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Increased Levels of Immunoreactive Leukotriene B_4 in Blister Fluids of Bullous Pemphigoid Patients and Effects of a Selective 5-Lipoxygenase Inhibitor on Experimental Skin Lesions

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The immunoreactive leukotriene B_4 (i-LTB_4) and i-LTC_4 content in the blister fluids of patients with bullous pemphigoid (BP) was determined by radioimmunoassay. Their amounts significantly exceeded those noted in superficial dermal burn patients and those in the fluids of suction blisters produced on normal human skin. When either BP blister fluids or BP-IgG from the patient sera were injected into guinea pig skin, neutrophil and eosinophil infiltrates were produced in the dermis. In addition, the dermis was noted to undergo marked edematous change. A single oral administration of a selective inhibitor of 5-lipoxygenase 1 hr before the intracutaneous injection of BP-IgG was found to significantly inhibit cell infiltrates. Furthermore, the inhibitor partly suppressed dermal epidermal separation. On the basis of these results, LTB_4 and LTC_4 appear to be generated in the skin lesions of BP, the former attracting granulocytes to the dermis and the latter, causing exudation. ***Key word: Leukocytes.***

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Chemotactic activity is present in the blister fluids of bullous pemphigoid (BP) (for review, see ref.1). Examination of these fluids has indicated various chemotactic factors to be involved in cell infiltration observed in skin lesions. Diaz-Perez & Jordon (2) detected complement C5a in blister fluids and confirmed it to contribute to chemotactic activity toward (polymorphonuclear leukocytes) PMNs. Chemoattractants from mast cells, such as the eosinophil chemotactic factor of anaphylaxis (ECF-A) (3) and histamine (4), are present at high concentrations in BP-blister fluids. However, the *in vivo* chemotactic activities of ECF-A and histamine are much less potent than those observed in vitro. Leukotriene B_4 (LTB_4) is a very potent leukocyte chemotactic factor released from mast cells, basophils, mononuclear cells and PMNs. The present study was conducted to determine whether LTB_4 is in some way related to the accumulation of neutrophils and eosinophils in BP skin.

MATERIALS AMD METHODS

Patients

Nine patients with BP were studied. None of the patients had received treatment previously. All cases showed infiltrate-rich dermis with neutrophils and eosinophils. Seven patients with superficial dermal burn and 3 normal volunteers with suction blisters on the skin were studied as controls. The suction blisters were produced by continuous suction at 180–200 mmHg below atmospheric pressure.

Measurement of immunoreactive leukotrienes (LTs) in blister fluids

The fluid of a new blister formed within 1 day on each patient with BP and burn and that of a suction blister on the normal control skin were individually collected into plastic tubes and mixed immediately with four volumes of cold ethanol. Each mixture was kept in an ice bath for 2 h and stirred occasionally. After centrifugating the mixture twice at 15,000 rpm for 45 min, the supernatant was evaporated *in vacuo*, and the residue dissolved in 5 ml of distilled water. After adjusting the pH to 3.5, each sample was applied onto a Sep-pak C18 (Waters Associates, Milford, Mass.). Extraction of LTs was performed with 20 ml each of water, petroleum ether and then methanol. Contaminants which may possibly interfere with this RIA system were previously confirmed to be eliminated from a sample by application of this procedure (5). Following evaporation of the methanol-eluted sample and dissolving it in 1 ml of 50 mM Tris-HCl buffer containing 0.1% gelatin, pH 8.6 (RIA-assay buffer), the amount of immunoreactive (i)-LTB_4 was measured with a radio-immunoassay kit from Amersham (Bucks, England) and i-LTC_4, with New

Table I. *Leukotriene concentrations (mean ± S.D.) in blister fluids*

Patient group	Immunoreactive LTB_4 (ng/ml)		Immunoreactive LTC_4 (ng/ml)		
Bullous pemphigoid ($n = 9$)	4.09±1.89	*	3.37±1.08	**	*
Superficial dermal burn ($n = 7$)	0.69±0.80		*1.70±1.55		
Normal skin ($n = 3$)	0.47±0.42		0.43±0.52		

*$p<0.01$; **$p<0.05$

England Nuclear (UK). The anti-LTB_4 antibody used in the RIA has been reported to crossreact with negligible amounts of other arachidonic acid metabolites (0.03% with 5-HETE, 2.0% with 12HETE and <0.03% with arachidonic acid). The anti-LTC_4 antibody, however, has been shown to react with considerable amounts of other peptide-LTs (55.3% with LTD_4 and 8.6% with LTE_4), and so consequently, the amount of i-LTC_4 may be considered as the sum of those of peptide-LTs.

In vivo chemotactic activity of blister fluids

One-tenth ml of the fluid of each blister from 3 patients with BP was injected intradermally into the shaved skin of the back of male Hartley guinea pigs, weighing 350–400 g, at 6–9 separate injection sites. The methanol-eluted RIA samples from the same patients diluted ten times with the RIA-assay buffer were also injected into the animals in the same manner. The controls were three blister fluids of burn and the RIA-assay buffer. The reaction sites were excised after 3, 6 and 24 h following injection and processed by histological (hematoxylin and eosin staining and Giemsa staining) and immunofluorescence methods. Neutrophils and eosinophils were counted in 10 random fields at each level of the upper, mid and lower dermis at high magnification (×400) in a light microscope and totalled for the 30 fields. The results were expressed as average cell numbers per field, and statistical analysis was performed by the Student's *t*-test.

Preparation of IgG fraction

IgG was purified from heat-inactivated (56°C, 30 min) patient sera collected from all patients with BP by precipitation at 50% saturation with ammonium sulfate followed by chromatography on DEAE-cellulose (DE52 Whatman, Clifton, New Jersey) with 0.01 M phosphate buffer at pH 7.20 and 4°C. The fractions of the first peak were collected, dialysed against three changes of 0.01 M PBS, pH 7.2 and concentrated at 4°C with an Amicon Diaflow membrane (YMlO). The protein concentration of BP-IgG was determined as 11.2 mg/ml and its pemphigoid antibody titre as 1:1280 by indirect IF staining using human foreskin as the tissue substrate. An identical dose of the IgG fraction from pooled normal human serum (NHS) (Gibco Laboratories) served as the control. One-tenth ml of either BP- or normal IgG was injected into guinea pig skin and skin specimens were taken from injection sites 1, 3, 6, 12 and 24 h after injection; histological and immunofluorescence examinations were subsequently conducted. The numbers of neutrophil and eosinophil granulocytes were counted in each of 10 random fields at the level of the upper dermis including the dermal epidermal junction (DEJ) at a high magnification of ×400 and expressed as the averaged value at each of the above times.

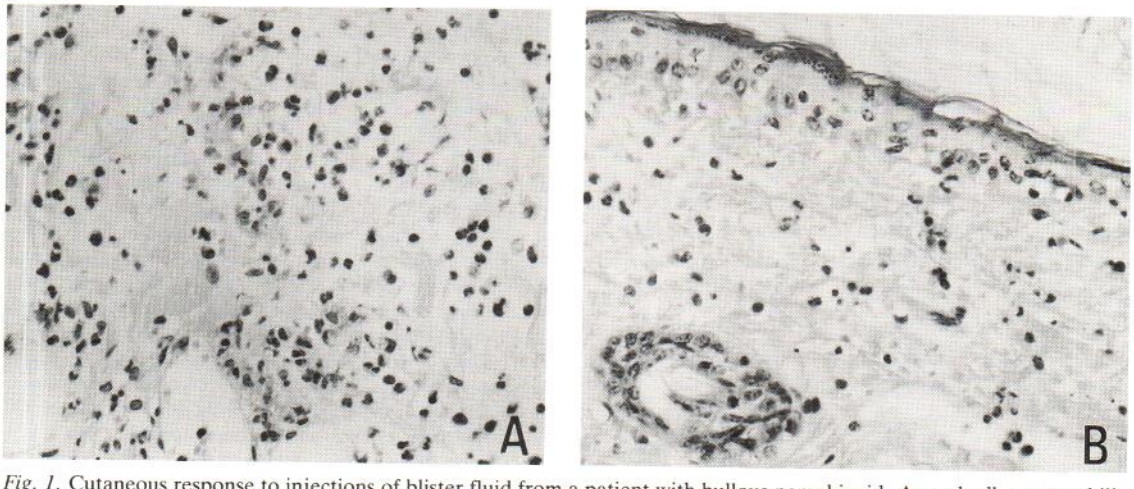

Fig. 1. Cutaneous response to injections of blister fluid from a patient with bullous pemphigoid. A markedly neutrophilic and eosinophilic infiltrate along with some mononuclear cells can be seen in the subcutaneous tissue (*A*), and in the upper and mid-dermis (*B*). (×200, H&E stain).

Table II. *Time course of neutrophil and eosinophil response (cells/field; mean ± SD) in guinea pig skin to blister fluids (n = 3)*

Diseases and materials examined	Time following injection		
	3 h	6 h	24 h
Bullous pemphigoid			
Neutrophils	5.0**±1.0	31.0*±1.7	21.0* ± 1.9
Eosinophils	5.2* ±0.4	43.4*±4.3	84.3* ± 5.1
Methanol eluted samples			
Neutrophils	5.0* ±0.2	37.0*±3.7	26.0**± 7.9
Eosinophils	5.4 ±2.1	40.3*±6.5	91.2* ±10.3
Superficial dermal burn			
Neutrophils	1.1**±0.3	0.4 ±0.1	1.1 ± 0.3
Eosinophils	0.5 ±0.3	0.4 ±0.2	0.7* ± 0.1
Assay buffer			
Neutrophils	0.3 ±0.2	0.3 ±0.2	0.3 ± 0.1
Eosinophils	0.3 ±0.1	0.3 ±0.1	0.2 ± 0.1

*$p<0.01$ and **$p<0.05$ compared with the response of skin treated with the assay buffer.

Inhibitory effect of a selective 5-lipoxygenase inhibitor

A 5-lipoxygenase inhibitor (AA-861), kindly provided by the Central Research Division of Takeda Chemical Industries, Ltd., was suspended at a concentration of 50 mg/ml in 0.9% saline containing 5% gum arabic and administered orally to each of 5 guinea pigs at 80 mg/kg 1 h before injecting patient and normal IgG into its skin. Skin biopsies were taken and examined histologically as mentioned above.

RESULTS

Immunoreactive LTB_4 and LTC_4 content in blister fluids

As shown in Table I, an elevated i-LTB_4 content in the blister fluids was observed in 9 patients with BP. The averages were 6- and 9-fold respectively that of blister fluids of the burn and suction blisters induced on normal skin ($p<0.01$). The i-LTC_4 content of BP-blister fluids was also 8 times that of the suction induced blisters on normal skin ($p<0.01$), and twice that of the burn ($p<0.05$) which also showed a moderately high level.

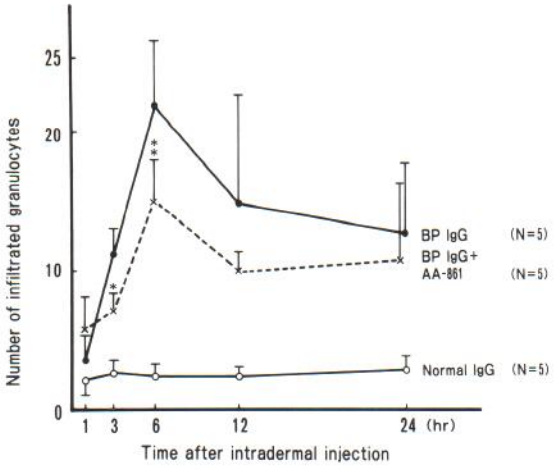

Fig. 2. Inhibition of bullous pemphigoid IgG-induced granulocyte infiltration in guinea pig skin by the selective lipoxygenase inhibitor. *$p<0.01$; **$p<0.05$ compared with bullous pemphigoid IgG-induced granulocyte response.

Neutrophil and eosinophil count

The *in vivo* chemotactic activity of blister fluids was studied in guinea pig skin. BP-blister fluids caused the formation of a massive neutrophilic and eosinophilic infiltrates along with some mononuclear cells in the lower dermis and the subcutaneous tissue into which the fluid had been injected, while moderate cell accumulation was noted in the upper and mid-dermis (Fig. 1). The time courses of the average cell numbers per field are shown in Table II. The numbers of neutrophils and eosinophils increased at the injection sites at 3 h and markedly so at 6 h. Neutrophils then gradually decreased at 24 h, but eosinophils continued to accumulate. The same cellular response was observed when 10-fold diluted RIA samples instead of BP-blister fluids were injected into the skin. Only a few cellular infiltrates

Table III. *Inhibition of bullous pemphigoid IgG-induced dermal epidermal separation in guinea pig skin by the selective 5-lipoxygenase inhibitor*

	Time following intradermal injection				
	1 h	3 h	6 h	12 h	24h
Normal IgG	0/5	0/5	0/5	0/5	0/5
BP IgG	0/5	1/5	3/5	3/5	3/5
BP IgG + AA-861	0/5	0/5	1/5	2/5	2/5

Data indicate the rates of dermal-epidermal separation.

were noted at the injection sites of blister fluids of burn and the buffer controls, even at 6 and 24 h following injection.

BP-IgG induced dermal infiltrates and dermal-epidermal separation

Binding of human IgG was observed at the DEJ of all skin specimens from the injection sites of BP-IgG from 1 h following injection and its extent lessened by 12–24 h. In contrast, guinea pig C3 was first detected at 3 h and continued to be in evidence up to 24 h. No IgG or C3 binding could be detected in any skin specimen treated with normal IgG.

All biopsied specimens from sites injected with BP-IgG showed neutrophil and eosinophil infiltrates associated with a number of mononuclear cells in the upper and mid dermis. Neutrophils and eosinophils had attached to the DEJ in some fields. The numbers of these granulocytes that infiltrated the upper dermis increased at 3 h, reaching a peak at 6 h after injection (Fig. 2). As shown in Table III, dermal-epidermal separation (DES) was shown histologically to occur at various rates 3, 6, 12 and 24 h after injection. In the skin specimens of normal IgG-injected sites, infiltration with mononuclear cells was observed, while only a few granulocytes were present and no dermal epidermal separation occurred.

Effect of the selective 5-lipoxygenase inhibitor

BP IgG-induced dermal infiltration of granulocytes at 3 and 6 h of injection was inhibited significantly by AA-861 by 39% ($p<0.01$) and 32% ($p<0.05$) respectively (Fig. 2). DES was also inhibited in some animals, although not to a statistically significant extent (χ^2-test) (Table III).

DISCUSSION

Our *in vivo* study demonstrated a chemotactic reaction to guinea pig neutrophils and eosinophils in both BP-blister fluids and those treated with methanol-elution. Since ECF-A as a low molecular peptide and complement C5a as glycoprotein are removed from samples during methanol-elution, they do not of course participate in this chemotaxis. LTB_4 is a specific chemotactic and chemokinetic factor toward neutrophils and eosinophils (6, 7). In *in vivo* studies using experimental animals and human volunteers, a single intradermal injection of LTB_4 has been shown capable of inducing dermal neutrophil and eosinophil infiltration (6, 8, 9). Thus, the blister concentration of LTB_4 was determined. RIA was conducted to measure the level of LTB_4 in the blister fluids, and so consequently, the term immunoreactive (i)-LTB_4 was used. The antibody used in this RIA study is highly specific, as described in Materials and Methods, and the method of extracting LTB_4 is an established one used by us for the simultaneous extraction of i-LTB_4 and i-LTC_4 and the elimination of substances that may impair the accuracy of RIA (5). Accordingly, LTB_4 concentrations in the blister fluids as determined by RIA may be considered accurate, though complete confirmation of this point has yet to be obtained.

The present RIA study showed the levels of i-LTB_4 in the blister fluids of 9 patients with BP to be nine-fold higher than those in suction blister fluids produced on normal control skin. LTB_4 has been shown to express maximum chemotactic activity *in vitro* (1 ng/ml) (10) and cause massive PMNs infiltration *in vivo* (subnanomolar (8) -several ng (11)). It would thus appear quite likely that the LTB_4 concentrations detected in the present study (1.06–7.14 ng/ml of BP-blister fluids) are sufficient to induce dermal infiltration of granulocytes. When BP-IgG was injected into guinea pig skin, neutrophil and eosinophil infiltrates were produced in the dermis of all the animals, thus being in agreement with Naito et al. (12). We found infiltrates of granulocytes situated close to DEJ where edematous change was conspicuous and DES was present in some areas. By immunofluorescence, the deposition of human IgG and guinea pig C3 was shown on BMZ. Neither inflammatory infiltrates nor DES formation could be found to any significant extent in skin specimens treated with normal IgG. The presence of complement deposits was noted to be closely correlated to the appearance of the dermal infiltrates of granulocytes. However, Gammon & Briggaman (13) recently reported the absence of any specific granulocyte infiltration, or any deposition of immuno-reactants on BMZ at most BP-IgG injected sites on guinea pig skin. This disparity may possibly arise from differences in affinity toward BMZ antigens and complement activating properties of the BP antibodies used. AA-861 is a selective inhibitor of 5-lipoxygenase (14) and effective in both *in vitro* (15) and *in vivo* systems (11). A single oral administration of 80 mg/kg of AA-861 1 h before an intracutaneous injection of BP-IgG, this amount being

sufficient for maximally suppressed LTB_4 generation and leukocyte infiltration in rat infarct cardiac tissue (11), was found in this study to significantly inhibit both neutrophil and eosinophil infiltrates at 3 and 6 h following injection. This inhibitor partially suppressed DES at both times. Thus LTB_4 possibly may function, as does the chemotactic complement, as a chemoattractant for granulocyte infiltration induced by BP-IgG injection.

Immunoreactive-LTC_4 was also detected at increased levels in the blister fluids of BP. Peptide LTs such as LTC_4, LTD_4 and LTE_4 induce dilation of microvasculatures, increase vascular permeability and elicit clinical erythema and edema formation (8, 16). Thus, LTC_4 in BP skin lesions may be reasonably considered to enhance exudate formation. The pathogenesis of BP that we propose is as follows: LTB_4 and LTC_4 are probably liberated from tissue mast cells through the action of C3a and C5a activated by BP-IgG. Locally accumulated granulocytes as well as macrophages may also be sources of LTs when stimulated by these complement components or by LTB_4 itself. LTB_4 may potentiate the exacerbation of BP-skin lesions by attracting granulocytes to the dermis, while at the same time, besides the action of complement as a chemoattractant or anaphylatoxin, LTC_4 causes the exudation of blister fluids. This in turn also potentiates the exacerbation of BP-skin lesions. The 5-lipoxygenase inhibitor may suppress LTs-induced exacerbation.

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