macule, we do not consider PUVA to be a useful treatment, as its effectiveness was minimal.

The facts, in most reported cases, that there were no melanocytes, as in the first case in this study, and that even though there was an area with regularly distributed melanocytes in the second case, there was also an area with no melanocytes in the same lesion, suggest that the primary defect in this disorder is the absence of melanocytes in the white macule. However, melanocytes may be present and regularly distributed in a small area of a white macule of piebaldism.

REFERENCES

1. Ortonne J-P, Mosher DB, Fitzpatrick TB. Piebaldism, Vitiligo and Other Hypomelanosis of Hair and Skin, 1st edn. New York: Plenum Medical Book Co., 1983: 310–336.
2. Pearson K. Note on the Honduras piebald. Biometrika 1913; 9: 330–331.
3. Davis BK, Verdol LD. Expansion and contraction of hypomelanotic areas in human piebaldism. Hum Genet 1976; 34: 163–170.
4. Hori Y. Partial albinism. Hifubyo-shinryo 1983; 5: 619–622 [in Japanese].
5. Ishii N, Sasaki T, Kawaguchi H, Uchiyama M, Nakajima H. Two cases of piebaldism. Jpn J Clin Dermatol 1985; 39: 469–473 [in Japanese].
6. Jimbow K, Fitzpatrick TB, Szabo G, Hori Y. Congenital circumscribed hypomelanosis: A characterization based on electron microscopic study of tuberous sclerosis, nevus depigmentosus and piebaldism. J Invest Dermatol 1975; 64: 50–62.
7. Comings DE, Odland GF. Partial albinism. JAMA 1966; 195: 111–115.
8. Breathnach AS, Fitzpatrick TB, Wyllie LM-A. Electron microscopy of melanocytes in human piebaldism. J Invest Dermatol 1965; 45: 28–37.
9. Kugelman TP, Lerner AB. Albinism, partial albinism, vitiligo. Yale J Biol Med 1961; 33: 407–414.
10. Grupper C, Pruniéras M, Hincky M, Garelly E. Albinisme partiel familial (piebaldisme): Étude ultrastructurale. Ann Dermatol Venereol 1970; 97: 267–286.
11. Hamada A, Nishida N. Partial albinism. Rinsho Derma (Tokyo) 1968; 10: 292–300 [in Japanese].
12. Tanaka H. Studies on pigmentation in nevoid, neoplastic, and other diseases of the skin. Jap J Dermatol 1958; 68: 1–59 [in Japanese].
13. Hayashibe K, Mishima Y. Tyrosinase-positive melanocyte distribution and induction of pigmentation in human piebald skin. Arch Dermatol 1988; 124: 381–386.
14. Mosher DB, Fitzpatrick TB. Piebaldism. Arch Dermatol 1988; 124: 364–365.

Plasmin-like Proteinase Associated with High Molecular Weight Complexes in Blister Fluid of Bullous Pemphigoid

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Blister fluid from tense bullae of 10 patients with bullous pemphigoid was investigated using a radial caseinolysis assay and zymography. Proteolytic activity, varying from 4.7 to 10 μg/ml, was found in 3 out of 10 patients, by using the caseinolysis assay. Zymography revealed that a major part of this caseinolytic activity co-migrated with plasmin standard. In addition, in the zymography, proteolytically active high molecular weight complexes were seen. This characteristic pattern was seen in the zymography of both positive and negative samples in the caseinolysis assay. These high molecular weight complexes were not seen in the zymography of the blister fluid of 2 patients with epidermolysis bullosa or in the suction blister fluid of 3 healthy control patients. These findings suggest that plasmin is generated at some phase of the blister formation, being possibly involved in the pathomechanism of bullous pemphigoid. ***Key words: Proteolysis; Zymography; Dermo-epidermal separation***

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Several studies (1, 2) have suggested that the blister formation in pemphigoid involves binding of the antibodies to the bullous pemphigoid antigen in the basement membrane which initiates complement activation. This leads to production of several inflammatory mediators and migration of inflammatory cells which release inflammatory mediators and proteolytic enzymes. The ultimate result is a subepidermal blister. This proposed model contrasts with theory of blister formation in pemphigus vulgaris, which

occurs in a complement-independent manner, through activation of plasminogen to plasmin (3).

The purpose of the present study was to investigate the involvement of proteolysis and especially the role of plasmin in the blister formation in bullous pemphigoid using radial caseinolysis assay and zymography. The radial caseinolysis assay is a sensitive method for detection of net proteolytic activity of the samples. The proteinases in the sample can be identified by zymography. Samples with no net proteolytic activity can also be studied since, during the process, proteinases are separated from their inhibitors and can be detected.

MATERIALS AND METHODS

Blister fluid from fresh tense bullae generated within 1–2 days in 10 patients with bullous pemphigoid was collected with a syringe for analysis. The samples were centrifugated in the cytocentrifuge for 10 min at 1000 rpm in order to study the cellular content of the blister fluid. The cells were stained using May-Grünwald and Giemsa stains. The blister fluid was stored in −20°C until analysed.

Protease assays

Net proteolytic activity of the blister fluid was determined using a modification of the radial caseinolysis procedure (4) described in detail elsewhere (5). In the assay, briefly, 5 µl specimens are added to wells in bovine milk casein-containing agarose using plasmin (25 casein units/mg; Kabi Diagnostica, Stockholm) as standard. The diameters of the lytic zones were recorded after incubation at 37°C for 48 h. The detection level of the assay is 0.1 µg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

Molecular weights of proteinases were determined using SDS–PAGE on slab gels *ad modum* Laemmli (6) and by zymography (7). The acrylamide concentration was 3.3% in the stacking gel and 5–16% in the separating gel. The samples dissolved in Laemmli's sample buffer were analysed under non-reducing conditions. After electrophoresis, the gels were thoroughly washed in Triton X-100 detergent-containing buffer and overlaid on casein–agarose indicator gel. The lytic zones developed within 24–48 h of incubation at 37°C. To determine the molecular size of proteinases commercially available, low molecular weight protein markers (Pharmacia, Uppsala, Sweden) and purified human plasmin (Kabi Diagnostica) were used.

RESULTS

Proteolytic activity was found in the blister fluid of 3 out of 10 patients with bullous pemphigoid. The proteolytic activities in these three samples were 10, 5.5 and 4.7 µg/ml. No net proteolytic acitivity was found in the other samples (the detection limit was 0.1 µg/ml of plasmin). Zymography revealed that the blister fluid of both patients with proteolytic activity and patients with no net proteolytic activity in the caseinolysis assay contained a proteinase co-migrating with plasmin standard (Fig. 1). In addition there was high molecular-weight proteinase-containing material which produced a characteristic pattern (Fig. 1).

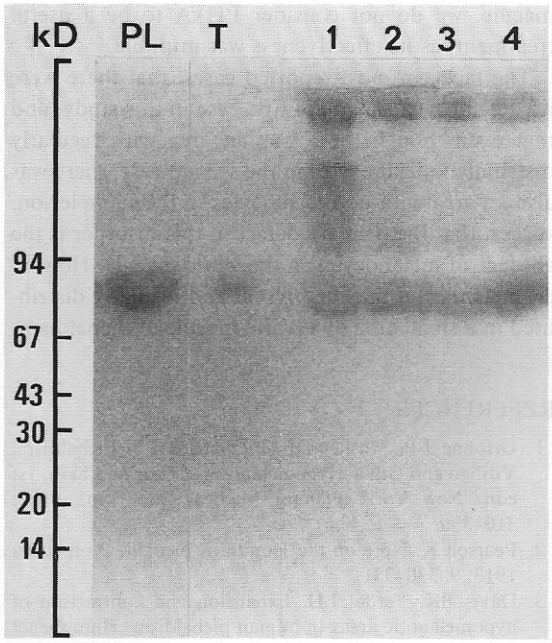

Fig. 1. Zymographic analysis of blister fluid of pemphigoid. *1–4*, indicate different samples. Sample 1 showed proteolytic activity in caseinolysis assay, but samples 2–4 were negative. *PL*, Plasmin standard and T plasmin in tear fluid after ocular challenge (used as a control). A major part of the proteinases detected co-migrated with plasmin standard. All blister fluid samples show the pattern of high molecular-weight proteinases. The molecular weights are expressed as kilodaltons (kD).

The suction blister fluid of 3 healthy control patients showed no caseinolytic activity. Zymography of these samples revealed a proteinase co-migrating with plasmin standard, but no high molecular weight complexes were seen. One blister fluid sample from 2 patients with epidermolysis bullosa showed a slight proteolytic activity of 0.5 µg/ml in the caseinolysis assay, but the other one was negative. Zymography revealed that both samples contained a proteinase co-migrating with the plasmin standard. However, no high molecular weight complexes were detected.

DISCUSSION

The present study showed that the blister fluid of 3 out of 10 patients with bullous pemphigoid possessed proteolytic activity, determined using a radial caseinolysis assay. This is in agreement with a previous observation that varying degrees of caseinolytic activity can be detected in bullous pemphigoid (8). A major part of the caseinolytic activity in the present study was due to plasmin-like proteinase, as revealed by zymography. Plasmin-like proteinase was also found in samples with no proteolytic activity in direct caseinolysis assay. In these blister fluids, plasmin and other proteinases were bound to inhibitors, giving negative results in the caseinolysis assay. In the SDS–PAGE preceding zymography, proteinases are separated from their inhibitors and can be detected even in samples with no net proteolytic activity. In all pemphigoid samples, plasmin was found to be associated with high molecular weight proteinases or proteinase aggregates. The nature and role of these high molecular weight proteinases is still obscure. It is possible that they represent complexes of plasmin and inhibitors, or they may be complexes of plasmin and some blister fluid components. It remains to be seen whether the pattern they formed is characteristic of pemphigoid. Such a pattern was not seen in the blister fluid of epidermolysis bullosa. Nor was it detected in the zymography of the suction blister fluid of 3 control persons, although a plasmin-like proteinase (obviously derived from the blood) was found in these samples.

The above findings suggest that the high molecular weight proteinase complexes are produced locally during the blister formation. Furthermore, since even in some fully developed blisters, proteolytic activity was still present, it is probable that plasmin detected in all samples is at least partly a result of local activation of plasminogen to plasmin and not only derived from the blood during blister formations. It is possible that this plasmin may be involved in dermo-epidermal separation in pemphigoid, as it is involved in acantholysis in pemphigus vulgaris.

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REFERENCES

1. Naito K, Morioka S, Ogawa H. The pathologic mechanism of blister formation in bullous pemphigoid. J Invest Dermatol 1982; 79: 303–306.
2. Jordon R, Kawana S, Fritz K. Immunopathologic mechanism in pemphigus and bullous pemphigoid. J Invest Dermatol 1985; 85 (Suppl): 72–78.
3. Hashimoto K, Shafran K, Webber P, Lazarus G, Singer K. Anti-cell surface pemphigus autoantibody stimulator plasminogen activator activity of human epidermal cells. J Exp Med 1983; 157: 259–272.
4. Salonen E-M, Tervo T, Törmä E, Tarkkanen A, Vaheri A. Plasmin in tear fluid of patients with corneal ulcers: basis for new therapy. Acta Ophthalmol 1987; 65: 3–12.
5. Saksela O. Radial caseinolysis in agarose: a simple method for detection of plasminogen activator in the presence of inhibitory substances and serum. Anal Biochem 1981; 111: 276–282.
6. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 1970; 227: 680–685.
7. Granelli-Piperno A, Reich E. A study of proteases and protease–inhibitor complexes in biological fluids. J Exp Med 1978; 146: 223–234.
8. Fräki J. Blister fluid proteinases in bullous skin diseases. Clin Exp Dermatol 1979; 4: 471–475.