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ISOLATION AND CHARACTERIZATION OF SOLUBLE EPIDERMAL ANTIGENS REACTIVE WITH PEMPHIGUS ANTIBODIES

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Abstract. The antigenic substance reactive with pemphigus antibodies was isolated from the soluble component of guinea pig epidermis by affinity chromatography. The substance was a protein of molecular weight 18 000 and consisted of RNA and hexose.

Key words: Pemphigus antigens; Affinity chromatography

Pemphigus has been defined as an autoimmune disorder since 1964 when Beutner (5) demonstrated skin antibodies in sera of pemphigus vulgaris patients. The pemphigus antibodies, mainly IgG class, are not specific for the species but for the organ and disease (6). The antigens reactive with the antibodies are localized upon the intercellular substance of the stratified squamous cell epithelium (5–7), but their detailed characterization has not yet been elucidated. Detection of the antigens was carried out by the immunoabsorption test after the consecutive fractionations of the epidermal components (2, 16), or by immunopathology using the competitive binding of antibodies vs. antigens (9).

In the present paper, the antigenic substance reactive with the pemphigus antibodies was isolated from the soluble component of guinea pig epidermis by means of affinity chromatography, and characterization of the antigenic substance was performed using ultraviolet absorption spectra, column chromatography and by quantitative analyses.

MATERIALS AND METHODS

1. *Preparation of soluble component from guinea pig epidermis*

About 60 female guinea pigs (Hartley), each weighing 350 to 600 g, were used. Their trunks were depilated and they were then exsanguinated by cardiac puncture. The epidermis was collected by the stretching method (4). The sample was frozen and thawed several times in acetone–dry ice, and ground in a mortar with about 8 wet weight volumes of phosphate-buffered saline (PBS) (pH 7.0, 0.02 M). The homogenate was filtered through bilayered gauze, and the crude fraction removed by centrifugation at 3 000 rpm for 20 min. The supernatant was ultracentrifugated at 105 000 *g* for 60 min, and white lipid on the top of the fluid was removed with a spatula. The residual supernatant was filtered. About 400 ml of the filtrate (OD 280 nm=40.6) obtained in this way was used as the soluble component of the guinea pig epidermis in the following experiments.

2. *Gamma-globulin from pemphigus patients and control sera*

A total of about 50 ml serum was collected over 3 months from a 46-year-old woman of blood group B who suffered from active pemphigus vulgaris. At each of 12 serum collections, antibody titers to the intercellular (IC) substance of human epidermis and to nuclei (AN) of guinea pig liver cells were determined by indirect immunofluorescence staining with FITC-labelled goat antiserum to human IgG (Hyland Laboratories, F/P (mol)=3.0, protein=2.0 mg/ml). The IC titer ranged from 1:64 to 1:1024, and the AN titer 1:1 on only two separate occasions.

Other laboratory data of the patient included: negative rheumatoid factor, positive C-reactive protein, 12 anti-streptolysin-O titer, normal liver function tests, serum total protein of 6.4 mg/ml (albumin 53.4%, α_1-globulin 4.4%, α_2-globulin 8.9%, β-globulin 11.1% and γ-globulin 22.2%).

The microscopic examination of the skin biopsy revealed suprabasal acantholytic bulla, and the direct immunofluorescence staining showed positive IC IgG deposit.

Control serum was obtained from a 20-year-old, healthy male of blood group B.

Crude γ-globulin was precipitated by ammonium sulfate (12) from the pemphigus as well as from the control sera.

3. *Isolation of soluble antigens by affinity chromatography* (Table I)

(*a*) *Conjugation of γ-globulin to carrier.* Gammaglobulins of the pemphigus patient or of the control, which

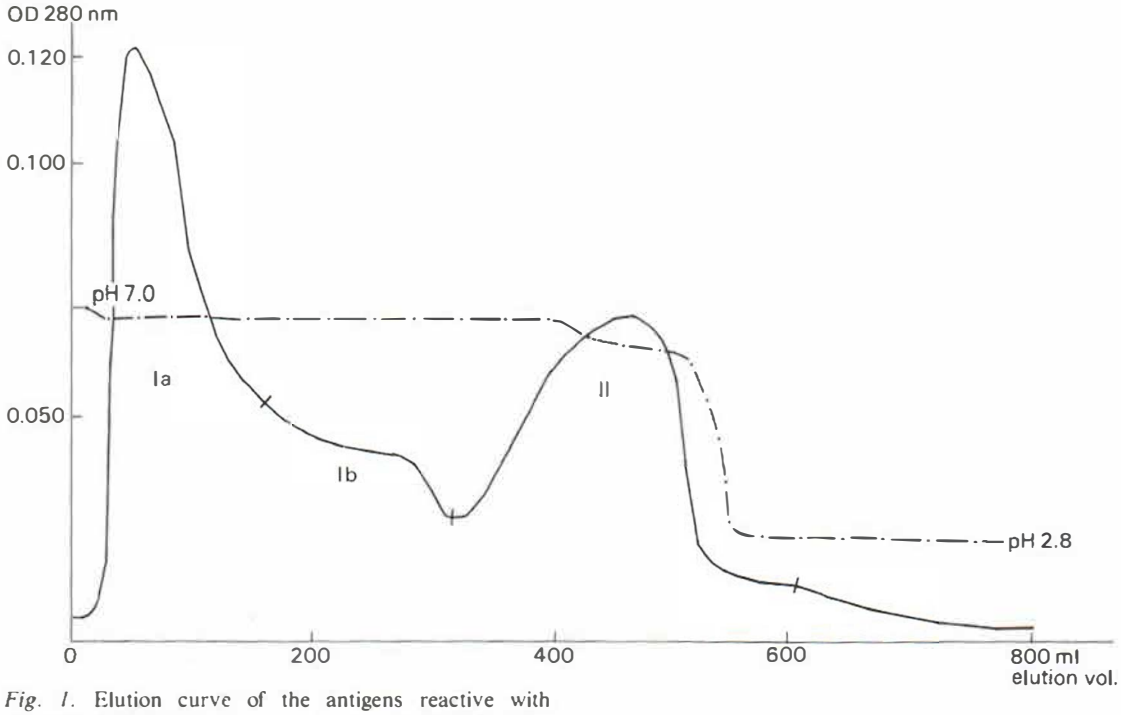

Fig. 1. Elution curve of the antigens reactive with pemphigus γ-globulin. Column size: 3.5×18 cm; fraction size: 10 ml; buffer: 0.1 M glycine-HCl buffer, pH 2.8.

were respectively suspended in an 0.2 M sodium citrate buffer (pH 6.6), and Sepharose 4B (Pharmacia) activated with CNBr, were conjugated at 4°C with continuous slow stirring. After conjugation, the mixture was washed with sodium citrate buffer until the optical density of the supernatant was less than 0.010 at 280 nm; 1 M ethanolamine (pH 8.0) was then added at room temperature and reacted for 2 hours to block the remaining active groups. After several washings with PBS, 0.1 N acetic acid, and 0.1 M borate buffer (pH 8.0), the mixture was suspended in PBS and stored at 4°C. The amount of conjugated protein thus obtained was equal to approximately 8 mg of γ-globulin per 1 g of Sepharose 4B, calculating from the optical density at 280 nm.

(*b*) *Isolation of antigenic substance from soluble component of epidermis.* Sepharose 4B-γ-globulin conjugate was reacted with 200 ml of the soluble component of guinea pig epidermis at 4°C overnight. After this mixture was thoroughly washed with PBS to remove the unconjugated substances, it was packed in a column, and the conjugate was eluted with 0.1 M glycine-HCl buffer at pH 2.8.

4. *Ultraviolet absorption spectra*

The sample was placed in a quartz cuvette with a 1 cm light path and the absorption spectrum was measured with a double beam spectrophotometer (Hitachi, Model 124), with glycine-HCl buffer as control.

Table 1. *Isolation of antigenic substance reactive with pemphigus antibodies by affinity chromatography*

γ-Gl
in sodium citrate buffer, 0.2 M, pH 6.6
Conjugate with CNBr-activated Sepharose 4B
wash with sodium citrate buffer
React with 1 M ethanolamine
wash with PBS, pH 7.0, 0.02 M acetic acid, 0.1 M borate buffer, pH 8.0, 0.1 M
React with soluble extract of guinea pig epidermis
wash with PBS
Elution glycine–HCl buffer, 0.1 M, pH 2.8

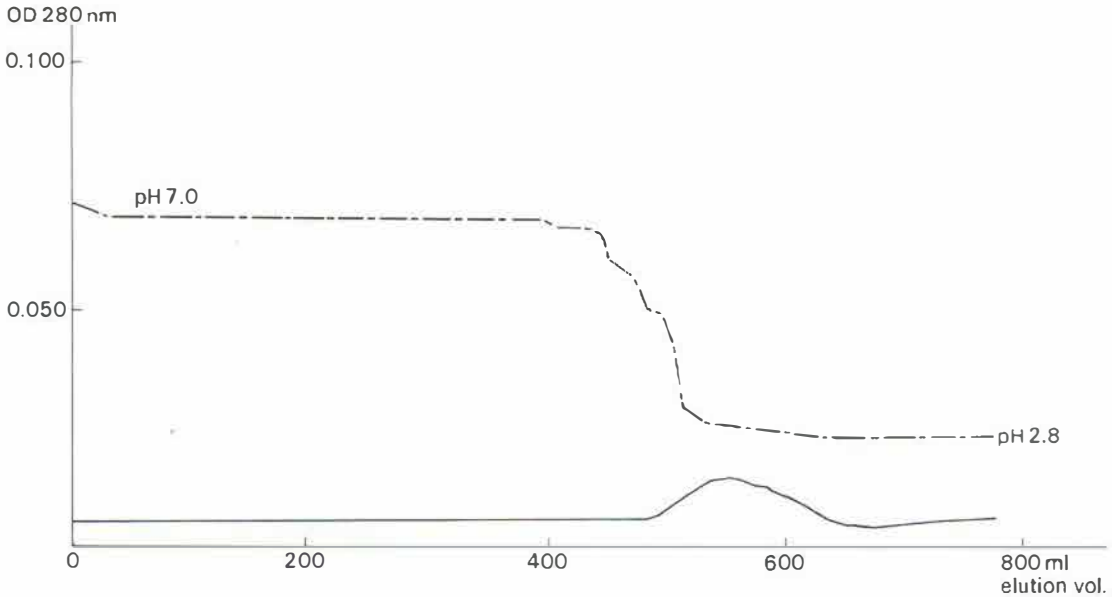

Fig. 2. Elution curve of the antigenic substance reactive with normal control γ-globulin. Column size: 2.5×35 cm; fraction size: 10 ml; buffer: 0.1 M glycine-HCl buffer, pH 2.8.

5. *Estimation of molecular weight by gel filtration* (3)

Sephadex G-200 was well swollen in PBS and packed in a Sephadex column (2.6×90 cm, Pharmacia). It was stabilized with a continuous flow of PBS for 24 hours and the sample was then applied to the top end of the column. The ultraviolet spectra of the effluents at 280 nm were automatically recorded with a spectrophotometer (Toyo-Uvicon, Model 540 M) and its molecular weight (MW) was estimated by comparison with the standard curve obtained with guinea pig IgG (MW=160 000), BSA (MW=67 000), and egg-white lysozyme (MW=14 700).

6. *Absorption test*

After 1 ml of serum from the pemphigus patient had been absorbed with an equal volume of the sample at 37°C for 1 hour, the reaction mixture was tested in serial two-fold dilutions for human epidermal IC and guinea pig liver cell AN antibodies by the indirect immunofluorescence technique.

As a control, serum from a 27-year-old female patient with systemic lupus erythematosus, whose AN titer was 1 : 160, was similarly examined.

7. *Quantitative analysis of chemical substances*

RNA, DNA, protein and hexose were quantitated by orcinol reaction (modified method of Zamenhof) (14), diphenylamine and indole reaction (17), microbiuret method (14), and Anthrone reaction (modified method of Jesaites) (1, 14).

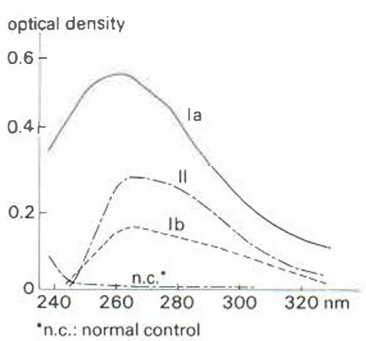

Fig. 3. Ultraviolet absorption curve.

RESULTS

1. *Elution profile of antigenic substance*

Fig. 1 shows the elution profile of the antigenic substance reactive with the pemphigus γ-globulin.

Table II. *Calculation of RNA/protein (%) by ultraviolet absorption*

	A_{260}[a]	A_{280}[a]	A_{280}/A_{260}	RNA/protein
I a	0.535	0.430	0.805	6.5 (%)
I b	0.145	0.144	0.993	3.4
II	0.260	0.272	1.05	2.8
Control[b]	0.020	0.008		

[a] Absorbancy at 260 nm and 280 nm, respectively.
[b] RNA per cent (%) is not calculable due to low A_{260} and A_{280} values.

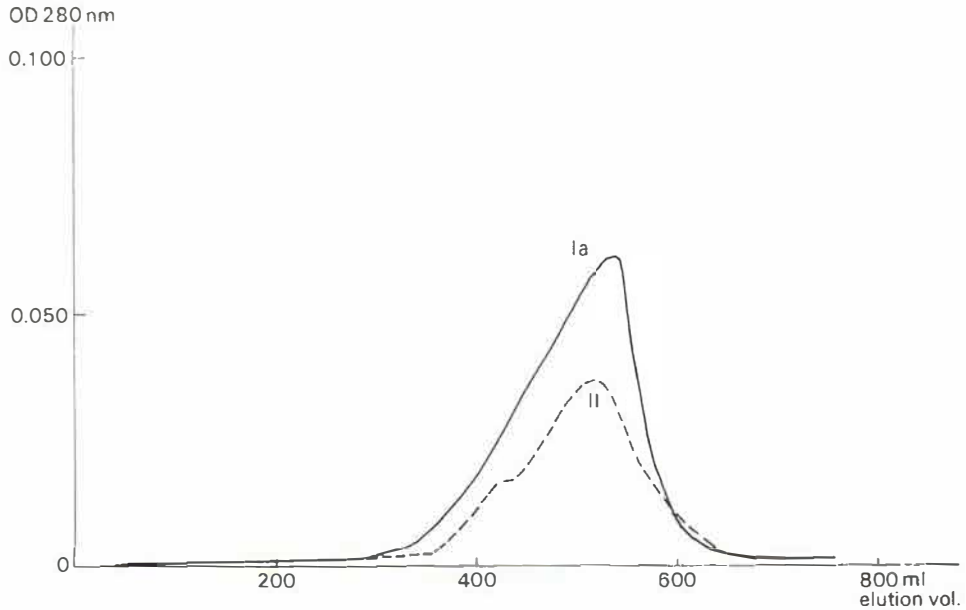

Fig. 4. Sephadex G-200 column chromatography. Column size: 2.6×90 cm; velocity: 45 ml/hour; buffer: PBS; load: 15 ml.

The profile was diphasic, with peaks at pH 6.8 and 6.3. The first peak was designated as I a, the following plateau as I b, and the second peak as II. Each fraction was collected and concentrated to 5-fold in a rotary evaporator (Yamato, Model RE-41).

Ultraviolet absorption spectra, various quantitative analyses and the absorption test by the indirect immunofluorescence antibody technique were carried out on each fraction.

The elution profile of antigenic substance reactive with the normal γ-globulin is shown in Fig. 2. Fractions corresponding to the above peaks were collected similarly, concentrated to 5-fold and analysed in the same way.

2. *Ultraviolet absorption spectra*

As shown in Fig. 3, fraction I a had a maximum absorption at 260 nm, and fractions I b and II at 265 nm. This was suggestive of their high nucleic acid content. The color reactions specific for DNA (the diphenylamine and indole reaction) were both negative. Therefore, the nucleic acid was assumed to be RNA only. Table II shows the ratio of RNA to protein, calculated from the optical density at 260 nm and 280 nm, according to the method of Warburg-Christian (11).

3. *Assessment of molecular weight by gel filtration on Sephadex G-200*

Fig. 4 shows the elution profile of I a and II fractions obtained by applying 15 ml of each fraction to the Sephadex G-200, with PBS as the eluent. Both I a and II fractions showed a wide distribution, and the peaks corresponded to the molecular marker of ca. 18000.

4. *Absorption test*

The patient's serum used for affinity chromatography (autologous to the absorbent) and other pemphigus vulgaris serum from a 31-year-old female (homologous to the absorbent) were both

Table III. *Indirect intercellular immunofluorescence absorption tests with each fraction*

Normal human skin sections served as testing antigens

	Staining with			
	Unabsorbed serum	Absorbed serum with		
Dilution		I a	I b	II
64	+	+	+	+
256	+	−	−	±
1 024	−	−	−	−

Table IV. *Indirect nuclear immunofluorescence absorption test with each fraction*

The sections of guinea pig liver served as testing antigens

Dilution	Staining with			
	Unabsorbed serum	Absorbed serum with		
		I a	I b	II
1	+	/	/	/
2	+	−	−	−
4	−	−	−	−

absorbed with each of I a, I b and II fractions respectively. As for the IC titers, there was no difference between the autologous and homologous sera. As shown in Table III, fractions I a and I b obviously absorbed pemphigus antibodies, while II fraction absorbed very little.

With respect to the detection of the AN antibodies, absorption test was not possible in the autologous antigen–antibody system as they were present only in undiluted serum. With the homologous system, the AN antibodies disappeared after the absorption with I a, I b and II fractions respectively (Table IV).

The systemic lupus erythematosus serum was not absorbed at all.

5. *Quantitative analysis of protein, RNA, DNA and carbohydrates*

Table V shows that the I a fraction contained 97.0 μg/ml protein and 8.0 μg/ml RNA, equivalent to 8.2% of protein content. The RNA content was slightly greater than the RNA content, as estimated by the ultraviolet absorption spectra. The RNA was not detected in either the I b or the II fraction. It was known from a preliminary experiment on purified yeast RNA that RNA of less than 5 μg/ml could not be detected by orcinol reaction. Each fraction was therefore assumed to contain 1 to 2 μg/ml RNA from the ultraviolet spectra.

As for the carbohydrate content according to the method of Anthrone, by which free and conjugated hexose could be quantitatively analysed, hexose was determined equivalent to 9.4%, 21.1%, and 7.6% of protein in I a, I b and II fractions respectively.

DNA was not found in any of the fractions and could not be detected even when the sample was further concentrated.

DISCUSSION

In the present experiment, affinity chromatography (8) was used to isolate the soluble antigenic substance from the guinea pig epidermis which reacted specifically with the pemphigus antibodies conjugated to Sepharose 4B.

Affinity chromatography is easy to handle and chemically stable. The specificity of the antigen obtained by affinity chromatography is considered to be excellent.

One possible problem with this technique might concern those pemphigus antibodies which are of mainly IgG class. Pemphigus antibodies are variable in their characteristics and are often of both IgA and IgM classes (6). In addition, contamination by other antibodies to other epidermal components in crude γ-globulin from the pemphigus patient could not be ruled out. A low AN antibody titer was shown in the patient's serum. Blood group substances reactive with the epidermal components might also cause contamination. Blood group substances are known to be insoluble glycolipid (10) and, in the present experiment, no attention was paid to the blood group substances as only soluble antigen was used. This could be proved by the

Table V. *Quantitative analysis of protein, RNA, DNA and hexose*

	Protein (μg/ml)	RNA (μg/ml (%))[b]	DNA	Hexose (μg/ml (%))[b]
I a	97.0	8.0 (8.2)	n.d.	9.1 (9.4)
I b	41.2	n.d.	n.d.	8.7 (21.1)
II	49.0	n.d.	n.d.	3.7 (7.6)
Control	n.d.[a]	n.d.	n.d.	n.d.

[a] Not detected.
[b] Percentage against the amount of protein.

control experiment in which no soluble antigens reactive with the γ-globulin of normal human serum (B blood group) were extracted.

The antigen(s) extracted was a soluble protein of molecular weight of 18000, containing RNA and hexose, but it was not considered a pure pemphigus antigen for the above reason. However, it was clear that this extract contained the pemphigus antigen(s), by the fact that this antigen(s) reacted not only with pemphigus serum used in the affinity chromatography, but also with serum from the other pemphigus patient. By indirect immunofluorescence staining with rat antiserum to this extract, antibodies not only to intercellular but also to intracellular substances were demonstrated, while no AN antibodies were detected. These microscopic findings were contradictory to the result obtained in the absorption test, in which AN antibodies in the pemphigus patient's serum were absorbed with the extract. Further investigation of these contradictory findings should be made. The extracts which were purified might contain intracellular substances of the guinea pig epidermis. The appearance of RNA in the extract may be due to the fact that glycoprotein (hexose+protein) can passively or ionically absorb RNA during homogenization and extraction, or that the extracellular portion of an antigen is associated with intracellular RNA through the cell membrane.

It is well known that the dissociation of antigen–antibody complex does not occur between pH 7.5 and 5.0, but does occur at pH values less than 3.5 (15). In the present experiment, as shown in Fig. 1, an antigenic substance was eluted at pH 6.8–6.3 and was not detected in the strong acid region. This finding demonstrates that the dissociated antigen in acid buffer in the upper portion of the column was again changed in acidity as it passed down through the lower part of the column.

The localization of carbohydrate to the intercellular portion of the epidermis was recently discovered in experimentation using concanavalin A (13), which inhibits the conjugation of pemphigus antibodies to intercellular substances (9). These findings lead to a conclusion that the combining site of pemphigus antibodies is the carbohydrate portion. This is consistent with the result of the present experiment where a large amount of carbohydrate was obtained in the antigenic extract.

Lipid analysis was not carried out because of the shortage of sample for the chemical analysis.

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