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Authors
Jeffes, Edward WB
Yamamoto, Robert S
Ahmed, A Razzaque
et al.

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Lymphotoxin Detected in the Blister Fluid of Bullous Pemphigoid Patients

EDWARD W. B. JEFFES,[1,2] ROBERT S. YAMAMOTO,[1] A. RAZZAQUE AHMED,[1] and GALE A. GRANGER[1]

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The role of lymphocytes in the pathogenesis of bullous pemphigoid was examined by assaying the blister fluid obtained from bullous pemphigoid patients for the presence of the lymphokine, lymphotoxin. Blister fluids from six bullous pemphigoid were assayed on L-929 target cells for the presence of cytolytic molecules in the standard lymphotoxin assay. Three of six blister fluids obtained from bullous pemphigoid patients and one linear IgA bullous dermatosis patient contained significant levels of cytolytic activity. Control blister fluids from suction blisters, herpes, pemphigus, and toxic epidermal necrolysis patients did not contain cytolytic activity. Serum from five bullous pemphigoid patients also had no cytolytic activity. Neutralization studies using rabbit anti-α-lymphotoxin demonstrated that 54 to 88% of the cytolytic activity found in bullous pemphigoid blister fluid was due to α-lymphotoxin. These results indicate that lymphotoxin is locally released in the skin of bullous pemphigoid lesions and is detectable in blister fluids.

KEY WORDS: Bullous pemphigoid; blisters; lymphocytes; lymphotoxin.

INTRODUCTION

The events initiating and regulating the disease processes causing bullous pemphigoid (BP) are not well understood. Although IgG and complement are found in BP lesions, they alone do not explain the pathogenesis of this disease (1, 2). Morphological studies of the bullous lesions seen late in the disease suggest a role for granulocytes and mononuclear cells (3). *In vitro* studies support this hypothesis (4). Similarly, morphological studies of the early erythematous papules seen in BP demonstrate that the lymphocyte is the earliest infiltrating cell (5), suggesting that these cells play role in pathogenesis of BP.

In this study, we describe a cytolytic activity antigenically similar to or identical to the lymphokine, lymphotoxin (LT), in the blister fluid from BP patients. We propose that local activation of lymphocytes and the release of lymphokines may play an important regulatory role in the pathogenesis of BP.

MATERIALS AND METHODS

Sera and Blister Fluids

Sera and blister fluid (BF) were obtained from patients admitted to the UCLA Medical Center with newly formed blisters. The diagnosis of BP was made by clinical presentations and confirmed by immunofluorescence (IF) examination of perilesional skin which demonstrated deposition of C3, or IgG and C3, in the basement membrane zone (BMZ).

Control blister fluid was obtained from two patients with pemphigus vulgaris (PV), one with toxic epidermal necrolysis (TEN), and one with herpes zoster. Suction blister fluid obtained from three healthy volunteers was generously donated by Dr. A. I. Oikarinen.

Sera and BF were tested for anti-BMZ antibodies of the IgG class by standard IF techniques using monkey esophagus as the substrate.

[1]Division of Dermatology, UCLA School of Medicine, Los Angeles, California 90024, and Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92664.
[2]To whom correspondence should be addressed at Division of Dermatology, UCLA School of Medicine, Los Angeles, California 90024.

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Lymphotoxin (LT) Assay and Anti-LT Antisera

The LT assay has been described in detail previously (6). Briefly, monolayers of an LT-sensitive murine L-929 fibroblast were established in glass culture tubes at a density of 100,000 cells/ml in RPMI-1640 medium containing 0.5 μg/ml mitomycin C during a 24-hr preincubation at 37°C. The material to be assayed for LT activity was added to these tubes and incubated 24 hr at 37°C. The number of viable adherent target cells was measured by trypsinizing the cells off the glass test tubes and using a Model F Coulter counter to count them.

A standard LT-containing supernatant (SAL) was prepared by using PHA-activated human lymphocytes as described previously (7) and was run as a positive control in all LT assays. Antibody neutralization tests were performed in the LT assay by adding antiserum [anti-α-LT or anti-whole supernatant (anti-WS)] at the same time the LT-containing sample was added to the target cells. Preparation and characterization of the anti-LT serum have been described in detail (7). Anti-α-LT antiserum can neutralize α-LT. The anti-WS serum was prepared with unfractionated LT-containing supernatants obtained from PHA-activated human lymphocytes and can neutralize α-, β-, and γ-LT activity. Percentage neutralization of LT activity was calculated by the following formula:

$$\text{percentage neutralization} = \left\{1 - \frac{[c - (s + Ab)]}{[c - s]}\right\} \times 100,$$

where c represents the cell number in the untreated control, s represents the cell number in the tube containing material being assayed for LT activity, and $(s + Ab)$ represents the cell number obtained when an LT-containing sample and antiserum to LT are mixed and then assayed.

RESULTS

Blister Fluid from BP Patients and One Linear IgA Bullous Dermatosis Patient Contains Cytolytic Activity When Assayed on L-929 Target Cells

Blister fluid from six BP patients and one linear IgA bullous dermatosis patient were assayed on L-929 in the standard lymphotoxin assay system as described in Materials and Methods. As shown in Table I, three of six BP patients and the one linear IgA bullous dermatosis patient contained significant cytolytic activity. Control blister fluid from suction blisters raised in three volunteers, two pemphigus vulgaris patients, one toxic epidermal necrolysis patient, and one herpes zoster patient did not contain cytolytic activity. All but one BP blister fluid collected in 1982 contained significant cytolytic activity. No correlations between the IF titer of serum or blister fluid and the presence of cytolytic activity in the blister fluid was noted.

Several possibilities may explain why previously collected and stored BP blister fluids did not contain cytolytic activity: (1) the blister fluid from large bullae may dilute the low levels of activity so that the assay is no longer sensitive enough to detect the cytotoxic activity. (2) The activity of the disease process may influence the amount of cytotoxic activity detected in the blister fluid. (3) The cyto-

Table I. Cytolytic Activity in the Blister Fluid from Bullous Pemphigoid Patients

Patient	Diagnosis[a]	IF titer[b]		Date collected	Cytolytic activity[c]
		Serum	Blister fluid		
DD	BP	BMZ 1280	BMZ 2560	7/82	(+)
TH	BP	BMZ 10	(−)	1/82	(+)
LW	BP	BMZ 640	BMZ 160	12/82	(+)
FP	Linear IgA BD	(−)	NT	11/82	(+)
BV	BP	ANA 320	ANA 160	4/80	(−)
LS	BP	BMZ 640	BMZ 2048	8/79	(−)
CW	BP	BMZ 1280	BMZ 512	8/78	(−)
TO	Suction blister	NT	NT	6/82	(−)
KO	Suction blister	NT	NT	6/82	(−)
SA	Suction blister	NT	NT	6/82	(−)
KS	PV	NT	(−)	6/82	(−)
RG	PV	ICS 160	ICS 16	10/78	(−)
MD	TEN	(−)	(−)	3/82	(−)
FP	Herpes	(−)	NT	11/82	(−)
—	SAL	NT	NT	10/80	(+)

[a]BP—bullous pemphigoid; linear IgA BD—linear IgA bullous dermatosis; TEN—toxic epidermal necrolysis; Herpes—herpes zoster; SAL—supernatant from activated lymphocytes.

[b]NT—not tested; BMZ—basement membrane zone antibody titer; ICS—intercellular substance antibody titer; ANA—antinuclear antibody titer. (−) indicates no membrane fluorescence observed; IF titer expressed as reciprocal of highest dilution of serum giving positive staining.

[c]LT assay done with 0.1–0.2 ml of blister fluid as described in Materials and Methods. (+) represents significant cytolytic activity on L-929 target cells. A control supernatant from activated lymphocytes (SAL) containing 200 units LT activity/ml was employed as the positive control.

Table II. Neutralization of Cytolytic Activity in Blister Fluid with Anti-α-LT Antisera

	Patient	Diagnosis	Blister fluid dilution	Antisera			Cell number ± SD	Percentage neutralization[c]
				Anti-α-LT	Anti-WS	Control sera		
Expt 1	—	—	—	—	—	—	25,844 ± 431	*
	FP	Herpes	1:5	—	—	—	28,173 ± 131	0
	FP	Herpes	1:5	0.05	—	—	25,261 ± 2703	0
	TH	BP	1:5	—	—	—	16,938 ± 183	0
	TH	BP	1:5	0.05	—	—	23,796 ± 491	77
	DD	BP	1:5	—	—	—	12,945 ± 532	0
	DD	BP	1:5	0.05	—	—	20,005 ± 867	54
	—	SAL	1:200	—	—	—	6,680 ± 418	—
Expt 2	—	—	—	—	—	—	17,944 ± 425	*
	DD	BP	1:7	—	—	—	15,223 ± 884	0
	DD	BP	1:7	0.05	—	—	17,231 ± 388	74
	DD	BP	1:7	—	0.05	—	15,501 ± 11	11
Expt 3	—	—	—	—	—	—	15,788 ± 310	*
	DD	BP	1:10	—	—	—	12,173 ± 955	0
	DD	BP	1:10	0.05	—	—	15,388 ± 176	88
	DD	BP	1:10	—	0.05	—	15,783 ± 98	100
	DD	BP	1:10	—	—	0.05 R	12,039 ± 1266	0
	DD	BP	1:10	—	—	0.05 G	11,337 ± 133	0

[a]BP—bullous pemphigoid; SAL—supernatant from activated lymphocytes; Herpes—herpes zoster.
[b]Antisera specificity described in Materials and Methods, amount of antisera given as ml/1 ml culture. Control sera are normal rabbit serum—R and normal goat serum—G.
[c]LT assay and calculation of percentage neutralizaion described in Materials and Methods. * indicates the control which represents 100% neutralization.

toxic activity in the blister fluid specimens may be unstable. β- and γ-LT cytolytic activities are labile in serum-containing medium and are not stable when stored (10, 13). α-LT is stable and can be stored frozen for long periods (13). Since α-, β-, and γ-LT are generally found in the same supernatants, it would seem reasonable to hypothesize their presence in BP blister fluid. Furthermore, the proportion of the different LT molecules produced by PHA-activated lymphocytes varies depending on the composition of the lymphocyte population being stimulated, the nature of the antigenic stimulus, and the duration of lymphocyte culture (9, 13). Thus, the bullous pemphigoid blister fluid specimens with no detectable cytolytic activity in Table I may have had mainly the unstable LT molecules which rapidly lost activity on storage.

Neutralization of Cytolytic Activity in Bullous Pemphigoid Blister Fluid with Antilymphotoxin Antisera

The bullous pemphigoid blister fluid contains immunoglobulins, complement, and other materials which could be cytotoxic. The data in Table II show that 50–88% of the cytolytic activity can be neutralized with anti-α-LT. Eleven to 100% of the activity was neutralized with anti-WS serum. These neutralization studies prove that the cytolytic activity found in these blister fluids shares antigenic determinants in common with LT and represents α-LT. Preliminary heat neutralization studied show that the cytotoxic activity found in patient DD's blister fluid is stable to heating 56°C × 45 min, again consistent with the hypothesis that the cytotoxic material represents primarily α-LT.

Serum from Bullous Pemphigoid Patients Assayed for Cytotoxic Activity

The sera of five bullous pemphigoid patients were assayed at a 1:2–1:5 dilution for the presence of cytolytic activity on L-929 targets cells. No cytotoxic activity was detected in the sera of BP patients with active disease even when assayed within 4–72 hr of collection. This demonstrated that the cytotoxic activity in the blister fluid of BP patients represents the products of a local process and is not due to the complement and immunoglobulins present in the blister fluid.

DISCUSSION

This study demonstrates a cytolytic lymphotoxin-like activity in the blister fluid from patients with bullous pemphigoid (BP) which is not present in the serum from patients with BP. The cytolytic activity is detected on L-929 target cells and is neutralized

by monospecific antisera to α-LT (6, 7). This implies that LT or LT-like molecules are present in the cytolytic blister fluid. Since LT is produced only by activated lymphocytes (8–10), activated lymphocytes may play an important role in the pathogenesis of BP. We suggest that the erythematous and indurated plaques that typically precede the bullae in BP patients represent the early local activation of lymphocytes in much the same way that activated lymphocytes generate a positive delayed-type hypersensitivity skin test reaction. The locally activated lymphocytes probably release LT and other lymphokines into the skin which diffuse into the blister fluid where it is measured.

Blister fluid from BP patients has been shown to contain several different bioactive molecules. Baba *et al.* (11) described an eosinophilic chemotactic activity in blister fluid from BP patients. The major portion of this activity was less than 1000 MW and had physical properties of the eosinophilic chemotactic factor of anaphylaxis (ECF-A). Blister fluid from BP patients has been shown to contain an eosinophilic-stimulating material similar or identical to colony-stimulating factor (CSF) (14) and a chemoattractant activity for lymphocytes (5).

The LT activity reported in this study represents a new class of molecules, the lymphokines, found in blister fluid of BP patients. The LT activity is mediated by a group of proteins with molecular weights of 25,000, 45,000, 80,000, and 150,000 and a complex >200,000. Some of these molecules are stable (e.g., α-LT) while others are unstable in serum (e.g., β- and γ-LT (13). Only lymphocytes have been shown to synthesize LT, and both T and B lymphocytes can synthesize LT molecules.

A current pathogenic model used to describe the phenomonen observed in BP patients proposes that IgG binds to the BMZ and fixes complement. Next, lymphocyte and granulocyte chemotactic factors are generated. Complement components or mast cells (5) appear to be involved at this stage. The recruited granulocytes bind to the BMZ, degranulate, and release proteases which digest the BMZ and form bullae (4).

Although this model explains much of the observed experimental data, it does not explain why passively transfused BP antibody does not cause disease (1) and why the serum titers of BP IgG do not correlate with disease activity (2). Anhalt *et al.* (15) have had limited success in passively transferring BP with IgG obtained from BP patients. They demonstrated that anti-BMZ IgG from BP patients injected into rabbit corneas caused inflammatory infiltrates and subepithelial blister formation. When the same BP IgG was injected into rabbit skin, no inflammatory lesions were produced. Thus, the anti-BMZ IgG found in BP patients is probably pathogenic under the correct conditions *in vivo*.

Although anti-BMZ IgG and complement are probably involved, a third component, not IgG or component, is found in blister fluid which is required to cause dermal epidermal separation (DES) (12). The material required from DES could theoretically be an enzyme derived from PMNs or eosinophils, a cytotoxic molecule derived from lymphocytes or a yet undescribed molecule. Naito *et al.* (12) reported that blister fluid from two BP patients caused DES both in human skin cultured *in vitro* and in guinea pig skin *in vivo*. Sera from BP patients containing anti-BMZ IgG were not effective in producing DES. This blister fluid activity required for DES *in vitro* does not appear to be a serine proteinase, carboxyl proteinase, or metaloprotease; however, DES is inhibited by 10 mg/ml of α-2-macroglobulin. The blister fluid activity detected *in vitro* is neutralized by heating and by antisera to human complement components. This suggests either that the material is unstable, and has antigenic determinants similar to complement, or that it requires complement and possibly IgG to cause DES. The relationship of the cytolytic material found in blister fluid and the material found in blister fluid required for DES is unknown. Further studies are in progress to examine the molecular characteristics of the cytolytic activity found in the blister fluid of BP patients and determine if unstable LT molecules are present in these blister fluids.

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