

# Immunoblot Analysis of Autoantigens in Localized Pemphigoid and Pemphigoid Nodularis

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We analyzed circulating antibodies in sera from patients with localized pemphigoid and pemphigoid nodularis, two variants of bullous pemphigoid, by means of western immunoblotting of human epidermal extracts and the recombinant protein of NC16a domain of the 180 kD bullous pemphigoid antigen. NC16a domain is now considered to be the most pathogenic site of bullous pemphigoid. Compared with the results of typical bullous pemphigoid patients, localized pemphigoid sera detected the 180 kD bullous pemphigoid antigen less frequently, and sera from both localized pemphigoid and pemphigoid nodularis showed a lower end point of titer of antibodies to NC16a domain. These results suggest that atypical clinical features of the two bullous pemphigoid variants may be related with low titer of autoantibodies to 180 kD bullous pemphigoid antigen, particularly to NC16a domain. **Key words:** BP230; BP180; NC16a domain.

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It is now well known that most bullous pemphigoid (BP) sera react with the two major BP antigens, the 230 kD BP antigen (BP230) and the 180 kD BP antigen (BP180). The BP230 is detected by the sera from 50-90% of typical BP patients, and the BP180 is detected by the sera from 35-50% of typical BP patients (1-5). In our previous study the sera from 74% of typical BP patients reacted with the BP230, and 51% reacted with the BP180 (6). Giudice et al. (7) have recently reported that BP180 NC16a domain is the most immunogenic site of the BP180. This domain is located between the membrane-spanning domain and the first collagen-like domain of the BP180. In the present study, we investigated immunologic characteristics of the two BP variants. We analyzed circulating antibodies in 8 localized pemphigoid and 6 pemphigoid nodularis patients with direct and indirect immunofluorescence and western immunoblotting of human epidermal extracts and the recombinant protein of BP180 NC16a domain. Subsequently, we compared the results with those of sera from patients with typical BP.

## PATIENTS AND METHODS

### Patients

Eight patients with localized pemphigoid and 6 patients with pemphigoid nodularis were studied. The diagnoses were made from clinical, histopathological and direct immunofluorescence findings. The specimens taken from all the cases showed a linear deposition of IgG and/or C3 along the basement membrane zone (BMZ) (data not shown). Localized BP case 5 also showed a linear deposition of IgM and fibrinogen along the BMZ.

Fig. 1a shows a clinical feature of localized pemphigoid case 1. One or two blisters appeared recurrently on almost the same region of the right forearm. Each of the localized pemphigoid cases 1-4 exhibited a few refractory blisters on restricted areas (case 1: right forearm;

case 2: chest; case 3: perianal region; and case 4: right ankle). The other 4 localized pemphigoid patients presented several blisters on their lower extremities. No patients showed scarring after bullae disappeared. Fig. 1b shows a clinical feature of pemphigoid nodularis case 6. Nodular lesions with excoriated surfaces associated with severe itching were present on the trunk and extremities. The other 5 pemphigoid nodularis patients also presented nodular lesions with itching on the trunk and extremities. The biopsy specimen revealed subepidermal split and acanthosis (data not shown). Pemphigoid nodularis case 4 revealed typical generalized BP lesions 3 years prior to the onset of nodular lesions. Ten sera from common type BP and 10 normal sera were used as controls.

### Indirect immunofluorescence studies

Indirect immunofluorescence of normal human skin was performed by standard method with anti-human IgG antiserum as a secondary antibody. Indirect immunofluorescence using 1M NaCl split skin was performed according to the method described by Woodley et al. (8), using anti-human IgG antiserum as a secondary antibody.

### Preparation of antigen sources for immunoblotting

Normal human epidermal extracts were prepared as follows. Briefly, normal human foreskin was incubated in 2 mM EDTA in phosphate-buffered saline with 2 mM phenylmethylsulphonyl fluoride (PMSF) at 4°C for 48 h to separate the epidermis from the dermis. Epidermal proteins were extracted with 1% sodium dodecylsulfate (SDS) and 5% β-mercaptoethanol in 0.1 M Tris HCl buffer (pH 6.8) including 2 mM PMSF and 5 mg/l each of 4 proteinase inhibitors.

cDNA encoding for the BP180 NC16a domain was amplified by polymerase-chain reaction using human keratinocyte cDNA library and specific primers selected from the cDNA sequence reported by Giudice et al. (9), subcloned into the bacterial expression vector pGEX-2T. Recombinant protein was induced by an addition of isopropyl-β-thiogalactopyranoside (10).

### Immunoblotting

Immunoblotting of epidermal extracts and recombinant protein was performed as described previously (6, 11). Briefly, electrophoresis for

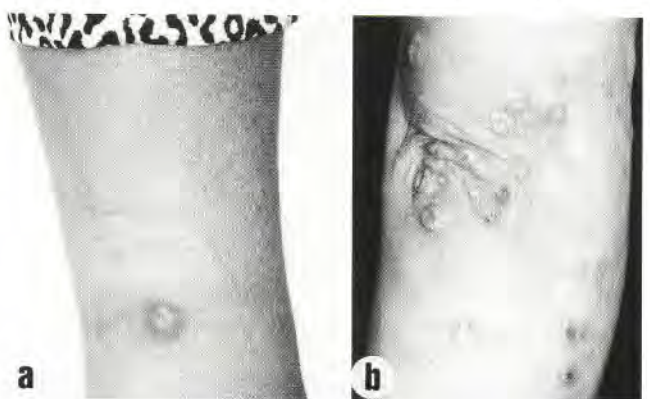


Fig. 1. (a) Clinical feature of localized pemphigoid in case 1. A blister is seen on the forearm. (b) Clinical feature of pemphigoid nodularis in case 6. Nodular lesions with excoriated surfaces.

epidermal extracts and the recombinant protein was performed using 6% and 15% separating gel, respectively. Subsequently, proteins were electrophoretically transferred to nitrocellulose sheets. Immunostaining was performed as described previously (11), using the 1:40 diluted sera for immunoblotting of epidermal extracts. Immunoblotting of recombinant protein was initially performed by using 1:20 diluted sera. Since NC16a domain is considered to be the most pathogenic site in BP, various dilutions of sera were also examined to obtain an end point of titer of each serum.

## RESULTS

All the results of indirect immunofluorescence and immunoblot analysis for the 14 cases are summarized in Table I.

### *Indirect immunofluorescence with normal human skin and 1M NaCl split skin*

Circulating anti-BMZ IgG antibodies were detected in all but one sera by indirect immunofluorescence using normal human skin, at a titer from 1:40 to 1:2560. No circulating anti-BMZ antibodies were detected in serum from localized pemphigoid in case 2. There was no apparent relationship between the immunofluorescence titer and the extent of lesions.

With indirect immunofluorescence of 1M NaCl split skin, 13 sera containing circulating anti-BMZ antibodies reacted with the epidermal side of the split (Fig. 2). The staining pattern was indistinguishable from that of typical BP sera. Sera from localized pemphigoid in case 2 did not react with either the epidermal or dermal side of the split.

### *Immunoblot analysis of normal human epidermal extracts*

The results are shown in Fig. 3. Seven out of the 8 sera from localized pemphigoid cases reacted with the BP230, which showed exactly the same mobility as control common type BP sera, although the reactivity of case 3 was very weak. In contrast, only one localized pemphigoid (case 3) serum reacted with the BP180, although 6 out of 10 control BP sera reacted



Fig. 2. Finding of indirect immunofluorescence using 1M NaCl split skin with serum of localized pemphigoid in case 4. Circulating IgG antibodies bound to epidermal side of the split.

with it (data not shown). All 6 pemphigoid nodularis sera reacted with the BP230. Four pemphigoid nodularis sera reacted with the BP180, but 2 of the 4 sera (sera from cases 4 and 5) reacted weakly with it.

### *Immunoblot analysis of the recombinant protein of BP180 NC16a domain*

The results of immunoblot analysis for 1:20 diluted sera are shown in Fig. 4. We usually see several bands with sera with high titer, and only the uppermost band is seen with sera of low titer. Therefore, we consider that the uppermost protein is probably an intact form of this recombinant protein, and degradation products of several sizes might be produced during our procedure. All the control common BP sera reacted clearly with the recombinant protein of the BP180 NC16a domain (lane 1). Seven of the 8 localized pemphigoid sera reacted with the recombinant protein but only 3 sera showed clear reactivity comparable with that of control common BP sera. Five of the 6 pemphigoid nodularis sera reacted with the recombinant protein but the reactivity was faint.

Table I. Summary of immunofluorescence (IF) and immunoblot studies

		Age/Sex	Indirect IF	Immunoblot		
				BP230	BP180	BP180RP <sup>a</sup> (titer)
Localized pemphigoid	Case 1	56F	1:640	(+)	(-)	1:320
	2	78M	(-)	(-)	(-)	(-)
	3	86M	1:40	(+)	(+)	1:40
	4	86F	1:640	(+)	(-)	1:40
	5	79F	1:640	(+)	(-)	1:320
	6	75F	1:640	(+)	(-)	1:20
	7	76F	1:2560	(+)	(-)	>1:320
	8	95F	1:320	(+)	(-)	1:40
Pemphigoid nodularis	Case 1	83F	1:640	(+)	(-)	1:40
	2	78F	1:640	(+)	(-)	(-)
	3	62F	1:160	(+)	(+)	1:40
	4	50F	1:640	(+)	(+)	1:20
	5	76F	1:160	(+)	(+)	1:40
	6	58F	1:640	(+)	(+)	1:20
Common type BP				8/10	6/10	10/10
Normal control				0/10	0/10	0/10

<sup>a</sup> BP180 NC16a domain recombinant protein.

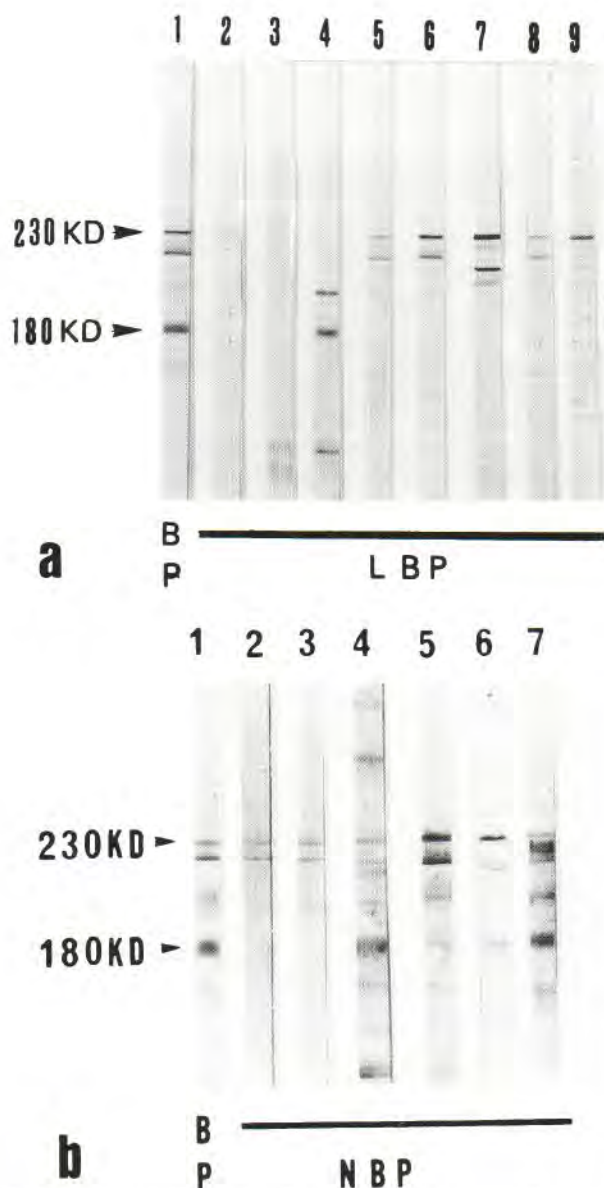


Fig. 3. Immunoblot analysis of normal human epidermal extracts. (a) Results of 8 localized pemphigoid cases (lanes 2-9). Reactivity with the BP230 and the BP180 is indicated by arrows. (b) Results of 6 pemphigoid nodularis cases (lanes 2-7). Lane 1: both panels show the reactivity of common generalized bullous pemphigoid serum.

Therefore, we performed further immunoblot analysis in order to obtain the end point of titer. The results are summarized in Table I. Five control common BP sera showed reactivity with the recombinant protein at a dilution of over 1:320. By contrast, the sera from the 14 cases, except for localized pemphigoid cases 1, 5 and 7, showed lower titers.

We obtained and examined a pair of sera from pemphigoid nodularis case 4 at both typical generalized BP and pemphigoid nodularis stages. Both sera contained circulating anti-BMZ IgG antibodies at 1:640 dilution by indirect immunofluorescence using normal human skin. However, it is interesting to note that the reactivity of the serum with the recombinant protein became weaker after the clinical features changed from common generalized BP into pemphigoid nodularis (Fig. 5; the end point of titer changed from over 1:320 to 1:20).

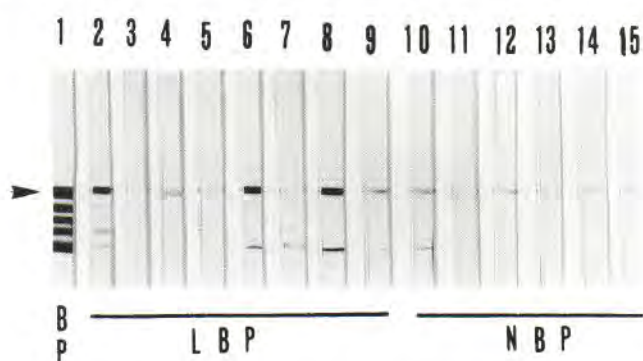


Fig. 4. Immunoblotting of the recombinant protein of BP180 NC16a domain. Results of 8 localized pemphigoid cases (lanes 2-9) and 6 pemphigoid nodularis cases (lanes 10-15). Lane 1: common generalized bullous pemphigoid serum. The most strongly stained band, indicated by an arrow, was considered to be intact recombinant protein. Ladder-like lower protein bands were probably degradation products of the intact protein.

## DISCUSSION

Several variants of BP have been described, including localized pemphigoid and pemphigoid nodularis. Person et al. (12) summarized the cases in the literature and their own cases and divided localized pemphigoid into two types: 1) the Brunsting and Perry type in which scarring bullous lesions occur on the head and neck, and 2) the Eberhartinger and Niebauer (or pretibial) type. In addition, localized pemphigoid cases in which various solitary regions were involved have been reported in the literature. Our localized pemphigoid cases 1-8 were included in this type. Pemphigoid nodularis was first described by Yung et al. (13). The patients develop pruritic, papular/nodular lesions resembling prurigo nodularis and tense bullae.

Five localized pemphigoid and 2 pemphigoid nodularis have been examined by immunoblot analysis (14, 15-17). All the sera, except for one localized pemphigoid case (17), reacted with the BP230. In the present study, with western immunoblot, 7 (87%) out of 8 localized pemphigoid patients and all the 6 (100%) pemphigoid nodularis patients reacted with the BP230. This frequency is almost the same as that of common BP (8/10 positive). As to the reactivity with the BP180, only one serum (13%) out of 8 sera from localized pemphigoid cases, and 4 (67%) out of 6 from pemphigoid nodularis reacted with the BP180. Since the number of sera investigated was small, we could not obtain statistical significance in these results. However, localized pemphigoid cases seemed to react less frequently with the BP180 in epidermal extracts.

The reactivity of sera from typical BP patients with the recombinant protein encompassing BP180 NC16a domain was about 80% in our previous study, and immunoblotting of this recombinant protein is considered to be the most useful technique for the diagnosis of BP (10). In addition, this domain has been considered as a pathogenic site of the BP180 (7, 18). In this study, all the 10 common BP sera reacted with it, and all the 5 common BP sera for which the end point of titer was determined showed a titer over 1:320. Seven (87%) of the 8 localized pemphigoid cases and 5 (83%) of the 6 pemphigoid nodularis cases reacted with the human BP180 NC16a domain recombinant protein. This frequency is almost

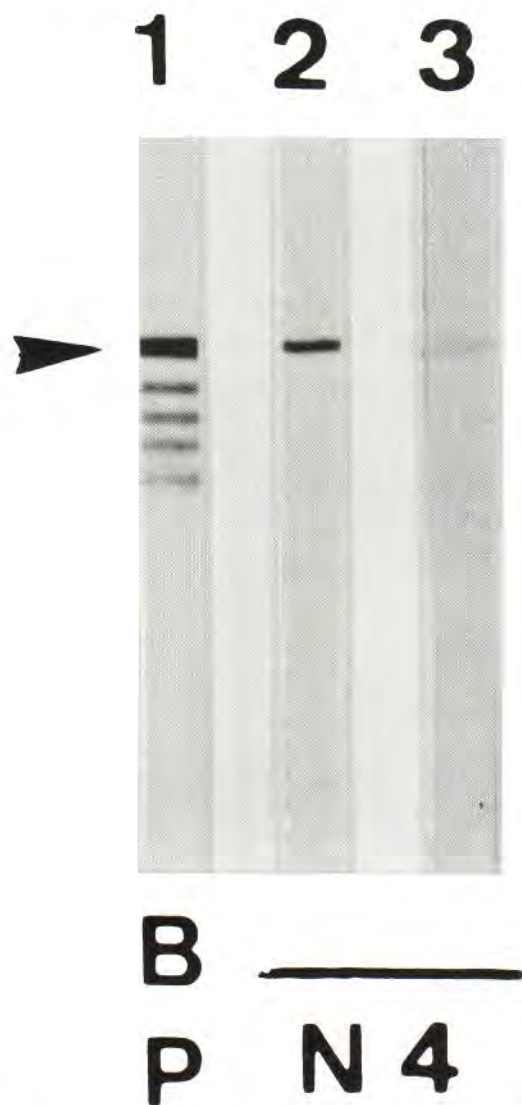


Fig. 5. Immunoblot results of the recombinant protein of BP180 NC16a domain for sera from pemphigoid nodularis case 4 (N4). Lane 1: common generalized bullous pemphigoid serum. Lane 2: serum from pemphigoid nodularis case 4 at the stage of typical generalized BP. Lane 3: serum from the same patient after the change into pemphigoid nodularis. An arrow indicates the position of the recombinant protein.

the same as that in our previous study (13). However, the sera from the 5 localized pemphigoid cases and all the 6 pemphigoid nodularis cases showed a very lower titer of antibodies reacting with this domain. Moreover, the titer of antibodies to this recombinant protein in the sera from pemphigoid nodularis case 4 turned out to be lower after the clinical features had changed from common generalized BP into pemphigoid nodularis. These results suggest that the atypical clinical features of the two types of BP are partly related with lower titer of antibodies to BP180 NC16a domain.

However, as the sera from localized pemphigoid cases 1, 5 and 7 showed a relatively high titer of antibodies to BP180 NC16a domain, there may be other factors. Further immunoblot analysis with more cases of the BP variants, using epidermal

extracts and the recombinant protein, will provide us with more precise information about their pathogenesis.

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