

INVESTIGATIONS ON THE BINDING SITES OF THE BASEMENT MEMBRANE ZONE FOR PEMPHIGOID ANTIBODIES IN VITRO

I. Enzymatic Action on the Active Groups of Human Skin¹

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Abstract. At the present time, nothing is known of the antigenic structures or the antibody binding sites for basement membrane zone antibodies of pemphigoid patients located in the basement membrane zone of the human skin. In the course of experiments to delineate this area, we have studied the action of various enzymes in vitro. These investigations showed that the exposure of cryostat sections of human skin to the action of proteolytic enzymes (papain/trypsin) produced an increase in antibody binding capacity of the basement membrane zone for basement membrane zone antibodies. This was demonstrated by an increased intensity of fluorescence of the basement membrane zone in 17/25 of the skin samples tested from different patients and by an increase of 1-7 titre dilution steps as compared with control sections not previously incubated. From various enzymes tested the α - and β -galactosidases in 19/21 skin samples also showed increased intensity of fluorescence, and titres increased by 1-6 dilution steps. The quantitative evaluation of our experimental results showed that incubation with galactosidases and proteolytic enzymes produced an activation independent of the titre, while there was, in addition, an inactivation dependent on the level of the initial titre produced by the action of papain and trypsin only. Furthermore, we believe these latest results, together with immunochemical studies on the basement membranes from the literature, allow conjecture on the possible site of enzyme action. This site must be in the immediate vicinity of the hydroxylysine on the molecule. In agreement with other authors and results, we have developed the hypothesis that the action of proteolytic enzymes causes the basement membrane zone to become antigenic, with the formation of basement membrane zone antibodies, "autoantibodies".

Key words: Bullous pemphigoid; Activation of basement membrane zone antigens; Proteolytic enzymes; α - and β -galactosidases; Molecular biology; Induction of autoantibodies

Since the discovery of the basement membrane zone (BMZ) antibodies in patients with bullous pemphigoid (1, 7) these "autoantibodies" have been investigated many times from the clinical and immunological points of view. According to electron microscopical findings the deposition of these immunoglobulins takes place in the space between the basal cells and basal lamina (14) or is confined to the basal lamina and lamina lucida (6). Whilst immunochemical studies of the basement membranes of varying origin indicate the occurrence of at least three different antigenic components (8, 9), nothing is so far known about the antigenic structures or binding sites for BMZ antibodies of pemphigoid patients. In the course of experiments to delineate this region, we have investigated the action of enzymes. In a previous publication (11) it has been reported that pretreatment of cryostat sections of human skin with proteolytic enzymes produced an increase in the binding capacity of the basement membrane zone for BMZ antibodies. In continuation of these experiments, various other enzymes have been investigated with respect to this effect and the results are reported here.

MATERIALS AND METHODS

Cryostat sections of healthy human skin from different persons were incubated with enzymes of varying specificity (Table I) and after several thorough washings with Coons' buffer, were examined by the indirect immunofluorescence method against experimental preparations which had not previously been incubated (normal series), with regard to the intensity of fluorescence and level of

¹ Some of the findings of this investigation are contained in the Doctoral Thesis of Mr Hartmut Kunow.

Table 1. List of all enzymes investigated with respect to their effect on the basement membrane zone—antigens of human skin and the results obtained

Enzyme	Source	Solution applied (amount of enzyme in mg/100 ml 0.1 M phosphate buffer)	Time of enzyme action (min)	No. of skin samples investigated from different persons	Alterations in titre steps compared with normal controls (= not previously incubated)	Remarks
α -Glucosidase	Boehringer Mannheim	20	1/5	3	None	None
β -Glucosidase	Serva	1 000	1/5	2	None	None
β -Glucuronidase	Serva	100	1/5	2	None	None
Collagenase	Boehringer Mannheim	10	1/15/60	2	None	Increasing detachment of epidermis after 15 min reaction time
Lysozyme	Mannheim	10/50	1/15	2	None	None
Arylsulfatase	Mannheim	10	1/10	2	None	None
Leucinaminopeptidase	Mannheim	10	1/5	2	None	None
Neuraminidase	Mannheim	2	1/10	2	None	None
Hyaluronidase	Serva	10	1/5	2	None	None
Papain	Serva	^a	1/5	12	Titre increase in 9/12 samples ^b	Detachment of epidermis in less than 5 min reaction time
Trypsin	Serva	^a	1/5/15	13	Titre increase in 8/13 samples ^b	Detachment of epidermis in less than 15 min reaction time
α -Galactosidase	Boehringer Mannheim	5/10/50	1/10/60/120	14	Titre increase in 13/14 samples ^b	None
β -Galactosidase	Mannheim	10	1/10	7	Titre increase in 6/7 samples ^b	None

^a For concentrations employed here see "Methods and Materials".

^b The results of individual skin samples (titre increase compared with the normal controls) are shown in Figs. 1 and 2.

titre. For the indirect immunofluorescence procedure (2, 4, 10), the cryostat sections were incubated with a serum containing BMZ antibodies (using guinea pig tongue as antigenic substrate, positive up to a dilution of 1:256), washed and covered with a layer of fluorescein-labelled antiserum (Behring Company AG, West Germany) in a dilution of 1:10. Characteristics of the conjugate: protein concentration of the FITC-conjugated gammaglobulin fraction: ca. 10 ± 3 mg/ml; total protein concentration (after addition of human albumin): ca. 40 ± 5 mg/ml; specific antibody content: ca. $10 \pm 5\%$ of the gammaglobulin concentration; molar F/P ratio: ca. 2.5 ± 1.5 .

Details of this experimental arrangement are described elsewhere (11). The individual enzymes investigated are listed in Table 1 with concentrations applied and times of action, the *Methoden der enzymatischen Analyse* (3) principally having been followed. As a supplement to the details given in Table 1, the solutions of the proteolytic enzymes which were used are as follows. Papain solution: to 8 ml of a 0.01 M cysteine hydrochloride solution (pH 7.2) were added 0.02 ml of a papain suspension (Serva Lab., purest crystalline form, 15.8 EU/mg); trypsin solution: 1 ml of a 0.001 N HCl solution containing 0.1 g % trypsin (Serva Lab., crystalline, salt-free, 160 EU/mg) was diluted with 99 ml 0.1 M phosphate buffer, pH 7.6.

RESULTS

The results of our experiments are summarized in Tables I and II, and the alterations in titre dilution steps due to the action of the proteolytic enzymes and the galactosidases compared with the non-

Table II. Average increase in titre (q ; given in titre steps) after the action of different enzymes, in comparison with normal controls, as well as the calculated increase in the slopes (m ; Figs. 1 and 2). For details see "Results".

Enzyme	Samples with moderate initial titre (from 1:4)		Samples with low initial titre (1:1/1:2)
	m	q	
Trypsin	0.6	3	6
Papain	0.8	2	5
α -Galactosidase	0.9	3	4
	(against 1.0)		
β -Galactosidase	1.0	3	5

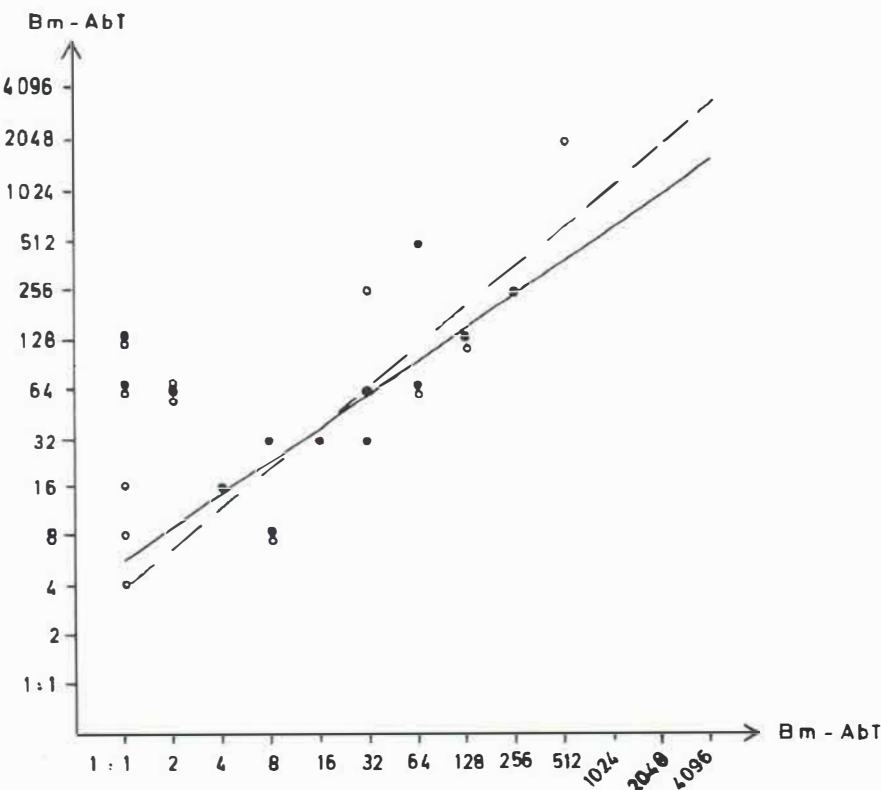


Fig. 1. Titres of basement membrane zone antibody binding (Bm-AbT), using cryostat sections of human skin, preincubated with trypsin (●—●) or papain (○—○) (ordinate) in comparison with the series not previously incubated (abscissa). Each plot (%) represents a test series using skin biopsy specimen from different patients as antigenic substrate. Thus, skin samples were inves-

tigated from 12 persons with papain (○) and 13 persons with trypsin (●). Skin samples with moderate initial titre values (from 1:4) yielded a constant activation (2 titre dilution steps with papain and 3 with trypsin) as well as a titre-dependent inactivation. Samples with low initial titres (1:1 and 1:2) were considerably more activated (5 titre steps with papain and 6 with trypsin).

incubated controls are shown in Figs. 1 and 2. According to these, the proteolytic enzymes produced an increase in the intensity of fluorescence of the basement membrane zone in 17/25 and the galactosidases in 19/21 of the skin samples tested from different patients (Figs. 3, 4). The titres increased by 1–7 dilution steps with the proteolytic enzymes and by 1–6 dilution steps with the galactosidases compared with the previously non-incubated controls. The quantitative evaluation of our experimental results is shown in Figs. 1 and 2.

Plotting the titres of the non-incubated series against the titres of the activated series shows that there is a mutual relationship of the two quantities which is particularly obvious in the trypsin curve. If the results of the experiments with small titres (1:1 and 1:2) in the non-incubated series are ignored, then a straight line ($y=mx+q$) can be con-

structed from the remaining values. The constants (m and q) resulting from the lines given are summed up in Table II. According to this, incubation with trypsin or papain produced an activation independent of the titre, whereas there was, in addition, an inactivation dependent on the level of the initial titre. With the α -galactosidase and perhaps with the β -galactosidase only an activation independent of titre was observed.

With a prolonged action of the proteolytic enzymes, a destruction of the BMZ and a detachment of the epidermis from the corium was observed, a finding with which the mathematical analytical results corresponded. With the galactosidases, even after a longer duration of action (up to 2 hours) there was no destruction of the antigenic area; furthermore, there was no mathematical suggestion of inactivation.

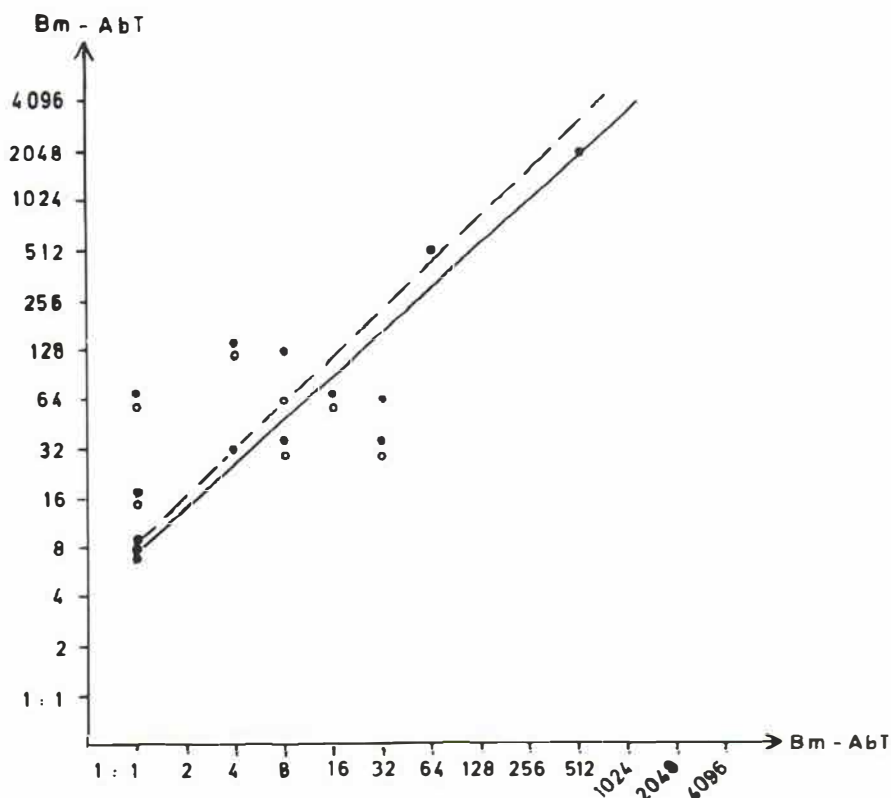


Fig. 2. Titres of basement membrane zone antibody binding (Bm-AbT), using cryostat sections of human skin, preincubated with α -galactosidase (●—●) or β -galactosidase (○—○) (ordinate) in comparison with the series not previously incubated (abscissa). Each plot (%) represents a test series using a skin biopsy specimen from different patients as antigenic substrate. Thus, skin samples were investigated from 14 persons with α -galactosidase

(●) and 7 persons with β -galactosidase (○). Skin samples with moderate initial titre values (from 1:4) yielded a constant activation with both enzymes (3 titre dilution steps). Samples with a low initial titre (1:1) were considerably more activated (4 titre steps with α -galactosidase and 5 with β -galactosidase). There was no evidence of inactivation, however.

The fact that detachment of the epidermis was initiated after incubation with collagenase without effect on the BMZ fluorescence will be discussed later. All other enzymes shown in Table I were without effect on the fluorescence of the BMZ or on the structure of the epidermis. The following controls were negative in every case: (1) samples which were incubated only with fluorescein-labelled antiserum; (2) various so-called "normal control sera" which contained no pemphigus, pemphigoid or nuclear antibodies. The following antigen-antibody systems were also examined: using sera containing nuclear or pemphigus antibodies, no activation of nuclear or pemphigus antigens could be recorded if the skin sections had been incubated with proteolytic enzymes, galactosidases or other enzymes listed in Table I.

DISCUSSION

In previous publications, we have reported that activation of antigens of the basement membrane zone is possible by X-ray and ultraviolet irradiation (10) and, enzymatically, by proteolysis (11). An attempt was made to explain this in terms of the law of mass action (10, 11). The results of the present work, according to which the action of α - and β -galactosidases also enhances the binding capacity of the antigenic structures of the basement membrane zones for BMZ antibodies, complement our previous results considerably.

Furthermore, we believe these latest results permit conjecture as to the possible site of enzyme action. Immunochemical studies by Kefalides (8, 9) on the basement membranes of various organs of different species demonstrated the presence of

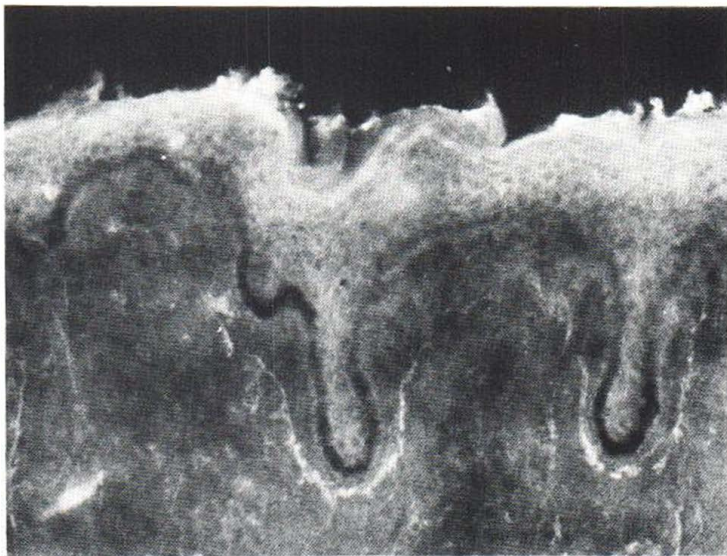


Fig. 3. Weak basement membrane zone fluorescence displayed by the indirect immunofluorescence method, using cryostat sections of human skin as antigenic substrate. Incubation with a serum containing basement membrane zone antibodies in a dilution of 1:1 (using guinea pig tongue as antigenic substrate positive up to a dilution of 1:256). Covering with a layer of fluorescein-labelled antiserum (Behring Company) in a dilution of 1:10. Objective $\times 10$.

three different antigenic components, of which one is probably collagen and the others are non-collagen glycoproteins. An essential component of this collagen is α -glucosyl- β -galactosylhydroxylysine, and, in much smaller quantities, a galactosylhydroxylysine, which cannot be more closely identified sterically. It is also known that the sites of action of papain and trypsin are carbonyl-bound lysine and arginine, respectively. Assuming that both delta-hydroxylysine and non-hydroxylated lysine react with the proteolytic enzymes mentioned, it is possible to focus attention on a rela-

tively limited region of the molecule in our search for the site of action of the enzymic reaction. This site must be in the immediate vicinity of the hydroxylysine on the molecule. The effect of α -galactosidase would be explained if the galactose were bound by α -glycoside to the galactosylhydroxylysine. However, the precise binding site of the antibody molecule cannot be deduced from our results.

It is quite possible that the protein and carbohydrate hydrolases actually have a different site of action in our model investigations. Thus, lysine and

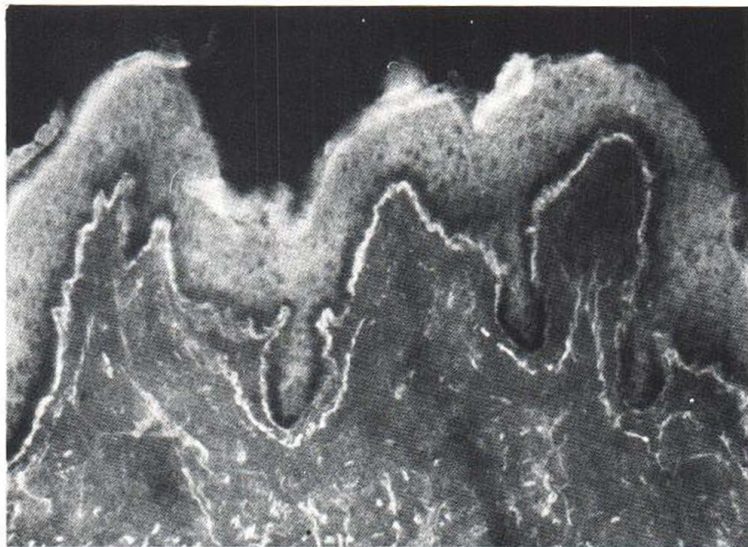


Fig. 4. Intensive basement membrane zone fluorescence displayed by the indirect immunofluorescence method, using cryostat sections of human skin as antigenic substrate which had been preincubated for 5 minutes with trypsin solution. Incubation with serum containing basement membrane zone antibodies in a dilution of 1:1 (same serum as Fig. 3), otherwise conditions as in Fig. 3.

arginine residues might be split off from other positions on the collagen or glycoprotein molecules.

Another publication (8) provides the explanation for the inactivation of the antibody binding sites in the BMZ and the morphologically manifest destruction of structures, which we observed after longer periods of incubation with the proteolytic enzymes. Collagen from the BMZ can be split into 80 polypeptides by the action of proteinases, particularly trypsin (8). We observed a similar effect on using collagenase, whose site of action must obviously lie too far away from the active centre to allow any demonstrable influence on the antigenic structure.

The activity of the galactosidases is probably limited to relatively isolated glycosidic bonds, so that the fact that these enzymes do not inactivate the antigen-antibody reaction in the basement membrane zone would not be surprising.

However, we are unable to explain why the individual skin specimens from different patients showed widely varying degrees of sensitivity to activation; in fact some samples could not be activated at all. It is uncertain whether this is a question of individual antigenic differences such as histocompatible antigens or whether an alteration of activation of antigens has already taken place in vivo.

The action of enzymes on the skin of pemphigoid patients should prove interesting. These investigations have as yet been unsuccessful due to technical difficulties, since in this case the antibodies are fixed in vivo in the basement membrane zone. Attempts to remove these antibodies by elution damaged the skin so severely that the preparations obtained were no longer of any practical value.

In agreement with other authors (8, 9, 12, 13) and results (5, 15, 16), we have developed the hypothesis that the action of proteolytic enzymes causes the basement membrane zone to become antigenic, with the consequent formation of BMZ antibodies—"autoantibodies". This development has been discussed in detail elsewhere (11).

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