 convertases and cleaving C5 compared to classical pathway activation by IgG and IgM [15–19]. At present, there is no direct experimental evidence that IgA deposits can mediate complement-dependent leukocyte recruitment. In our studies

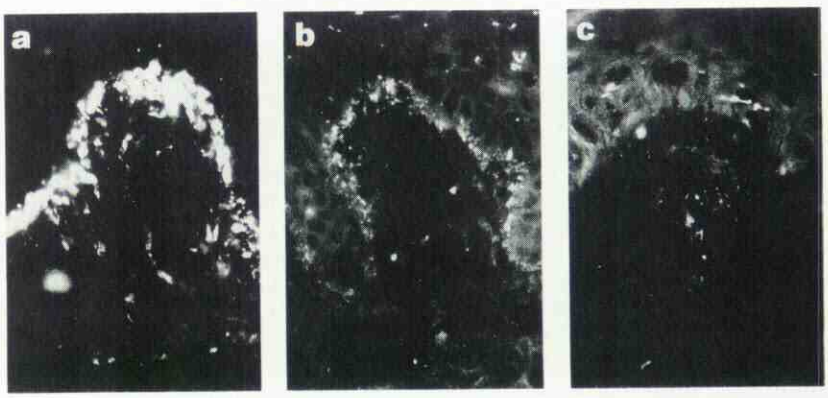

Figure 6. Direct IgA immunofluorescence of DH skin biopsies showing 3+ (*a*), 2+ (*b*), and 0–1+ (*c*) IgA deposits in dermal papillae (magnification ×400).

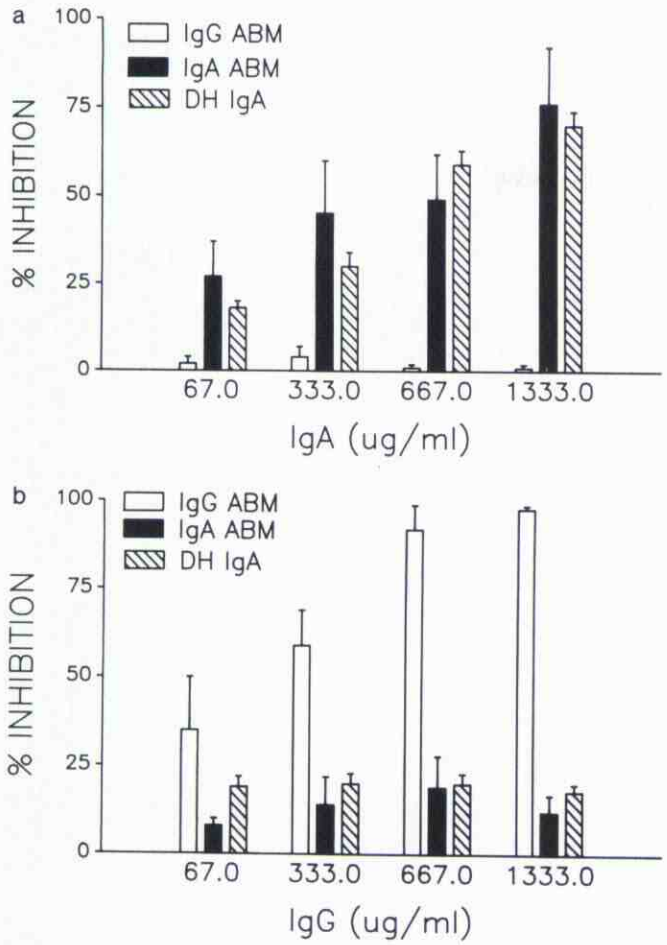

Figure 8. Results of inhibition of NA with exogenous purified human IgA or IgG. Substrates used in these studies included: 1) normal human skin pretreated with 10% serum containing IgG ABM (titer 1:160) from a patient with epidermolysis bullosa acquisita (IgG ABM); 2) 10% serum containing IgA ABM (titer 1:80) from a patient with LABD (IgA ABM); or DH skin with 2–3+ IgA in dermal papillae (DH IgA). Sections were incubated with 67.0 ng/ml rGM-CSF and neutrophils in the presence of increasing concentrations (67.0–1333.0 μg/ml) of either purified secretory human IgA (*a*) or purified human IgG (*b*). Note that exogeneous IgA significantly inhibits NA to IgA ABM and DH IgA in a dose-dependent manner but does not inhibit NA to IgG ABM. Conversely, exogenous IgG inhibits NA to IgG ABM significantly better than to IgA ABM or DH IgG.

we have observed that tissue deposits of IgG ABM are capable of recruiting leukocytes to the BM by C5-dependent directed migration; however, we have not found that IgA deposits in LABD or DH are capable of demonstrating that activity ([20,21] and unpublished observations). Those observations suggest that localization of neutrophils at sites of IgA deposits may involve mechanisms of leukocyte recruitment other than IgA-mediated complement activation and/or immune adherence.

In this study, we examined the ability of cutaneous IgA deposits to mediate adherence of neutrophils. The substrates used included normal human skin with in vitro bound IgA ABM, perilesional skin from LABD patients with in vivo bound IgA ABM, and skin from DH patients with granular deposits of IgA in dermal papillae. We used rGM-CSF as a neutrophil-activating agent because of a previous study showing it can increase the affinity of neutrophil IgA Fc receptors. We found that incubation of rGM-CSF-treated neutrophils with skin substrates containing IgA deposits resulted in significantly greater NA to sites of IgA deposition than to corresponding sites in normal human skin.

Several lines of evidence suggest that tissue IgA in those substrates was at least partly responsible for NA. First, by immunofluorescence analysis, the only Ig class specifically deposited in the tissues was IgA. No IgG or IgM was observed. We did not stain for IgE and IgD class deposits but neutrophils do not appear to have receptors for those Ig classes. Second, there was a perfect correlation between the sites of IgA deposits (BM and dermal papillae) and the site of optimal NA. Third, there was a dose-response relationship between the concentration of serum IgA ABM used to pretreat skin and NA to the BM, and a positive relationship between the intensity of IgA staining and NA in dermal papillae. Fourth, we observed that the addition of exogenous secretory IgA purified from human colostrum could significantly inhibit NA in a dose-dependent manner. The specificity of IgA-mediated inhibition of NA to tissue IgA deposits was demonstrated in experiments showing that exogenous secretory IgA could not inhibit NA to IgG ABM and that exogenous human IgG could inhibit NA to tissue-bound IgG ABM but not IgA ABM and IgA deposits in dermal papillae.

Other molecules, such as matrix and complement proteins, that are capable of mediating neutrophil adherence were present in these tissues; however, we do not believe they could have accounted for all the adherence observed in these studies. When tissues with in vivo bound IgA were examined by immunofluorescence for deposits of C3, the major complement-derived neutrophil adherence ligand, we observed minimal or no C3 staining in 6/12 DH biopsies and 2/3 LABD biopsies. In addition, the IgA ABM used in this study was not capable of activating complement as assayed by indirect C3-binding immunofluorescence. Furthermore, exogenous IgA which inhibited adherence in these studies would not be expected to inhibit adherence mediated by C3. Adherence mediated by matrix proteins such as laminin, type IV collagen, and fibronectin would not explain increased adherences to tissues containing IgA deposits because those proteins are also present in normal human skin.

The results of this study suggest that IgA deposits in LABD and DH may contribute to the localization of neutrophils at the BM and tips of dermal papillae, respectively, by functioning as adherence ligands. The results do not rule out the possibility that other factors, particularly chemoattractants, are also involved. The observation that optimal adherence in vitro required a neutrophil-activating agent suggests that neutrophil activation may be a prerequisite for neutrophil adherence to IgA deposits in vivo. Additional evidence suggesting that neutrophil activation may be important in IgA interactions in vivo is provided by the observation that circulating neutrophils from patients with LABD are in a primed or activated state [22]. The nature and source of putative chemoattractants and neutrophil activators in these diseases are unknown. Although GM-CSF was used as the activator in these studies, it is possible, even likely, that other biologic response modifiers can function to stimulate neutrophil adherence to tissue IgA deposits. Studies to investigate that possibility are in progress.

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