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

II. *Immunohistochemical Reactions of the Reactive Groups*

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Abstract. In continuing our studies on the characteristics of the antigenic structures of the basement membrane, procedures used in histochemistry for the identification and blocking of reactive groups have been adapted in this present work to the pretreatment of cryostat sections of guinea-pig tongue. These sections have then been studied by the indirect immunofluorescence method. By means of reagents which produce a reversible extinction of the basement membrane fluorescence produced with pemphigoid sera, it proved possible to determine the existence of certain chemical groupings which bind the anti-basement membrane antibodies, namely: free carboxyl groups deriving from amino acids, hydrogen bridges in which the hydroxyl groups from the carbohydrates in the antigen are probably involved, bonds involving the free aldehydes of the antigen (either cross-links from aldehydes, or formation of Schiff's bases with amino groups of the antibodies), and lastly, to a lesser extent, disulphide bonds. The antigen/antibody reaction probably occurs in the neighbourhood of the alpha-glucosido-beta-galactosido-hydroxylysine. A reaction model has been developed from these findings. There were also some indications that the antigenic structures of the intercellular substance are chemically related to those of the basement membrane. The cell nucleus antigens, on the other hand, appear to possess different chemical properties.

Key words: Bullous pemphigoid; Immunohistochemistry; Molecular biology; Structure of the antigenic basement membrane zone; Chemical bindings in the antigen/antibody complex on the basement membrane; Induction of autoantibodies

When antibodies directed against certain skin structures were discovered in bullous dermatoses (1, 13), a new era in clinical and immunological dermatology began. Subsequently, much attention was devoted to the chemical and the physicochemical properties of these protein bodies (3, 4, 9, 15, 19), although a systematic investigation of the antigens was not undertaken until fairly recently. The properties of the antigens in pemphigus vulgaris (5, 15, 17) have been studied in this connection. The reaction of the antigenic structures with lectins has been found to be especially fruitful (10, 12, 16). For example, in the cell surfaces of the Malpighian layer, polysaccharides and mucopolysaccharides—such as presumably immunogenic compounds—could be detected (8). The biochemical structures of the basement membranes, on the other hand, were not investigated until very recent times. On the basis of experiments which suggest that the binding capacity of the basement membrane zone for anti-basement membrane antibodies is increased by proteolytic enzymes, it has become possible to concentrate on the binding region responsible for the binding of these antibodies, from the viewpoint of molecular biology. In this present paper, the results of the first paper (18) are corroborated by immunohistochemical methods, and, so far as is possible, the chemical combinations required for the formation of the antigen/antibody complex are characterised.

METHODS

Cryostat sections of guinea-pig tongue were used as antigen substrate. The pemphigoid sera used were sera with a titre of 1 : 256 or more, and in which cell nucleus antibodies were detected on slight dilution (up to about 1 : 9); before the pemphigoid serum was applied, the sec-

Table 1. *Reaction schemes (indirect immunofluorescence method) of the experiments described in this paper*

The reaction with acidic methanol has been chosen as an example of the extinction of the basement membrane fluorescence, and the reaction with potassium permanganate/oxalic acid as an example of the reversion of the extinction effect. For further details and other chemical reactions, see Methods

Normal series	Extinction series	Reversion series
←	Cryostat sections	→
↓	← methanol	→
↓	← washing 3×	→
↓	↓	Potassium permanganate
↓	↓	washing once
↓	↓	oxalic acid
↓	↓	washing 3×
←	← Pemphigoid serum	→
←	← washing 4×	→
←	← fluorescein-conjugated anti-IgG	→
←	← washing 4×	→
←	← embedding	→

tions were incubated with the chemicals in the sequence given as an example in Table 1. In the experiments with reversibly acting reagents, three series were always used, namely: a normal series, an extinction series and a reversion series; with the irreversible reagents, however, only a normal series and an extinction series were used. In the various series, the patient's serum was applied in the following dilutions: undiluted, 1:3, 1:9, 1:81. We used the fluorescein-labelled antiserum in the dilution 1:10. With regard to the details of the indirect immunofluorescence method, reference may be made to Beutner et al. (2) and also to one of our own earlier papers (4).

Techniques for the chemical pretreatment of the cryostat sections:

1. *Reaction with acetic anhydride/caustic potash*

(*a*) *Extinction series:* incubation of the cryostat sections for 1 min with 2.5% acetic anhydride in anhydrous pyridine, and then rinsing for ×5 min with "salt solutions",[1] pH 5.0, and 3×5 min with Coons' buffer.

(*b*) *Reversion series:* as extinction series, then incubation for 30 min with 0.01 N caustic potash, finally rinsing 3×5 min with Coons' buffer.

2. *Reaction with sodium bisulphite/hydrochloric acid*

(*a*) *Extinction series:* incubation of the cryostat sections for 1½ min with 40% sodium bisulphite in 20% ethanol (wt./vol), pH 5.0, finally rinsing 4×5 min with Coons' buffer.

(*b*) *Reversion series:* as extinction series, then incubation with "salt solution",[1] pH 2.0, finally rinsing with Coons' buffer 4×5 min.

3. *Reaction with methanol/potassium permanganate*

(*a*) *Extinction series:* incubation of the cryostat slices for 20 min with acidic methanol, pH 1.2, finally rinsing 3×5 min with Coons' buffer.

(*b*) *Reversion series:* as extinction series, then incubation for 15 min with 0.5% potassium permanganate, rinsing for 5 min with Coons' buffer, finally incubation for 4 min with 1% oxalic acid and rinsing 3×5 min with Coons' buffer.

4. *Reaction with formalin/water*

(*a*) *Extinction series:* incubation of the cryostat sections for 30 min with 1% formalin, then rinsing 1×5 min with Coons' buffer.

(*b*) *Reversion series:* as extinction series, then rinsing 4×5 min with Coons' buffer.

5. *Reaction with periodic acid*

Extinction series: incubation of the cryostat sections for 3 min with 0.8% periodic acid, then rinsing with Coons' buffer for 3×5 min.

6. *Reaction with mono-iodoacetic acid*

Extinction series: incubation of the cryostat sections for 10 min with 0.1 M sodium mono-iodoacetate (pH 8.0), then rinsing 3×5 min with Coons' buffer.

7. *Reaction with 2,4-dinitrobenzenesulphonic acid (6, 7)*

Extinction series: incubation of the cryostat sections for 30 min with 1 M dinitrobenzenesulphonic acid sodium (pH 7.2), then rinsing 4×5 min with Coons' buffer.

During the rinsings with Coons' buffer (pH 7.2), the prevailing pH of the rinsing liquid was determined with indicator paper, and the rinsing was continued until a pH of 7.2 was attained. To prevent the cryostat sections from becoming depleted of cations, we included washing processes with a "salt solution"[1], whenever many rinsings were required.

In addition to the controls usually undertaken for the indirect immunofluorescence method, tests with sera from healthy subjects were also performed. No fluorescence of the basal membrane or of the intercellular substance could be induced by chemical pretreatment of the cryostat sections.

The anti-IgG antiserum was supplied by Behringwerke, Marburg. The characteristics of this antiserum are: protein concentration of the gammaglobulin fraction conjugated with FITC=10±3 mg/l; specific antibody fraction=10±5% of the gammaglobulin concentration; molar F/P ratio=about 2.5.

The chemicals used for the pretreatment of the cryostat sections were commercial analytical grade preparations. We are particularly indebted to Bayer laboratories, Inc. for the supply of the sodium salt of the 2,4-dinitrobenzenesulphonic acid.

[1] The "salt solution": 9 g NaCl, 0.42 g KCl, 0.24 g $CaCl_2$, 0.03 $MgCl_2$ dissolved in 1 litre distilled water; the pH adjusted with N/10 HCl to the value specified in the procedures.

Table II. *Summary of the experimental results*

On the left side of the Table are the fluorescence patterns (BM = fluorescence of basement membrane; CN = fluorescence of cell nuclei; PFL = fluorescence pattern as in pemphigus vulgaris) after pretreatment of the cryostat sections with the stated reagent, and on the right side, the fluorescence patterns after further treatment with a second reagent are shown. The end point titre of the BM fluorescence relates to the observations made after restoration of fluorescence. In the "normal series", the sera showed basement membrane fluorescence down to a serum dilution of at least 1:256. For further details, see Results

Pretreatment of the preparation with	Immuno-optical findings after pretreatment			Further treatment of preparation with	Immuno-optical findings after further treatment			End point titre of the BM fluorescence
	BM	CN	PFL		BM	CN	PFL	
Acetic anhydride	–	–	–	Caustic potash	+	–	+	1:27
Sodium bisulphite	–	+	–	Hydrochloric acid	+	–	+	1: 9
Methanol	–	–	–	Potassium permanganate	+	–	–	1:27
Formalin	–	–	–	Water	+	–	–	1:27
Periodic acid	–	–	–					
Mono-iodoacetic acid	–	+	–	No further treatment was undertaken (irreversible reactions).				
2,4-dinitrobenzene-sulphonic acid	+	+	+					

RESULTS

The methods used in this work are techniques well known in the field of histology where they are used to identify, or even to block, certain chemical reactions. For our purpose, it was essential that the reactions should, by alteration of the concentrations and the reaction times of the chemicals, be adapted for the immunofluorescence of native cryostat sections. For the purpose of the fixation of anti-basement membrane antibodies from pemphigoid sera, it was necessary to exclude reactive groups by the most careful possible pretreatment of the preparations, and, in a subsequent reaction, to achieve this blocking reversibly and in such a way that the reactivity of the antigenic basement membrane should be retained. Since many substances cause an irreversible destruction of the morphological structures in the region of the basement membrane, if the strictest criteria for the detection of certain active groups are applied, then only those reactions provide proof in which the maintained antigenic functional capacity of the basement membrane can be guaranteed by a final antibody fixation. Furthermore, histochemical processes to which the cryostat sections are subjected before the start of the antigen/antibody reaction on the basement membrane, and which have no influence on this reaction, can in certain instances be regarded as proof that certain reactive groups do not take any part in these reactions.

The results of this work are summarised in Table II. They may be supplemented by the following details: It is noteworthy that, in some test series in which the fluorescence of the basement membrane had been eliminated by chemical action, some fluorescent cell nuclei were observed. It should also be noted that all the pemphigoid sera that we used exhibited a slight cell nucleus fluorescence even in the "normal series". It is also interesting that even the sera from non-pemphigoid patients, which also exhibited a slight fluorescence of the cell nuclei in the normal indirect immunofluorescence test, reacted with chemically pretreated antigenic substrates in such a manner that all the cell nuclei present in the epidermis showed extraordinarily intense fluorescence, whereas the end titre of this reaction was not affected.

It is especially noteworthy that, after the action of acetic anhydride/caustic potash and sodium bisulphite/hydrochloric acid after reversion of the extinction effect, a fluorescence was observed not only of the basement membrane but also of the intercellular substance, such as normally occurs only with pemphigus sera. The small end-point titre found after restoration of the basement membrane fluorescence (1:9 to 1:27) in comparison with the non-pretreated antigenic substrates (1:256 and more) can be explained as a consequence of the chemical actions and the large number of rinsing processes.

Table III. *Survey of the reactive groups determined with the reagents that we used, made as described in the literature (11)*

Only corresponding groupings of the proteins and the carbohydrates were considered. For further details, see Discussion

Chemical groups	Acetic anhydride/ caustic potash	Sodium bi-sulphite/ hydrochloric acid	Methanol/ potassium permanganate	Formalin/ water	Periodic acid	Mono-iodo-acetic acid	2,4 Dinitro-benzene-sulphonic acid
Amino	+			+	α-amino-alcohols		+
Imino				+			
Carboxyl			+	+			
Sulphonic acid			+				
Sulphydryl	+			+		+	+
Hydroxyl	+			+	α-glycols		
Carbamyl (aldehyde + ketone)		+					

The extinction of basement membrane fluorescence, observed after pretreatment of the cryostat sections with periodic acid and with mono-iodoacetic acid, seems rather less clear-cut in the evaluation. In both test series, care was taken to ensure that the morphological structure of the epidermis was preserved intact. Even in the case of mono-iodoacetic acid, despite the elimination of the antigenicity of the basement membrane, a cell nucleus fluorescence was observed. On the basis of these findings, it is not possible to evaluate the extent to which the fluorescence extinction phenomenon in these two test series is specific. After the action of 2,4-dinitrobenzenesulphonic acid, the immunological findings of the antigen/antibody reaction on the basement membrane were markedly intensified. The preparations appeared generally brighter. The cell boundaries of the stratum basale and spinosum, and the cell nuclei in higher sections, were also sharply defined. The corium, too, exhibited an enhanced fluorescence.

Table III gives information about the possible action sites of the blocking reactions induced by various chemicals. This table contains data derived from the literature (11), the active groups of the carbohydrates and the aminoacids being considered mainly for reasons that are mentioned in the discussion. In the discussion, moreover, the many groups that are theoretically capable of reaction are considered and an attempt is made, by a process of exclusion, to identify those that are probably involved in the fixation of the anti-basement membrane antibodies.

DISCUSSION

Our experimental results must be discussed against the background of Kefalides' recent review (14) of the biochemistry of the basement membrane. According to this review, three antigenic components must be considered, one of them being a collagen and the other two containing glucoproteins. The structures are stabilised by hydrogen and disulphide bridges, and also by cross-linkages derived from aldehydes. A noteworthy feature is the high proportion of hydroxylysine and hydroxyproline, and also the presence of alpha-glucosido-beta-galactosido-hydroxylysine whereas amino sugars and imino acids are practically absent.

In our first paper (18), we reported that the antigenic determinant for the binding of the anti-basement membrane antibodies in pemphigoid is, very probably, the alpha-glucosido-beta-galactosido-hydroxylysine, but that the question of the possible binding sites still remained unresolved. On the basis of the studies described in this present paper, a more exact identification of the binding mechanism of the antibodies will now be attempted.

The fact that the pretreatment of the cryostat sections with 2,4-dinitrobenzenesulphonic acid in high concentration does not suppress the immunofluorescence of the basement membrane, shows that $-NH_2$ and $-NH_3^+$ groups are not involved in the antibody fixation. Quite the contrary! The fluorescence pattern is qualitatively and quantitatively increased after the action of this reagent, and this provides an indication that the anti-basement membrane antibodies also carry

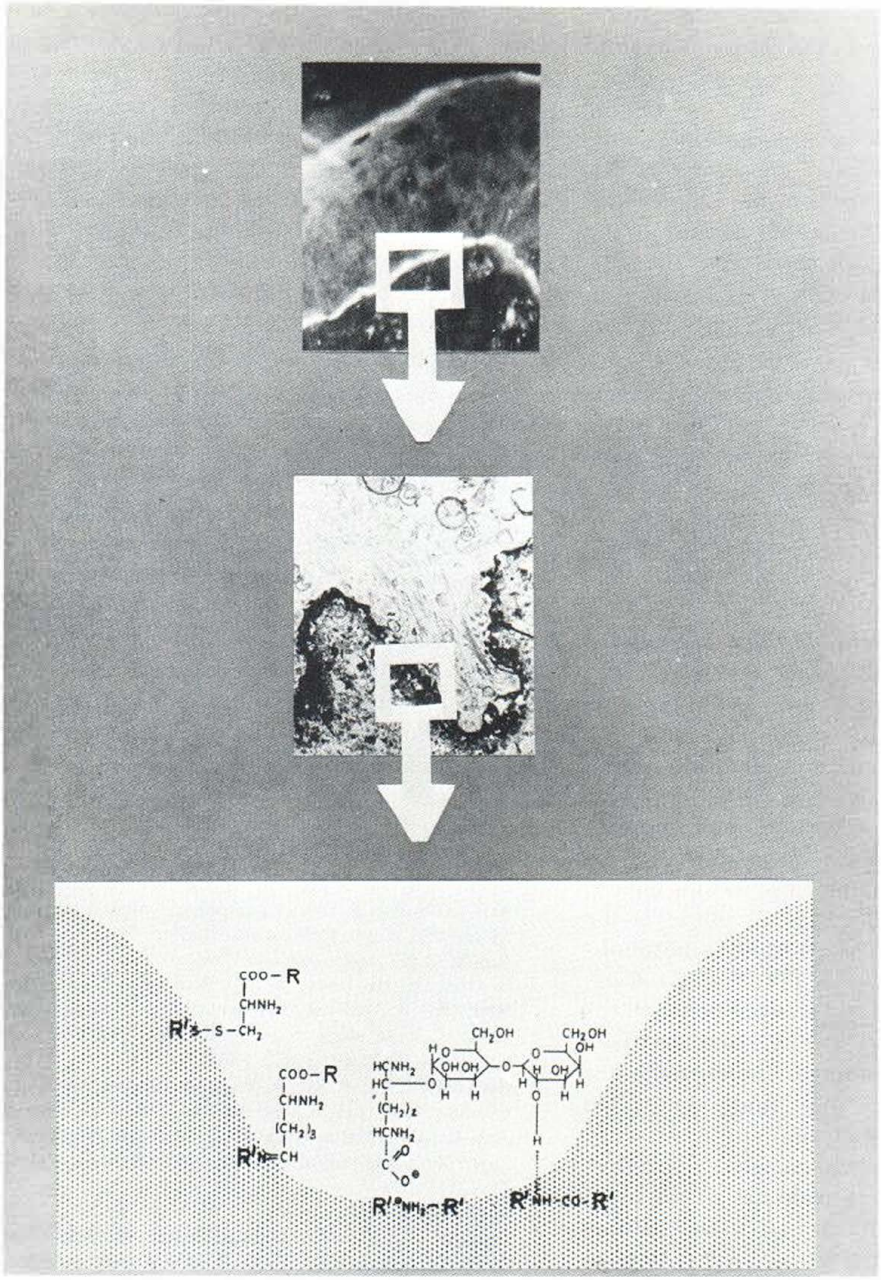

Fig. 1. (*a*) Immunfluorescent staining of the basement membrane zone using the indirect method. ×100. (*b*) Immuno-electron microscopy of the basement membrane zone. Arrows indicate peroxidase activity and thus the site of IgG deposits. ×22200; (from Holubar et al., J Invest Dermat *64*, 224, 1975). (*c*) Model presentation of antigen/antibody reaction in the basement membrane zone on molecular biological base. Lower, grey part: Antibasement membrane antibody with pocket; above it, basement membrane zone (white background). The model takes account the following kinds of bonds (from left to right): (1) a disulphide bridge, (2) the formation of Schiff's base, (3) an electrostatic bond, (4) a hydrogen bridge. ●, electrostatic charges.

positively charged binding sites and that their repulsion by the amino group of the antigen is reduced after reaction with the dinitrobenzenesulphonic acid.

The positive outcome of the methoanol/potassium permanganate reaction implicates the involvement of acidic groups, which, in the light of our present knowledge about the structure of the basement membranes, comprise the terminal carboxyl groups of amino acids. This provides yet further proof of positively charged adhesion sites for the antibodies. The result of the formalin/water reaction can be interpreted in the same way. Admittedly, still other reactive groups are detected in this way, but the only other groups that need be considered in this context are hydroxyl groups and possibly (on account of their relatively rare occurrence) also sulphydryl groups. The results obtained with acetic anhydride/caustic potash also point in this same direction.

The sodium bisulphite/hydrochloric acid-induced inhibition of basement membrane fluorescence must be attributed to the involvement of carbonyl groups. According to recent publications (20, 21), the collagen of the basement membranes has been shown to contain not only cross-links formed by aldehydes, but also free aldehyde groups, which are formed enzymatically by the lysyl oxidase.

In summary, then, the probable site for binding the antibodies to the antigenic structures of the basement membrane can be envisaged in the following way: In the neighbourhood of the alpha-glucosido-beta-galactosido-hydroxylysine, and perhaps also in the region of free hydroxylysine, and with the participation of electrostatic bonds from free carboxyl groups of terminal amino acids, the antigenic basement membrane reacts with the antibodies, and the resultant complex is stabilised by hydrogen-bridges from hydroxyl groups (from glucose, galactose, and perhaps from free hydroxylysine), by cross-linds from aldehydes (derived from alpha-amino-adipic acid-ε-semialdehyde), or by formation of Schiff's bases, and, to a smaller extent, by disulphide bridges (derived from the cysteine). Fig 1 shows a model presentation which takes account of all the above-mentioned kinds of bonds. In this model is shown the binding of the antibodies to the basement membrane, starting from the fluorescence microscopy findings, through the electron-microscopy photographs, to the situation in the molecular region.

The antigenic structures of the intercellular substance appear to possess a certain relationship to the antigen of the basement membrane, for in several test series we observed, after chemical pretreatment of the cryostat section, not only the fluorescence of the basement membrane zone but also a fluorescence like that found in pemphigus vulgaris. The cell nucleus antigens, on the other hand, appear to possess different chemical properties, as can be deduced from the fact that cell nuclei repeatedly fluoresced under conditions where all other fluorescence patterns had disappeared; and even after incubation with chemical substances, when sera from non-pemphigoid patients were used, the cell nuclei of the epidermis showed an intensified fluorescence.

We ourselves have already reported (18) that the antigenicity of the basement membrane is increased by preincubation of the cryostat sections with proteolytic enzymes. The results of this present work indicate that the intercellular substance of the epidermis of guinea-pig tongue can be antigenically modified by chemical action, and this finding can similarly be interpreted in the sense of a "secondary antibody formation" (18).

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