Acta Derm Venereol (Stockh) 1983; 63: 495–500

Pemphigus Antibodies Mediate the Development of an Inflammatory Change in the Epidermis

A Possible Mechanism Underlying the Feature of Eosinophilic Spongiosis

KEIJI IWATSUKI, HACHIRO TAGAMI and MIZUHO YAMADA

Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Iwatsuki K, Tagami H, Yamada M. Pemphigus antibodies mediate the development of an inflammatory change in the epidermis. A possible mechanism underlying the feature of eosinophilic spongiosis. Acta Derm Venereol (Stockh) 1983; 63: 495–500.

The interaction between complement-fixing pemphigus antibodies and leukocytes was investigated by the in vitro leukocyte attachment test. Attachment of eosinophils as well as netrophils was induced by pemphigus antibodies at the intercellular space of epidermis in the presence of complement. This phenomenon was found to be produced by 7 out of 10 pemphigus sera in the indirect method using non-lesional skin as a substrate, and in 2 out of 7 biopsy specimens taken from perilesional skin without addition of any sera by the direct method. Those leukocytes bound specifically to the epidermis showed an enhanced function, i.e., vigorous incorporation of nitroblue tetrazolium dye complexes and formation of formazan crystals in the cytoplasms. The results of the present studies suggest that complement-fixing pemphigus antibodies mediate the development of an inflammatory change by inducing an infiltration of polymorphs in the epidermis, which is noted as eosinophilic spongiosis histologically. *Key words: Pemphigus; Complement-fixing antibodies; Immune adherence; Eosinophilic infiltration.* (Received May 30, 1983.)

K. Iwatsuki, Department of Dermatology, Hamamatsu University School of Medicine, 3600, Handacho, Hamamatsu, 431-31, Japan

A prominent leukocytic infiltration with exudative changes, designated as eosinophilic spongiosis, can also be found in the early stage of pemphigus (1) and in patients with herpetiform pemphigus (2). Although previous reports (3, 4) have demonstrated that pemphigus antibodies have the capability of fixing complement in vivo as well as in vitro, the role of complement system in the pathogenesis of pemphigus has been controversial, because the hallmark of pemphigus, an acantholytic, noninflammatory bulla in the epidermis, can be induced in vitro by only IgG fraction of the sera taken from pemphigus patients without any complement sources (5, 6).

In the present study, we investigated the interaction between complement-fixing pemphigus antibodies and leukocytes to clarify the inflammatory aspects noted in pemphigus.

MATERIALS AND METHODS

Preparation of skin samples

Skin samples for substrates were obtained from hypertrophic scar of healthy, human blood group O persons. For the direct leukocyte attachment test, 7 biopsy specimens were taken form the perilesional skin or fresh lesions of either pemphigus vulgaris or pemphigus foliaceus; histologically there were few demonstrable leukocytes in the epidermis. Deposits of IgG and C3 in the intercellular space (ICS) of epidermis were detected by the standard direct immunofluorescence (IF) study. Those samples were snap-frozen in liquid nitrogen, and stocked at −70°C until use.

Preparation of serum samples

Ten serum samples containing antibodies to the ICS of epidermis were obtained from 10 patients with pemphigus. Prior to use, all serum samples were heat-inactivated at 56°C for 30 min, and stocked at

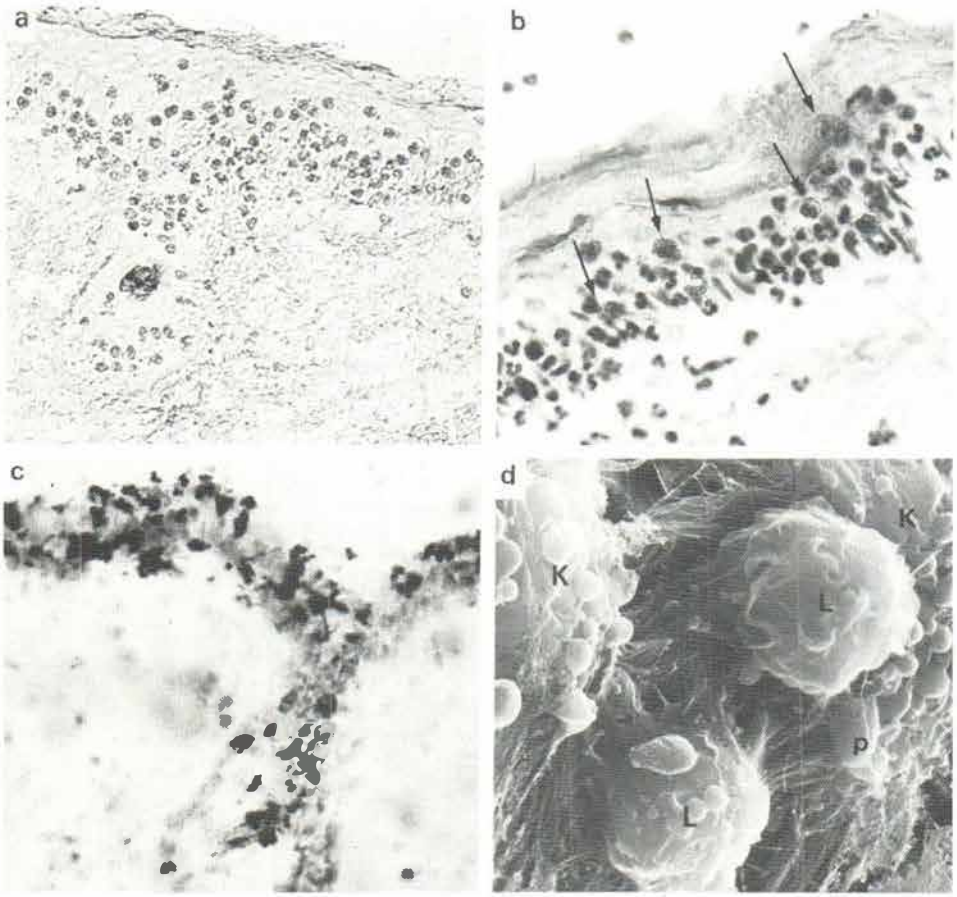

Fig. 1. (a) Leukocytes attach specifically to the epidermis in the tissue sections pretreated with pemphigus antibodies and complement (indirect method, unstained, ×100). (*b*) Eosinophils (*arrows*) as well as neutrophils attach to the epidermis in the lesional skin biopsy (direct method, stained wtih HE, ×200). (*c*) Leukocytes bound to the epidermis in the lesional skin incorporate NBT dye complexes into the cytoplasms and formed blackish blue formazan crystals. Note the other leukocytes randomly adherent to the tissue section, showing little incorporation of NBT dye complexes (direct method, stained with methyl-green, ×200). (*d*) Leukocytes (*L*) attach to the ICS between keratinocytes (*K*). Platelets (*p*) also adhere to the surface of keratinocytes (×4.000).

−70°C. Antibody titers and complement-fixing antibody titers of those sera were determined by the indirect IF staining and by the complement IF staining test (7), respectively. Human skin specimens described above were utilized as substrates and fluorescein isothiocyanate-labelled antihuman IgG and C3 sera produced by MBL (Nagoya, Japan) were used for the staining. 1 : 4 diluted fresh normal human serum was used as a complement source for the complement IF study. Control serum samples obtained from healthy volunteers were stocked in the same manner.

Preparation of leukocytes

Peripheral blood leukocytes were obtained from normal volunteers and patients with non-pemphigus dermatoses by dextran sedimentation. Leukocyte-rich plasma was removed and centrifuged at 1000 rpm for 5 min. The supernatant was used later as a complement source. Residual red blood cells were shock-lysed by 0.2% aqueous sodium chloride, and then isotonicity was reestablished by adding 1.6% sodium chloride. After centrifuging, leukocytes were resuspended in Hanks' solution at a concentration of $2–3 \times 10^7$/ml, with and without fresh plasma as a complement source, for the indirect leukocyte attachment test and for the direct method, respectively. Viability of isolated leukocytes examined by trypanblue-dye exclusion was greater than 97%.

Leukocyte attachment test

Indirect method: The leukocyte attachment test was carried out on normal human skin specimens as previously described (8, 9, 10). In brief, 8 μm cryostat specimens were placed on glass slides. The tissue specimens were allowed to dry for 30 min and then washed for 15 min in phosphate buffered saline (PBS). They were incubated with 2 drops of 1 : 2 diluted pemphigus serum or control serum for 30 min in a moist chamber, and rinsed in PBS for 15 min. After incubation with 20% fresh plasma in PBS for 30 min, vinyl tape strips were taped around the tissue specimens, and then a cover glass was placed on the vinyl tape strips to make a gap between the tissue specimens and the glass. The cell suspension was poured into the gap to cover the tissue specimens, and then 45 minutes' incubation was performed at 37°C in a moist chamber.

Direct method: Eight μm crystat sections of the perilesional skin biopsies were placed on the glass slides and allowed to dry. After rinsing in PBS for 15 min, the tissue sections were incubated with leukocytes in the same manner as described above, but adding no complement source in this study.

Following incubation, the tape strips and cover glasses were removed and nonadherent cells were washed away with PBS.

Nitroblue tetrazolium (NBT) test

The NBT test was performed to evaluate both phagocytotic activity and respiratory burst of leukocytes bound to the epidermis by complement-fixing pemphigus antibodies. Following 45 minutes' incubation in the process of leukocyte attachment test, excess cell suspension was removed gently. The solution containing 0.6 ml of 0.28% NBT in saline, 0.5 ml of heat-inactivated serum and 0.3 ml of saline was poured into the gap between the cover glass and the glass slides, and incubated further for 15 min at 37°C. Stimulated leukocytes incorporated the NBT dyes into phagosomes and, after lysosomal fusion, intracellular reduction results in the formation of blackish blue formazan crystals. Formazan-forming leukocytes attached to the ICS of epidermis were easily detectable under a light microscope in contrast to those which adhered nonspecifically to the glass slides and to the other portions of the tissue specimens.

Scanning electron microscopic study

After finishing the leukocyte attachment test, the tissue specimens were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide. The tissue sections were coated with Au and observed under JEM-35 scanning electron microscope.

RESULTS

Induction of leukocyte attachment at the epidermis

In the indirect method, when leukocytes suspended in Hanks'solution with fresh plasma were incubated with tissue specimens pretreated with pemphigus antibodies and complement, they were noted to attach specifically to the epidermis (Fig. 1a). When they were

Table I. *Results of the indirect leukocyte attachment test*

HP: herpetiform pemphigus, PF: pemphigus foliaceus, PV: pemphigus vulgaris, ICS-Ab: antibodies to the ICS of epidermis, C': complement

Cases	ICS-Ab titer	C'-fixing Ab titer	Leukocyte attachment
1. Y. M. (HP)	10>	10>	−
2. M. M. (PF)	80	20	+
3. A. S. (PF)	80	40	+
4. K. S. (PF)	20	10>	+−
5. M. K. (PF)	320<	80	+
6. T. N. (PV)	320<	80<	+
7. K. T. (PV)	80	10>	+
8. K. H. (PV)	160	10	+−
9. T. K. (PV)	320<	20	+
10. N. K. (PV)	160	20	+

incubated with tissue specimens treated with control sera or incubated with those not treated with complement sources, leukocytes could not attach to the epidermis specifically. The induction of specific leukocyte attachment was observed in 7 out of 10 serum samples taken from the patients. A comparison between pemphigus antibody titers, complement-fixing antibody titers, and leukocyte attachment was summarized in the Table I. In general, the higher the titers of pemphigus antibodies, the more effective in inducing leukocyte attachment in vitro.

In the direct method, although deposits of both IgG and C3 were detected in all the seven biopsy specimens, 5 of them could hardly mediate attachment phenomenon. The remaining 2 biopsy specimens induced leukocyte attachment at the epidermis, but nonspecific adhesion of leukocytes to the whole tissue specimens was rather prominent compared with those of the indirect method (Table II).

Hematoxylin-eosin staining of these sections showed that the leukocytes attached to the epidermis were neutrophils, eosinophils and a small number of monocytes. Platelets were also found to attach to the epidermis (Fig. 1*b*).

The NBT test

The NBT test was performed right after both the direct and indirect leukocyte attachment test, which revealed that leukocytes bound specifically to the epidermis were found to have blackish blue formazan crystals in the cytoplasms. However, leukocytes randomly adherent to the glass slides and tissue specimens showed hardly any such crystals (Fig. 1*c*).

Scanning electron microscopic study

It was found that when there took place specific attachment of leukocytes, they attached to the ICS of keratinocytes, where IgG and C3 were detectable, by their pseudopodia (Fig. 1*d*). Platelets also adhered to the epidermis but not specifically to the ICS.

DISCUSSION

Acantholysis constitutes an essential histologic change in pemphigus. It can be produced in vitro by only IgG fraction of pemphigus antibodies without complement or any preceding inflammatory processes (5, 6). In contrast, eosinophilic spongiosis (1), which consists of a prominent infiltration of both eosinophils and neutrophils in the epidermis, is one of the characteristic histologic patterns noted in pemphigus. The results of the present study

Table II. *Results of the direct leukocyte attachment test*

PV: pemphigus vulgaris, PF: pemphigus foliaceus

Cases	ICS deposits IgG	C3	Leukocyte attachment
1. K. O. (PV)	+	+	−
2. M. T. (PV)	+	+	+−
3. T. N. (PV)	+	+	+−
4. K. S. (PF)	+	+	−
5. A. S. (PF)	+	+	+
6. M. K. (PF)	+	+	−
7. M. M. (PF)	+	+	+

suggest that complement activation by pemphigus antibodies mediates the induction of an inflammatory change in the epidermis and that subsequent interaction between complement components and polymorphonuclear leukocytes produces epidermal changes designated as eosinophilic spongiosis.

As shown previously (3, 4), the present study also confirmed that pemphigus antibodies have the capability of fixing complement. Moreover, the leukocyte attachment test demonstrated clearly that pemphigus antibodies bound to the ICS of epidermis in vivo as well as in vitro mediate attachment of leukocytes to the epidermis via complement activation in the same way as noted in bullous pemphigoid (8, 9).

The scanning electron microscopic study showed that the binding site of the leukocytes is the ICS of epidermis, the site of reaction of pemphigus antibodies and complement. Both neutrophils and eosinophils were found to attach specifically to the ICS by their complement receptors. These findings suggest that when pemphigus antibodies bound to the ICS of epidermis activate complement sufficiently, they attract leukocytes into the epidermis and mediate immune adherence of leukocytes at the ICS. We confirmed by in vitro chemotaxis study using such skin samples that they actually provoke chemotaxis of neutrophils and eosinophils in vitro (unpublished data). Furthermore, the results of the NBT test demonstrated that leukocytes bound to the ICS were stimulated to phagocytosis with subsequent production of superoxide. This enhanced leukocyte function may cause a tissue damage in the leisonal epidermis to produce such a change as eosinophilic spongiosis.

As shown in the Table I, sera containing high titers of pemphigus antibodies were more effective in inducing leukocyte attachment in vitro. Nevertheless, eosinophilic spongiosis is known to develop in patients with a relatively mild form of pemphigus (1). The rather low incidence of positive leukocyte attachment in the direct method when compared with that of the indirect method, suggest that in vivo some inhibitory factors such as β1H globulin (11) may down-regulate the complement activation in actual lesional skin. Recently, Anhalt et al. (12) reported interesting findings that both eosinophilic spongiosis and acantholytic, noninflammatory pemphigus lesions were observed in the same mice experimentally given lower doses of pemphigus antibodies intraperitoneally than those required to produce solely the noninflammatory acantholytic lesions.

Further investigation on the interaction between pemphigus antibodies, complement and some inhibitory or amplifying factors occurring in the epidermis is required for the clarification of the pathomechanism underlying the inflammatory processes in pemphigus.

ACKNOWLEDGEMENT

This work was supported by Grant-in-Aid for Scientific Research 457269 and 557252, and Grant-in-Aid for Encouragement of Young Scientist 58770730 from the Ministry of Education, Japan.

REFERENCES

1. Emmerson RW, Wilson-Jones E. Eosinophilic spongiosis in pemphigus. A report of an unusual histologic change in pemphigus. Arch Dermatol 1968; 97: 252–267.
2. Jablonska S, Chorzelski TP, Beutner EH, Chorzelska J. Herpetiform pemphigus. A variable pattern of pemphigus. Int J Dermatol 1975; 14: 353–359.
3. Hashimoto T, Sugiura M, Kurihara S, Nishikawa T. In vitro complement activation by intercellular antibodies. J Invest Dermatol 1982; 78: 316–318.
4. Nishikawa T, Kurihara S, Harada T, Sugiura M, Hatano H. Capability of complement fixation of pemphigus antibodies in vitro. Arch Dermatol Res 1977; 260: 1–6
5. Barnett ML, Beutner EH, Chorzelski TP. Organ culture studies of pemphigus antibodies. 2.

Ultrastructural comparison between acantholytic changes in vitro and human pemphigus lesions. J Invest Dermatol 1977; 68: 265–271.
6. Schilz JR, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. J Invest Dermatol 1976; 67: 254–260.
7. Jordon RE, Sams WM Jr, Beutner EH. Complement immmunofluorescent staining in bullous pemphigoid. J Lab Clin Med 1969; 74: 548–556.
8. Gammon WR, Lewis DM, Carlo JR, Sams WM Jr, Wheeler CE Jr. Pemphigoid antibody mediates attachment of peripheral blood leukocytes at the dermal-epidermal junction of human skin. J Invest Dermatol 1980; 75: 334–339.
9. Iwatsuki K, Tagami H, Yamada M. Induction of leukocyte adherence at the basement membrane zone with subsequent activation of their metabolic pathway by pemphigoid antibodies and complement. Acta Derm Venereol (Stockh) 1983; 63: 290–295.
10. Yamamoto T, Kihara I, Morita T, Oite T. Attachment of polymorphonuclear leukocytes to glomeruli with immune deposits. J Immunol Method 1979; 26: 315–323.
11. Nishikawa T, Sugiura M, Hashimoto T Kurihara S, Tamura N. Demonstration of β1H globulin in pemphigus. Acta Derm Venereol (Stockh) 1983; 63: 39–43.
12. Anhalt GJ, Labib RS, Voorhees JJ, Beals TF, Diaz LA. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N Engl J Med 1982; 306: 1189–1196.