0022-202X/83/8103-0261$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:261–266, 1983

Vol. 81, No. 3
Printed in U.S.A.

Demonstration of Collagenase and Elastase Activities in the Blister Fluids from Bullous Skin Diseases. Comparison Between Dermatitis Herpetiformis and Bullous Pemphigoid*

AARNE I. OIKARINEN, M.D., PH.D., JOHN J. ZONE, M.D., A. RAZZAQUE AHMED, M.D., URPO KIISTALA, M.D., PH.D., AND JOUNI UITTO, M.D., PH.D.

Division of Dermatology, Department of Medicine, University of California School of Medicine, Los Angeles, Harbor-UCLA Medical Center (AIO, JU), Torrance, California; Division of Dermatology, Department of Medicine, University of Utah (JJZ), Salt Lake City, Utah; Combined UCLA-VA Dermatology Programs, University of California (ARA), Los Angeles, California, U.S.A.; Department of Dermatology, University of Helsinki (UK), Helsinki, Finland

We have investigated potential mechanisms for blister formation by assaying proteolytic enzymes in the blister fluids of patients with various bullous diseases. Blister fluids were obtained from patients with dermatitis herpetiformis (DH), bullous pemphigoid (BP), chronic bullous disease of childhood (CBDC), and pemphigus vulgaris (PV). The cells were recovered by centrifugation, and the supernatants as well as the cell pellets were assayed first for collagenase activity using [^{3}H]proline-labeled type I collagen as substrate. Collagenase activity could be detected in most cases with DH, BP, and CBDC, while no activity was found in 2 cases of PV or in 5 control blister fluids obtained from suction blisters induced in healthy control subjects. Elastase activity was assayed in the same blister fluids by using a synthetic substrate succinyl-(L-alanyl)$_3$-paranitroanilide or soluble [^{14}C]valine-labeled tropoelastin. High levels of elastase activity were present in all DH patients, while lower, but clearly detectable, levels were found in BP, CBDC, and PV. The enzyme activity in BP was inhibited by Na_2EDTA, but not by phenylmethylsulfonyl fluoride (PMSF), and Ca^{2+} stimulated the activity, suggesting that the enzyme in BP was a metalloproteinase. In cell-free supernatants of the DH blister fluids, the elastase activity was markedly decreased by PMSF, indicating that most of the enzyme activity was due to a serine protease. The cells recovered from DH blister fluids also contained high levels of elastase activity which could be inhibited by PMSF but not by Na_2EDTA. Thus, in DH, the elastase activity is probably derived from polymorphonuclear leukocytes abundantly present in the lesions. The results indicate that active proteases are present in the blister fluids of skin diseases, and they may play a mechanistic role in the blister formation by degrading connective tissue components of the dermis and the dermal-epidermal junction.

Manuscript received February 23, 1983; accepted for publication May 4, 1983.

This work was supported by U. S. Public Health Service, National Institutes of Health grants GM-28833, AM-28450, AG-03172, AM-28412, and 5S-07-RR-05354, by a Health Resources grant RR-64, by a grant from the March of Dimes–Birth Defect Foundation, and by the Medical Research Council of the Academy of Finland. Dr. Oikarinen is a UCLA Silbert International Fellow and Dr. Uitto is a recipient of the Research Career Development Award 5-K04-AM-00897 from the National Institutes of Health.

* A preliminary report of this study was given at the 44th Annual Meeting of The Society for Investigative Dermatology, Inc., Washington, D.C., April 1983 [1].

Reprint requests to: Dr. Aarne I. Oikarinen, Division of Dermatology, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, California 90509.

Abbreviations:
BDE: bullous drug eruption
BMZ: basement membrane zone
BP: bullous pemphigoid
CBDC: chronic bullous disease of childhood
DH: dermatitis herpetiformis
DTT: dithiothreitol
ICS: intercellular substance
Na_2EDTA: disodium ethylenediaminetetracetic acid
NEM: N-ethylmaleimide
PMSF: phenylmethylsulfonyl fluoride
PV: pemphigus vulgaris
SAPNA: succinyl-(L-alanyl)$_3$-paranitroanilide
SBTI: soybean trypsin inhibitor
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEN: toxic epidermal necrolysis

Bullous diseases are a group of clinical conditions characterized by destruction of various components of the epidermis or dermis [2,3]. This destruction leads to separation of tissues, resulting in the formation of fluid-filled blisters or bullae. The events leading to bulla formations are frequently mediated by immunologic factors [3,4]. For example, previous studies have shown that an IgG fraction purified from the sera of patients with pemphigus vulgaris (PV) is able to elicit acantholysis in vitro, mimicking the histopathologic changes found in this disease in vivo [5,6]. It has been suggested that the acantholysis in vitro is independent of complement, and may be mediated by activation of skin proteinases [6–9]. In contrast to PV, the IgG fraction isolated from the sera of patients with bullous pemphigoid (BP) has failed to produce the dermal-epidermal separation in vitro [10]. However, incubation of skin explants with pemphigoid blister fluid results in the separation of the epidermis and dermis [11]. This separation was shown to be complement-dependent, and it could be inhibited by α_2-macroglobulin, again suggesting that proteolytic enzymes may participate in the reaction [11]. Similar in vitro data are not available on dermatitis herpetiformis (DH), although the alternative pathway for complement activation has been shown to occur in the skin of these patients [12]. In addition to these studies, increased collagenase activity may play a role in tissue destruction in recessive dystrophic epidermolysis bullosa, a heritable disease without immunologic aberrations [13–15].

In this study, we have approached the mechanisms of blister formation by assaying proteolytic enzymes in the blister fluids of patients with various bullous diseases. The results indicate that particularly high levels of collagenase and elastase activity are present in DH. Characterization of these enzymes suggested that they are derived from polymorphonuclear leukocytes present in the lesions. Thus, these enzymes may directly participate in the blister formation in DH.

PATIENTS AND METHODS

Patients

A total of 22 patients with various forms of bullous diseases were included in the study. The clinical data on these patients are presented

TABLE I. *Clinical data on patients with bullous disease*

Patient number	Diagnosis[a]	Age (years)/sex	Immunofluorescence findings		Clinical activity[c]
			Direct (pattern/AB)[b]	Indirect (titer)	
1	DH	45/M	Granular/IgA	—	Mild
2	DH	62/F	Granular/IgA	—	Moderate
3	DH	32/F	Granular/IgA	—	Severe
4	DH	28/F	Granular/IgA	—	Severe
5	DH	68/M	Granular/IgA	—	Severe
6	DH	56/M	Granular/IgA	—	Severe
7	BP	72/F	BMZ/IgG + C_3	1:1280	Severe
8	BP	74/F	BMZ/IgG	0	Mild, localized to 1 foot
9	BP	72/M	BMZ/IgG + IgM	1:640	Severe
10	BP	49/M	BMZ/IgG	1:320	Moderate
11	BP(C)	66/F	BMZ/IgG	0	Severe
12	BP	84/M	BMZ/IgG + C_3	1:160	Moderate
13	BP	83/F	BMZ/IgG	1:1280	Moderate
14	BP	76/M	BMZ/IgG + C_3	1:640	Moderate
15	PV	60/F	ICS/IgG	1:320	Severe
16	PV	51/F	ICS/IgG	1:640	Moderate
17	CBDC	6/M	Linear/IgA	—	Severe
18	CBDC	2/M	Linear/IgA	—	Moderate
19	BDE	39/F	—	—	Severe
20	TEN	42/M	—	—	Severe
21	Burn blister	26/F	—	—	
22	Burn blister	60/M	—	—	

[a] DH, dermatitis herpetiformis; BP, bullous pemphigoid; BP(C), cicatricial bullous pemphigoid; PV, pemphigus vulgaris; CBDC, chronic bullous disease of childhood; BDE, bullous drug eruption; TEN, toxic epidermal necrolysis.

[b] The pattern indicates positive immunofluorescence staining with the antibody (AB) tested; BMZ, basement membrane zone; ICS, intercellular substance.

[c] Clinical activity in DH patients was graded on the basis of the severity of the flare after withdrawal of medication. In PV, BP, CBDC, TEN, and BDE patients, clinical activity was graded before treatment.

in Table I. All of the DH patients had been off all suppressive therapy for at least 72 h before collecting the blister fluid samples. From BP, PV, and chronic bullous disease of childhood (CBDC) patients blister fluids were collected before any specific treatment. For comparison, blister fluids were obtained from suction-induced blisters in 5 healthy volunteers [16,17]. Fluids from 2 patients with burn blisters, 1 with toxic epidermal necrolysis (TEN), and 1 with bullous drug eruption (BDE) were also used as controls.

Preparation of Samples

The blister fluids were collected with a sterile syringe and stored at −20°C. In cases of DH, the blister fluids were collected with a capillary tube and pooled from several blisters. The cells were immediately separated by cytocentrifugation, and the relative number of polymorphonuclear leukocytes was determined by counting the cells on microscope slides stained with Wright's stain. The cell pellets and the supernatants were stored frozen.

For enzyme assay, the unfractionated blister fluids or the supernatants obtained by a centrifugation were thawed and aliquots were taken for collagenase and elastase assays. The cell pellets were homogenized with a glass-glass homogenizer in 50 mM Tris-HCl, pH 7.8, at 4°C, and the homogenates were centrifuged at 15,000 *g* for 20 min. The supernatant was removed and aliquots were taken for enzyme assays.

Enzyme Assay

Collagenase activity was determined by incubation of blister fluids with ^{3}H-labeled type I collagen, prepared as described previously [18]. In a standard incubation, 3–40 μl of the blister fluid was incubated in 10 mM $CaCl_2$, 0.15 M NaCl, and 50 mM Tris-HCl, pH 7.6, and containing 20 μg/ml bovine serum albumin, in a final volume of 100 μl, for 4 h, at 37°C. The reaction was terminated by the addition of Na_2EDTA to a final concentration of 40 mM. The collagen degradation was determined by a method recently developed in our laboratory [18]. This method is based on digestion of the collagen cleavage products by a mixture of trypsin and α-chymotrypsin into trichloroacetic acid (TCA)-soluble peptides, using conditions under which the undegraded collagen substrate resists proteolysis. The collagen cleavage products, following incubation with blister fluids, were also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19]). The ^{3}H-labeled collagen peptides were visualized by fluorography [20].

For assay of elastase activity, 3 independent methods were used. First, a synthetic substrate, succinyl-(L-alanyl)$_3$-paranitroanilide (SAPNA) in 1.25 mM concentration, in 50 mM Tris-HCl, pH 7.8, was incubated with the enzyme preparations at 37°C for varying time periods [21]. The reaction was monitored by the change in the absorbance at 410 nm, and the enzyme activity was expressed as hydrolysis of SAPNA, nmol/h/ml blister fluid. Blanks without added enzyme were included in all incubations. The second method for detection of elastase activity employed as substrate ^{14}C-labeled tropoelastin, prepared by incubation of pig aorta explants with [^{14}C]valine. The ^{14}C-labeled tropoelastin was purified by propanol-butanol extractions [22]. Twenty-microliter aliquots of the substrate, containing 3.2×10^3 cpm, were incubated with 3–10 μl of the enzyme preparation in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.8, in a final volume of 70 μl, for 1–8 h, at 37°C. The degradation of the substrate was monitored by SDS-PAGE, followed by fluorographic visualization of the radioactive peptides [19,20]. The ^{14}C-peptides were then studied by scanning the fluorograms with an integrating densitometer (Gelman, ACD 18). The third method for detection of elastase activities consisted of incubation of blister fluid with orcein-elastin (Millipore Corp.), 2 mg/ml, at 37°C [23]. The degradation of the insoluble elastin was monitored by release of orcein-containing peptide fragments to 3000 *g* supernatant of the incubate, as detected by absorbance at 590 nm.

Statistics

The statistical analyses were performed using Student's two-tailed *t*-test.

RESULTS

In the first set of experiments, the activity of collagenase was measured in blister fluids employing a highly sensitive and specific assay method. The results indicated that significant levels of collagenase activity could be detected in blister fluids in 3 of 6 patients with DH. Similar levels of collagenase activity could be detected in 1 of 2 patients with CBDC, in 5 of 8 patients with BP, and in the 1 patient with TEN. No detectable activity was found in the 2 cases of PV, 1 case of BDE, nor in any of the suction blister specimens.

In 2 cases with DH, the cells were immediately separated from the blister fluid by centrifugation. The cell pellets contained over 95% neutrophils in both cases. The collagenase was then separately measured in the cell pellet and the supernatant.

TABLE II. *Distribution of collagenase and elastase activity between cell pellet and the supernatant of blister fluid samples obtained from two patients with dermatitis herpetiformis*

Patient number[a]	Sample fraction[b]	Collagenase activity[c]			Elastase activity[d]		
		Units × 10^{-3}/mg protein	Units × 10^{-3}/ml[e]	% of total	nmol/h/mg protein	nmol/h/ml[e]	% of total
DH 5	Supernatant	2.2	24.2	36.4	14.1	165.0	77.6
DH 5	Cell pellet	16.7	42.3	63.6	18.6	47.7	22.4
DH 6	Supernatant	4.4	63.8	29.4	13.3	193.0	91.7
DH 6	Cell pellet	67.8	153.1	70.6	7.7	17.4	8.3

[a] The numbers refer to Table I.

[b] The blister fluids were collected by capillary tubes and the cells and the supernatant were immediately separated by centrifugation as indicated in *Patients and Methods*. Cell pellets consisted of over 95% neutrophils in both cases.

[c] Collagenase activity was assayed by using ^{3}H-labeled type I collagen as substrate; the values refer to the amount of collagen degraded; mean of two parallel assays.

[d] Elastase activity was assayed by using SAPNA as substrate; the values indicate the hydrolysis of this substrate, as detected by absorbance at 410 nm; mean of two parallel assays.

[e] The values indicate the activity in either cell fraction or in the supernatants recovered from 1.0 ml of the original blister fluid.

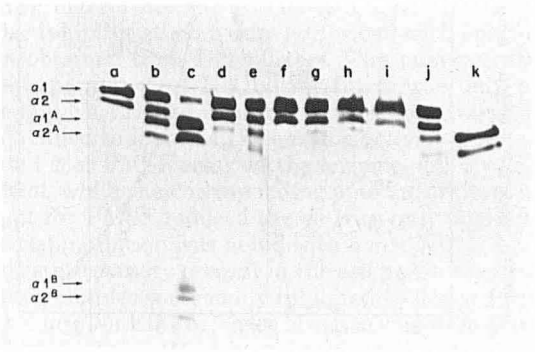

FIG 1. Demonstration of collagenase activity in blister fluids. [^{3}H]-Proline-labeled type I collagen, purified from chick embryo tendons, was incubated with blister fluid or cell homogenate recovered from blister fluids by centrifugation. The digests were electrophoresed on 8% polyacrylamide gels in SDS, and the radioactive peptides were visualized by fluorography. The *arrows* indicate the position of α1(I) and α2(I) chains as well as those of the specific degradation products, $\alpha1^A$ and $\alpha2^A$, as well as $\alpha1^B$ and $\alpha2^B$, representing 3/4 and 1/4 fragments of the corresponding α-chains, respectively. *a*, Control without enzyme. *b*, Cell homogenate from patient no. 5 with DH. *c*, Cell homogenate from patient no. 6 with DH. *d*, Cell-free supernatant from patient no. 5 with DH. *e*, Cell-free supernatant from patient no. 6 with DH. *f*, Blister fluid from patient no. 14 with BP. *g*, Blister fluid from patient no. 8 with BP. *h*, Blister fluid from patient no. 9 with BP. *i*, Blister fluid from a patient with TEN. *j*, Blister fluid from patient no. 18 with CBDC. *k*, Control digestion with partially purified leukocyte collagenase.

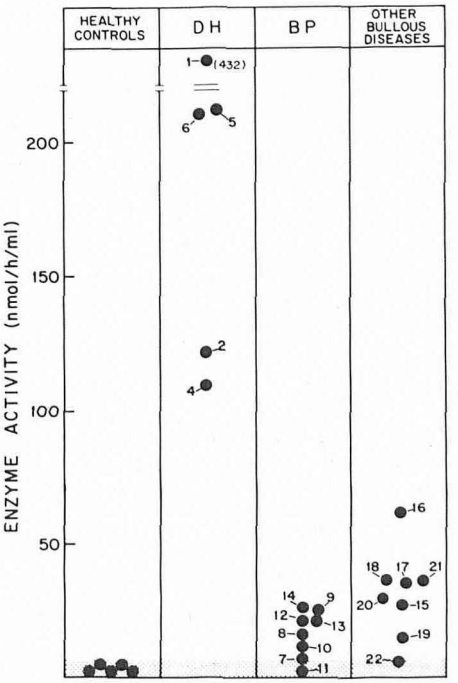

FIG 2. Elastase activity in the blister fluids from patients with bullous diseases and in suction blisters induced in healthy controls. Blister fluids were incubated with SAPNA as substrate, and the enzyme activity is expressed as hydrolysis of SAPNA, nmol/h/ml blister fluid, as monitored by absorbance at 410 nm. The individual values are means of 2–4 parallel samples. The numbers by individual values refer to patients in Table I. The mean + 2 SD limit of the healthy controls is indicated by the *hatched area.*

The results indicated that significant activity could be detected in both fractions (Table II). Calculations of the relative activity indicated that 63.6% and 70.6% of the total activity was associated with the cell pellet.

The specificity of the collagenase activity was determined by examining the cleavage products by PAGE in SDS. The results indicated that collagenase found in patients with DH, BP, TEN, or CBDC cleaved the ^{3}H-labeled collagen substrate into fragments representing 3/4 and 1/4 length of the intact molecule (Fig 1). The activity found in the cell pellets of DH blister fluids demonstrated the same specificity (Fig 1, lanes *b* and *c*).

The collagenase was further characterized by determining the effects of various protease inhibitors. Using both assay methods, a complete inhibition of the enzyme activity was noted in the presence of 20 mM Na_2EDTA and 10% human serum. No inhibition was noted in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF).

The activity of elastolytic enzymes in the blister fluids was first measured by using a synthetic substrate, SAPNA. Significant enzyme activity was detected in 5 patients with DH studied (Fig 2). Control experiments indicated that the enzyme activity in DH samples measured by hydrolysis of SAPNA, was linearly proportional to the amount of blister fluid assayed. The enzyme activity in the DH group was 219 ± 127 nmol/h/ml (mean ± SD); this value is significantly higher than that of 5 suction blister controls, the latter value being 1.8 ± 2.5 ($p < 0.005$). Detectable enzyme activity was also present in BP blister fluids (Fig 2), and the activity linearly increased with increasing amounts of sample assayed. The mean ± SD of the enzyme activity in the BP group was 16.2 ± 8.3; the value is statistically different from the suction blister controls ($p < 0.005$). Elevated activity was also found in 2 cases with PV, 2 cases with CBDC, and in 1 case each of burn blister, BDE, and TEN (Fig 2).

In 2 patients with DH, the distribution of elastase activity between the cell pellet and cell-free supernatant was also determined. The results indicated that significant activity was detected in both fractions, the relative activity in the cell pellet being 22.4% and 8.3% of the total in the 2 cases, respectively (Table II).

Since the lack of detectable enzyme activity in suction blister controls could be explained by the presence of an inhibitor, mixing experiments were subsequently performed. Addition of an equal volume of suction blister fluid to either DH or BP blister fluid did not change the activity measured without added suction blister fluid. Thus, no evidence for an inhibitor for the elastase activity was found.

The elastase activity was also assayed by incubating blister fluids with purified tropoelastin, the 68,000 dalton precursor of insoluble elastin. Incubation with samples from either DH or BP patients, resulted in degradation of tropoelastin (Fig 3). Finally, the elastase activity in BP blister fluid from patient no. 14 was demonstrated by incubation with insoluble elastin substrate containing orcein. Incubation of 60 μl of blister fluid in a final volume of 1.0 ml released orcein-containing elastin fragments, during a 24-h incubation; the increase in the absorbance at 590 nm was 0.046 per h/ml blister fluid.

The elastases detected in DH and BP blister samples were characterized first by determining the pH optimum of the enzymic reaction using SAPNA as substrate. The results indicated that the enzymes from both sources had the highest activity at the neutral pH around 7.4–7.6 (Fig 4).

The elastases were further characterized by testing the effects of various protease inhibitors on their activity. The activity found in BP blister fluids was readily inhibited with 2 mM Na_2EDTA and 1.0 mM *o*-phenanthroline (Table III). No inhibition was noted with 0.3 mM PMSF. Addition of 1 mM PMSF resulted in an apparent decrease in the enzyme activity, but

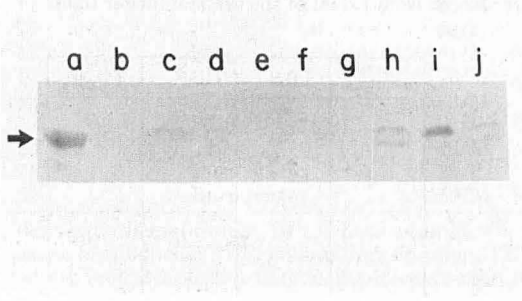

FIG 3. Degradation of tropoelastin by blister fluids from patients with DH and BP. Blister fluids were incubated for 120 min at 37°C with [^{14}C]valine-labeled tropoelastin purified as described in *Patients and Methods*. The samples were electrophoresed on 12% polyacrylamide gels in SDS, and the radioactive peptides were visualized by fluorography. The migration position of tropoelastin (68,000 daltons) is indicated by an *arrow*. *a*, Control without enzyme. *b*, Cell-free supernatant from patient no. 5 with DH. *c*, Same as *b*, but with 20 mM Na_2EDTA. *d*, Same as *b*, but with 1 mM PMSF. *e*, Cell homogenate from patient no. 5 with DH. *f*, Cell-free supernatant from patient no. 6 with DH. *g*, Cell homogenate from patient no. 6 with DH. *h*, Blister fluids from patient no. 14 with BP. *i*, Same as *h*, but with 20 mM Na_2EDTA. *j*, Same as *h*, but with 1 mM PMSF.

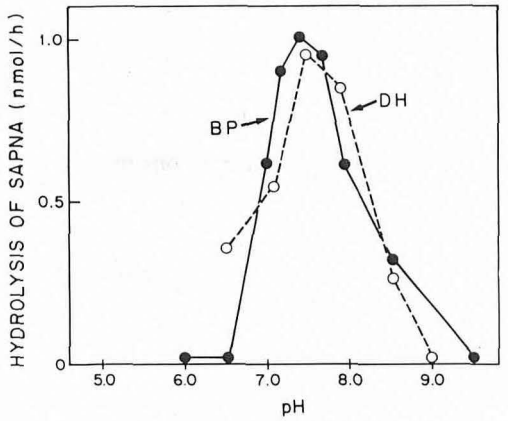

FIG 4. The effect of pH on the activity of elastase in blister fluids from patient no. 5 with DH or patient no. 14 with BP. The buffer in pH range 7.0–9.5 was 50 mM Tris-HCl, and pH 6.5 and 6.0 were adjusted by the addition of 0.1 M citrate (see [41]). In DH samples, 10-μl aliquots of cell-free supernatant were used for each point. In BP samples, 30-μl aliquots of blister fluids were used for each point. The values are expressed as hydrolysis of SAPNA, nmol/h; the individual points are means of 2 parallel assays.

TABLE III. *Effects of various protease inhibitors and calcium on the activity of elastase in blister fluids from bullous pemphigoid and dermatitis herpetiformis lesions*

Test compound added	Concentration (mM)	Elastase activity[a]					
		BP blister fluid		DH blister supernatant		DH cell pellet	
		(nmol/h/ml)[b]	(% of control)	(nmol/h/ml)[b]	(% of control)	(nmol/h/mg)[c]	(% of control)
None	—	20.4	100.0	164.0	100.0	18.6	100.0
Na_2EDTA	1.0	12.0	58.8				
Na_2EDTA	2.0	5.7	27.9	110.0	67.1	14.9	80.0
Na_2EDTA	4.0	5.1	25.0	70.5	43.0		
o-Phenanthroline	1.0	1.0	4.9				
Ca^{2+}	1.0	22.8	111.8				
Ca^{2+}	5.0	30.8	151.0	163.5	99.7		
Ca^{2+} + Na_2EDTA	5.0 + 20.0	6.9	33.8				
PMSF in propanol	0.3	19.8	97.1				
1% propanol[d]		20.4	100.0				
PMSF in propanol	1.0	11.6	56.9	55.8[e]	34.0	5.6[e]	30.0
3% propanol[d]		11.9	58.3	136.0[e]	82.9	16.5[e]	88.7
NEM	1.0	19.0	93.1				
NEM	5.0	13.3	65.2	109.9	67.0		
DTT	0.2	21.1	103.4				
DTT	1.0	16.9	82.8				
DTT	4.0	12.6	61.8				
SBTI	(100 μg/ml)	20.0	98.0				
Human serum	(10%)	19.7	96.6				

[a] Elastase activity was assayed by incubation of SAPNA in 50 mM Tris-HCl, pH 7.8.

[b] The values represent hydrolysis of SAPNA, as monitored by absorbance at 410 nm, × h^{-1} per ml of the original blister fluid.

[c] The values are elastase activity in cell pellet isolated from the DH blister fluids, as indicated in *Patients and Methods*, and are expressed per mg soluble protein.

[d] The final concentration of propanol is the same as in the corresponding sample with PMSF.

[e] In this experiment, the final concentration of propanol in both samples was 1%.

this reduction could be explained by the presence of n-propanol used as solvent for the inhibitor (Table III). No inhibition was noted with 10% normal human serum, soybean trypsin inhibitor (SBTI) (100 μg/ml), or with N-ethylmaleimide (NEM) or dithiothreitol (DTT) in 1.0 mM and 0.2 mM concentrations, respectively. A small inhibition with higher concentrations of NEM or DTT was noted, however (Table III).

Since the inhibition of the BP enzyme activity by Na_2EDTA or *o*-phenanthroline suggested that the enzyme may be a metalloproteinase, the effects of Ca^{2+} on the activity were studied. The addition of Ca^{2+} in 5 mM concentration into the standard incubation which did not contain added Ca^{2+} markedly stimulated the enzyme activity. The Ca^{2+}-dependent enhancement of the enzyme activity could be abolished by the addition of 20 mM Na_2EDTA (Table III). The inhibition of BP elastase activity by Na_2EDTA was also verified by using tropoelastin as substrate. The addition of 20 mM Na_2EDTA clearly prevented the degradation on tropoelastin to smaller peptides, while 1 mM PMSF had no effect (Fig 3, lanes *h*, *i*, *j*).

Similar inhibitor studies were performed with enzyme preparations obtained from DH blisters. The enzyme activity in cell-free supernatant of DH blister fluids was only partially inhibited by Na_2EDTA in concentrations up to 4.0 mM, and 5 mM Ca^{2+} failed to increase the enzyme activity (Table III). In contrast, 1 mM PMSF reduced the enzyme activity to 34% of the control, while the corresponding amount of propanol used as solvent for PMSF reduced the activity only slightly (Table III). Partial inhibition was noted with 5 mM NEM.

The elastase activity present in the cell pellet recovered from DH blister samples was readily inhibited by 1 mM PMSF, but not with 4 mM Na_2EDTA. These observations were noted using both SAPNA (Table III) or tropoelastin as substrate.

DISCUSSION

In this study, we have demonstrated that blister fluids obtained from lesions of various bullous diseases contain active proteases capable of degrading collagen and elastin. Particularly high levels of proteases were present in DH blister fluids. Histologically, the DH lesions are characterized by destruction of dermal components leading to formation of papillary microabscesses and subepidermal blisters [2]. Thus, it is conceivable that the active proteases demonstrated in this study mediate the tissue destruction and participate in blister formation in some bullous diseases; in others, alternate mechanisms may exist.

Histologically, the DH lesions contain abundant numbers of polymorphonuclear leukocytes [2]. Polymorphonuclear leukocytes have been shown to contain a collagenase, which cleaves collagen molecules at a specific locus located at three-quarter distance away from the amino-terminal end of the molecule [24–26]. The leukocyte collagenase is a metalloproteinase which is inhibited by metal chelators, such as Na_2EDTA, and by human serum. Similar characteristics were also present in the collagenase demonstrated in the DH blister fluids, and consequently, it is possible that polymorphonuclear leukocytes are the main source of collagenase activity in these lesions. In support of this possibility was the observation that the cell pellet, which predominantly consisted of polymorphonuclear leukocytes, contained similar collagenase activity as did the cell-free supernatant.

Polymorphonuclear leukocytes have also been shown to contain an elastase [27–28]. This enzyme is a serine protease and can be inhibited by PMSF. In this study, the elastase activity found in DH cell pellet was markedly inhibited by PMSF. However, the elastase activity detected in the cell-free supernatant of DH blister specimens was only partially inhibited by PMSF, and a significant reduction in elastase activity was noted with Na_2EDTA. Thus, it appears that the cell-free blister fluid contains 2 kinds of elastases. One of them is a serine protease probably derived from polymorphonuclear leukocytes. The other elastase may be a metalloprotease, but its origin is currently unknown (see below).

The release of proteases from polymorphonuclear leukocytes can be elicited by a variety of factors including immune complexes and immunoglobulins [29,30]. The mechanisms of the enzyme release from leukocytes in DH are not known, but it is possible that local deposition of immunocomplexes containing IgA may trigger leukocyte chemotaxis and, subsequently, induce the release of the proteases into the extracellular milieu [31,32]. Previous studies have demonstrated that collagenase and elastase are located in separate subcellular storage granules in polymorphonuclear leukocytes [33]. Thus, the release of these 2 proteases may be regulated by different factors, and such differential release may occur at different stages of the disease process. In support of such differential release mechanism is our observation that when cells were separated from the extracellular fluid in 2 samples from DH lesions, relatively more elastase compared to collagenase was found in the extracellular space. It should be noted that leukocyte elastase, in addition to degrading elastin, cleaves type III and type IV collagens, which are present in dermis and the dermal-epidermal basement membrane, respectively [34–36]. The leukocyte elastase may thus contribute to the destruction of the collagen matrix in the DH lesions.

The elastase detected in BP blister fluids was different from that found in DH, in that it was readily inhibited by metal chelators, but not by PMSF. The activity was also stimulated by the addition of 5 mM Ca^{2+}, suggesting that this enzyme is a metalloprotease. The origin of this enzyme in BP blister fluid is unknown at this point. Previously, an elastase-like metalloprotease has been demonstrated in cultured macrophages [37], and cultured skin fibroblasts have been shown to secrete a similar enzyme [38]. Also, serum from several patients with liver disease [39] and some patients with rheumatoid arthritis [40] have been shown to contain a Ca^{2+}-dependent elastase-like enzyme activity. Similar elastase-like metalloprotease has also been suggested to play a role in the degradation of elastic fibers in a family with cutis laxa and severe pulmonary emphysema [41]. It is possible, therefore, that the elastase activity in BP blister fluids is derived from locally proliferating cells, such as macrophages or fibroblasts [42]. Alternatively, the enzyme is derived from serum of the same patients. These possibilities are currently under investigation.

The authors thank Leslie Anderson for expert technical assistance.

REFERENCES

1. Oikarinen AI, Zone JJ, Ahmed AR, Kiistala U, Uitto J: Connective tissue degrading enzymes in the blister fluids from bullous skin diseases: demonstration of two separate elastolytic enzymes in bullous pemphigoid and dermatitis herpetiformis. Clin Res 31:593A, 1983
2. Lever WF, Schaumburg-Lever G: Histopathology of the Skin. Philadelphia, JB Lippincott, 1975, pp 95–132
3. Anhalt GJ, Diaz LA: The dermal-epidermal junction in normal and disease states, Progress in Diseases of the Skin, vol 1. Edited by R Fleischmajer. New York, Grune & Stratton, 1981, pp 161–176
4. Beutner ER, Jordon RE, Chorzelski TP: Autosensitization in pemphigus, bullous pemphigoid and other chronic bullous diseases, Textbook of Immunopathology, vol 2. Edited by PA Miescher, HJ Muller-Eberhard. New York, Grune & Stratton, 1976, pp 931–946
5. Schiltz JR, Michel B: Production of epidermal acantholysis in normal human skin in vitro by IgG fraction from pemphigus serum. J Invest Dermatol 67:254–260, 1976
6. 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 306:1189–1196, 1982
7. Farb RM, Dykes R, Lazarus GS: Antiepidermal cell surface pemphigus antibody detaches viable epidermal cells from culture plates by action of proteinase. Proc Natl Acad Sci USA 75:459–463, 1978
8. Schiltz JR, Michel B, Papay R: Pemphigus antibody interaction with human epidermal cell in culture. A proposed mechanism for pemphigus acantholysis. J Clin Invest 62:778–788, 1978
9. Hopsu-Havu VK, Fräki JE: Proteinases and their inhibitors in

skin diseases. Int J Dermatol 20:157–163, 1981
10. Pehamberger H, Gschnait F, Konrad K, Holubar K: Bullous pemphigoid, herpes gestationes and linear dermatitis herpetiformis: circulating anti-basement membrane zone antibodies; in vitro studies. J Invest Dermatol 74:105–118, 1980
11. Naito K, Morioka S, Ogawa H: The pathogenic mechanisms of blister formation in bullous pemphigoid. J Invest Dermatol 79:303–306, 1982
12. Jordon RE: Complement activation in bullous skin diseases. J Invest Dermatol 65:162–169, 1975
13. Eisen AZ: Human skin collagenase: relationship to pathogenesis of epidermolysis bullosa dystrophica. J Invest Dermatol 52:449–453, 1969
14. Lazarus GS: Collagenase and connective tissue metabolism in epidermolysis bullosa. J Invest Dermatol 58:242–248, 1972
15. Bauer EA, Eisen AZ: Recessive dystrophic epidermolysis bullosa. Evidence for increased collagenase as a genetic characteristic in cell culture. J Exp Med 148:1378–1387, 1978
16. Kiistala U: Suction blister device for separation of viable epidermis from dermis. J Invest Dermatol 50:129–137, 1968
17. Kiistala U: Dermal-epidermal separation. II. External factors in suction blister formation with special reference to the effect of temperature. Ann Clin Res 4:236–246, 1972
18. Ryhänen L, Rantala-Ryhänen S, Tan EML, Uitto J: Assay of collagenase activity by a rapid, sensitive, and specific method. Collagen Rel Res 2:117–130, 1982
19. King J, Laemmli UK: Polypeptides of the tail fibres of bacteriophage T_4. J Mol Biol 62:465–477, 1971
20. Bonner WM, Laskey RA: A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46:83–92, 1974
21. Bieth J, Spiess B, Wermuth CG: The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem Med 11:350–357, 1974
22. Sandberg LB, Bruenger E, Cleary EG: Tropoelastin purification: improvements using enzyme inhibitors. Anal Biochem 64:249–254, 1975
23. Stone PJ, Franzblau C, Kagan HM: Proteolysis of insoluble elastin. Methods Enzymol 82(part A):588–605, 1982
24. Lazarus GS, Daniels JR, Brown RS, Bladen HA, Fullmer HM: Degradation of collagen by a human granulocyte collagenolytic system. J Clin Invest 47:2622–2629, 1968
25. Ohlsson K, Olsson I: The neutral proteinases of human granulocyte: isolation and partial characterization of two granulocyte collagenases. Eur J Biochem 36:473–481, 1973
26. Turto H, Lindy S, Uitto V-J, Wegelius O, Uitto J: Human leukocyte collagenase: characterization of enzyme kinetics by a new method. Anal Biochem 83:557–569, 1977
27. Janoff A, Scherer J: Mediators of inflammation in leukocyte lysosomes. IX. Elastinolytic activity in granules of human polymorphonuclear leukocytes. J Exp Med 128:1137–1155, 1968
28. Ohlsson K, Olsson I: The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. Eur J Biochem 42:519–527, 1974
29. Weissman G, Zurier KB, Spieler PJ, Goldstein IM: Mechanisms of lysosomal enzyme release from leukocytes exposed to immunocomplexes and other particles. J Exp Med 134:1495–1501, 1971
30. Gammon WR, Merritt CC, Lewis DM, Sams WM Jr, Carlo JR, Wheeler CE Jr: An *in vitro* model of immune complex-mediated basement membrane zone separation caused by pemphigoid antibodies, leukocytes, and complement. J Invest Dermatol 78:285–290, 1982
31. Zone JJ, LaSalle BA, Provost TT: Circulating immune-complexes of IgA type in dermatitis herpetiformis. J Invest Dermatol 75:152–155, 1980
32. Spilberg I, Gallacher N, Melita JM, Mandel B: Urate crystal-induced chemotactic factor. J Clin Invest 58:815–819, 1976
33. Murphy G, Bretz U, Baggiolini M, Reynolds JJ: The latent collagenase and gelatinase of human polymorphonuclear neutrophil leukocytes. Biochem J 192:517–525, 1980
34. Gadek JE, Fells GA, Wright DG, Crystal RG: Human neutrophil elastase functions as a type III collagen collagenase. Biochem Biophys Res Commun 95:1815–1822, 1980
35. Mainardi CL, Dixit SN, Kang AN: Degradation of type IV (basement membrane) collagen by proteinase isolated from human polymorphonuclear leukocyte granules. J Biol Chem 255:5435–5441, 1980
36. Uitto J, Ryhänen L, Tan EML: Collagen: its structure, function, and pathology, Progress in Diseases of the Skin, vol 1. Edited by R Fleischmajer. New York, Grune & Stratton, 1981, pp 103–141.
37. Werb Z, Gordon S: Elastase secretion by stimulated macrophages. Characterization and regulation. J Exp Med 142:361–377, 1975
38. Bourdillon MC, Brechemier D, Blaes M, Derouette JC, Hornebeck W, Robert L: Elastase-like enzymes in skin fibroblasts and rat aorta smooth muscle cells. Cell Biol Int Rep 4:313–320, 1980
39. Katagin K, Ito K, Miyaji M, Takeuchi T, Yoshikane K, Sasaki M: Succinyl trialanine p-nitroanilide-hydrolytic enzymes in human serum. An improved method for clinical diagnosis. Clin Chim Acta 95:401–404, 1979
40. Saklatvala J: Hydrolysis of the elastase substrate succinyltrialanine nitroanilide by a metal-dependent enzyme in rheumatoid synovial fluid. J Clin Invest 59:794–801, 1977
41. Oikarinen AI, Anderson LL, Anderson CE, Uitto J: Characterization of an elastase-like neutral protease in serum of a patient with cutis laxa and pulmonary emphysema. Clin Res 31:266A, 1983
42. Fräki JE, Lazarus GS, Hopsu-Havu VK: Protein catabolism in the skin, Biochemistry and Physiology of the Skin. Edited by LA Goldsmith. New York, Oxford Univ Press, 1983, pp 338–362