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THE ACTIVATION OF ANTIGENS OF THE BASEMENT MEMBRANE ZONE BY PROTEOLYTIC ENZYMES IN VITRO

Wolfgang Remy, Hans Bockendahl, Günter Stüttgen and Gisela Petersen

From the Department of Dermatology of the Free University, Rudolf-Virchow Hospital, Berlin

Abstract. Following brief exposure of cryostat sections of human skin to the action of proteolytic enzymes (papain or trypsin), there was a pronounced increase in the antibody binding sites of the basement membrane zone, shown in indirect immunofluorescence by an increased intensity in fluorescence of the basement membrane zone as compared with preparations which had not previously undergone incubation, and by an increase of 5–6 (papain) or 4–5 (trypsin) titre dilution steps. This effect was practically absent when guinea-pig tongue was used as the antigenic substrate. In conjunction with findings published in the literature, our results can be interpreted as indicating that the activation of the basement membrane zone antigens by proteolytic enzymes is associated with an increase in antigenicity which results in the formation of "autoantibodies" of the basement membrane zone antibody type.

Key words: Basement membrane zone; Bullous pemphigoid; Activation of antigens; Antigen-antibody reaction; Proteolytic enzymes; Human skin

In contrast to the numerous studies on the significance in pathological mechanisms of basement membrane zone antibodies in bullous pemphigoid (4, 9, 13), nothing is known about the antigens located in the basement membrane zone of the epidermis. We have attempted, in a series of experiments begun recently, to define more clearly the sessile antigens located in the basement membrane zone of the human skin. Since, in the view of some authors (8, 11), the reticulum of fine filaments of the "electron microscopic basement membrane" is probably composed of tropocollagen aggregates, we have investigated the protein properties of the antigenic basement membrane zone. In the course of these investigations we discovered that proteolytic enzymes produce quantitative changes in the antigenicity of the basement membrane zone. We too attempted to confirm this effect with another substrate. For this we selected the basement membrane zone antibody itself, which after incubation for fluorescein-conjugated antiserum represents the equivalent of an antigen.

MATERIALS AND METHODS

Cryostat sections of healthy human skin were incubated with papain or trypsin and compared by indirect immunofluorescence with preparations not previously incubated (Table 1). We used the following solutions of papain (1): To separate preparations of 8 ml of a 0.01 M solution of cystein hydrochloride (pH 7.2) were added 0.005, 0.01, 0.02, 0.04 and 0.08 ml of the following suspension of papain: papain (Serva Lab.), purest crystalline form, about 15.8 EU/mg, or 39 mg/ml, in suspension in 0.05 M sodium acetate solution at pH 4.5. The cryostat sections were incubated for 1 and 5 minutes in these different concentrations of papain solution. The trypsin solution was made up as follows (2): 1 ml of an N/1000 HCl solution containing 0.1 g% of trypsin (Serva, 1× crystalline, salt-free, pharmaceutical: 160 EU/mg) was diluted with 99 ml of 0.1 M phosphate buffer at pH 7.6. The sections underwent 5 and 15 min of preliminary incubation in this solution (1 mg% trypsin).

For indirect immunofluorescence (for details see 3, 5, 6, 10), the cryostat sections were incubated with serum containing basement membrane zone antibodies (using guinea-pig tongue as antigenic substrate, positive up to a dilution of 1 : 256), then washed and flooded with fluorescein isothiocyanate-conjugated antiserum (Behring Company AG, West-Germany) at a dilution of 1 : 10 ("normal series" in table). The characteristics of the conjugate were: Protein concentration of the FITC conjugated gamma-globulin fraction: ca. 10±3 mg/ml. Total protein concentration (after addition of human albumin): ca. 40±5 mg/ml. Specific antibody content: ca. 10±5% of the gamma-globulin concentration. Molar F/P ratio: ca. 2.5±1.5. The details of the experiments, including the modifications for the effect of the proteolytic enzymes on the basement membrane zone antibodies (II and III in the table) are shown in Table I.

Table 1. *Details of experiments for indirect immunofluorescence (normal series) and modifications for the effect of proteolytic enzymes (I–III)*

Normal series	I	II	III
Frozen section →	"	"	"
	Papain/trypsin 4× washed		
Pemphigoid serum → (1 : 1–1 : 1 024)	(1 : 1–1 : 1 024)	(1 : 1–1 : 1 024) 4× washed 0.02 ml papain, 1 min	(1 : 1) 4× washed 0.02 ml papain, 1 min 4× washed Pemphigoid serum (1 : 1–1 : 1 024)
4× washed →	"	"	"
Fluorescein-conjugated Anti-IgG →	"	"	"
4× washed →	"	"	"
Mounted →	"	"	"

RESULTS

Following preliminary incubation with papain (for 1 min) and with trypsin (for 5 min), there was a much greater intensity in fluorescence of the basement membrane zone as compared with previously non-incubated control sections at the same dilutions (1 in the table). There was in addition an increase of 5–6 titre dilution steps (factor 2, geometric progression) in all the concentrations of papain investigated (0.005–0.08 ml of papain suspension) and in 4–5 dilutions of the trypsin. After one minute's incubation with papain solution (0.01 ml of papain suspension) or after 5 min action of the trypsin solution, there was an increasing degree of subepidermal separation of the epidermis, at first localised (Fig. 1), while the effect of the solution of papain which contained 0.08 ml of the papain suspension was to produce more pronounced separation of the epidermis at multiple sites (Fig. 2). The epidermis was completely separated from the corium after incubation for 5 min with the papain and 15 min with the trypsin.

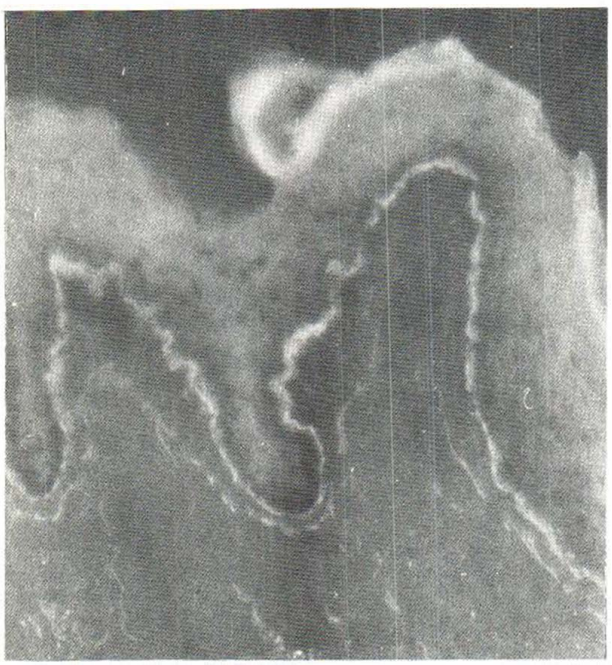

Fig. 1. Subepidermal band of fluorescence and incipient loosening of epidermis from corium, shown by indirect immunofluorescent staining using cryostat sections of human skin pre-incubated for 1 min with a solution of papain (0.01 ml of papain suspension), and then flooded with a patient's serum containing basement membrane zone antibodies diluted to 1 : 32, and incubated, after washing, with diluted 1 : 10 commercially available fluorescein isothiocyanate-conjugated antiserum (for characteristics of conjugate, see: Materials and Methods). ×10 objective.

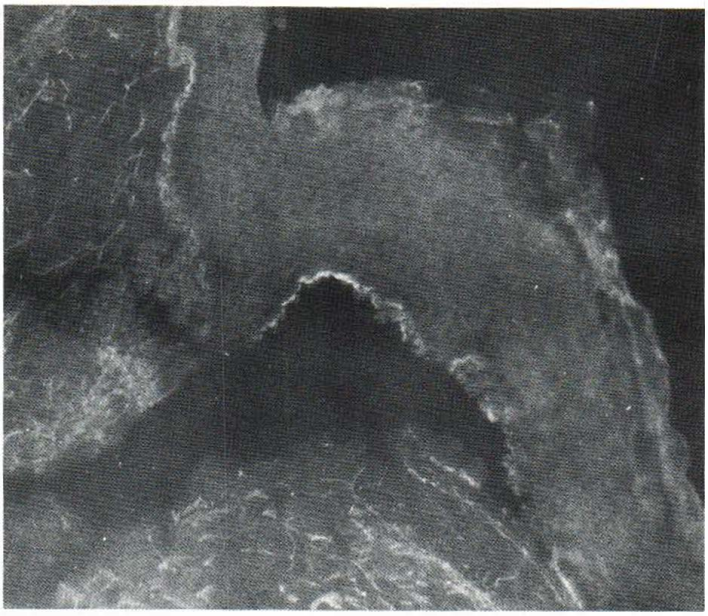

Fig. 2. Localised subepidermal band of fluorescence with evident loosening of epidermis from corium, shown by indirect immunofluorescence using cryostat section of human skin pre-incubated for 1 min with a solution of papain (0.08 ml of papain suspension). Details otherwise as for Fig. 1. ×10 objective.

In experiment II in the table, where the papain acted only after fixation of the basement membrane zone antibodies to the basement membrane zone, there was an increase of three titre dilution steps compared with the normal control series. A ninefold increase in titre dilution steps was seen in experiment III in the table.

The following controls were examined: 1. Tests with fluorescent conjugate were negative in the absence of serum. 2. Several so-called "normal control sera" (these sera did not contain nuclear, pemphigus or basement membrane zone antibodies) were tested. Some of these sera had elevated antibody titres against measles, rubella or *T. pallidum*. Using these sera the results were always negative. 3. The following other antigen-antibody systems were tested: Using sera containing nuclear or pemphigus antibodies there was no activation of nuclear or pemphigus antigens after exposure of skin sections to proteolytic enzymes.

On the other hand, when guinea-pig tongue was used as the antigenic substrate, there was practically no increase in the antigenicity of the basement membrane zone.

DISCUSSION

Our results support the assumption that the antigens located in the basement membrane zone are proteins. However, it is a matter for discussion whether protein structures are formed as a result of the enzyme action, with the release of free, included functional groups of different chemical nature.

We found in the course of this experiment that brief exposure to the action of proteolytic enzymes results in a marked increase in the antibody binding sites of the basement membrane zone. We showed in the case of papain (with the test modifications shown as II and III in the table) that this enzyme is also active against the fixed basement membrane zone antibodies. The sharp increase in titre dilution steps in the series III experiments is due to the combination of both sites of action of papain.

The effect of increase in the intensity of fluorescence seen in these experiments, and the increase in titre dilution steps, can be explained by the law of mass action and has been extensively discussed elsewhere (10) in a similar context—the activation of basement membrane zone antigens by high-energy irradiation. Since guinea-pig tongue scarcely shows this effect at all, it clearly contains antigenic structures which, while related to human skin (both structures react with basement membrane zone antibodies), differ, since in effect only the basement membrane zone antigens of the human epidermis were activated by the enzymes.

As regards the significance of proteolytic enzymes in the pathogenesis of bulla formation, Stütt-

gen et al. (15) were able to demonstrate di- and tri-peptidases regularly in blister fluid, as well as a proteolytic effect of endopeptidases in the epidermis and corium (14). According to the studies of Braun-Falco (7), the action of trypsin and chymotrypsin on fresh, living skin results in separation of the epidermis and cutis, and the histological findings resemble those of bullous pemphigoid (7). Hence in this disease there are clearly marked changes in the basement membrane and in the cutaneous mesenchymal matrix as a result of enzymatic processes (7).

This enables us to extend the view of Braun-Falco (7) on the basis of recent immunological discoveries in pemphigoid patients, to say that a moderate action of proteolytic enzymes on basement membrane zone antigens results in a marked increase in the basement antibody binding sites. An increase in antigenicity which may be associated with this could lead to the formation of "autoantibodies" of the basement membrane zone antibody type. This agrees with the hypothesis of Sams (12) and Sams & Gleich (13), that the basement membrane has become antigenic, so that specific basement membrane zone antibodies are formed and bound there.

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W. Remy, M.D.
Department of Dermatology
Rudolf-Virchow Hospital
1 Berlin 65
Augustenburger Platz 1
FRG