An Enzyme-Linked Immunosorbent Assay for Desmosine

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SUMMARY

A sensitive, reproducible and rapid analytical method that is capable of identifying the presence of lung damage at an early stage is required. We have developed a sensitive enzyme-linked immunosorbent assay for desmosine in human body fluids. An amino group of desmosine was exclusively linked to bovine gamma globulin and the high-titer antibody to this conjugate was prepared. This antibody was used in this assay. This method enables the routine analysis of large numbers of samples with a detection limit of 0. 4 ng desmosine. Using this method, we quantitatively measured desmosine in the urine of 8 normal controls and 25 patients with chronic obstructive lung disease. The results were expressed as desmosine/creatine ratio. On average, a statistically significant higher level of desmosine/creatinine was found in the patients compared to the controls.

INTRODUCTION

Pulmonary emphysema usually develops insidiously over many years, and significant damage to lung tissue has already occurred before the chest X-ray and the pulmonary function tests become abnormal. For this reason, an analytical method that is capable of identifying the presence of lung damage at an early stage, before conventionary detectable symptoms develop, is required. It could be useful in identifying those people who are at risk and possibly in monitoring the progression of the disease.

This disease is characterized by a progressive destruction of alveolar walls. Elastin is one the major components of lung connective tissue and is responsible for maintaining its elasticity (1, 2). When the connective tissue is destroyed, elastin is released from the lungs to the body fluids as elastin peptides (2, 3). Unusual changes in the concentration of elastin degradation products in body fluids may indicate the progression of the pulmonary destruction. Therefore, sensitive assay methods are necessary to monitor the concentration of these products in body fluids. So far, the approaches which have been used for this purpose are tests for the elastin-specific crosslinking amino acids, desmosine (4, 5) and isodesmosine (6), or elastin peptides

Key words: Enzyme immunosorbent assay, Desmosine, Isodesmosine, Chronic obstructive lung disease.

(7, 8).

Darnule et al. (7) developed a radioimmunoassay to determine the elastin peptide content of human serum. Kucich et al. (8) made antibodies for peptides derived from human lung parenchymal elastin and used these antibodies in an enzyme-linked immunosorbent assay (ELISA)[1] to quantify elastin peptides in human serum. However, the structures and properties of elastin peptides in human body fluids are not yet clear. Desmosine and isodesmosine are known as specific crosslinking amino acids of elastin, and thier structures and properties have been identified (9-12). Therefore, the measurement of urinary desmosine could provide a true monitor of elastin turnover. Harel et al. (5) described a radioimmunoassay for detecting desmosine in acidhydrolysates of urine. Skinner et al. (6) developed a radioimmunoassay for the determination of isodesmosine. Urinary desmosine excretion is known to rise as a result of increased elastin degradation in the body.

Expensive equipment and radioactive materials have been used in the immunoassays performed for the determination of desmosine (4, 5) or isodesmosine (6). ELISA, on the other hand, is cheaper and safer, since the assay does not require radioactive materials. We have avoided the use of radioisotopes and developed a double antibody ELISA for the determination of desmosine in an acid hydrolysate of urine.

For this purpose, we prepared the desmosine derivative of which an amino group was substituted exclusively by a heterobifunctional coupling reagent (13). The antiserum for the conjugate obtained from this derivative and a carrier protein showed high-titer and good specificity in the ELISA. The assay was found to be suitable for accurate estimation of elastin degradation products in human urine. We measured desmosine excretion in normal controls, and in a group of patients with chronic obstructive lung disease (COLD).

MATERIALS AND METHODS

Chemicals. Bovine ligamentum nuchae elastin was purchased form Worthington (USA); bovine gamma globulin (BGG, labile enzyme free fraction II) from Miles Labs (USA); keyhole limpet hemocyanin (KLH) from Calbiochem-Behring Corp. (USA); N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) from Pharmacia Fine Chemicals (Sweden); alkaline phosphatase labeled goat antirabbit IgG from Miles-Yeda (USA). All other chemicals were of the highest analytical grade available.

The type of microtitration plates used was flatbottom Nunc-Immuno plate I. Corona MTP-12 two waves microplatephotometer was used for the measurement of uv-absorption.

Purification of desmosine and isodesmosine. The fraction containing desmosine and isodesmosine was obtained from acidhydrolysates of elastin using Franzblau's method (14). Elastin was hydrolyzed in 6. 0 M hydrochloric acid under reflux for 48 h. The hydrolysate was evaporated to dryness, redissolved in water and placed on a Dowex 50-X8 column (4. 5×17 cm). Elution was carried out with 1 liter of 1. 5 M hydrochloric acid, followed by 5 liters of 2. 5 M hydrochloric

[1] Abbreviations used: BGG, bovine gamma globulin; COLD, chronic obstructive lung disease; D/C, desmosine/creatinine; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PT, pyridine-2-thione; SIMS, secondary ion mass spectrometry; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate; TCA, trichloroacetic acid.

acid, and finally 6 liters of 4.0 M hydrochloric acid in which the eluted desmosine and isodesmosine were in solution.

Isolation of desmosine and isodesmosine from this mixture was carried out using Ledvina and Bartos' method (15) by which the mixture was repeatedly evaporated to dryness, dissolved in 0.38 M citrate buffer (pH 4.35) and applied to an AG50WX4 column (0.6×60 cm) equilibrated with the buffer. Elution was performed with the buffer at a flow rate of 0.1 ml/min. Eluant fractions were monitored by uv-absorption spectrophotometry, and the isodesmosine fraction and the desmosine fraction were obtained. Each fraction was desalted on an AG50W X8 column (1.6×30 cm) using 1 M ammonium hydroxide as the eluting solvent, and evaporated to dryness. After that each compound was dissolved in 0.1 M acetic acid, applied to a Bio-Gel P-2 column (2.5×91 cm), eluted with the solvent, and lyophilized to give desmosine acetate or isodesmosine acetate as a yellowish powder.

These compounds were identified by ^{1}H-NMR spectroscopy (500 MHz) and secondary ion mass spectrometry (SIMS), and their purities were checked by HPLC (Partisil 10SCX, 4.6×250 mm ; 10% acetonitrile-0.05 M ammonium acetate, pH 4.0). This highly purified desmosine was used for the next conjugation step and as a reference standard. Highly purified isodesmosine was used for testing cross-reaction.

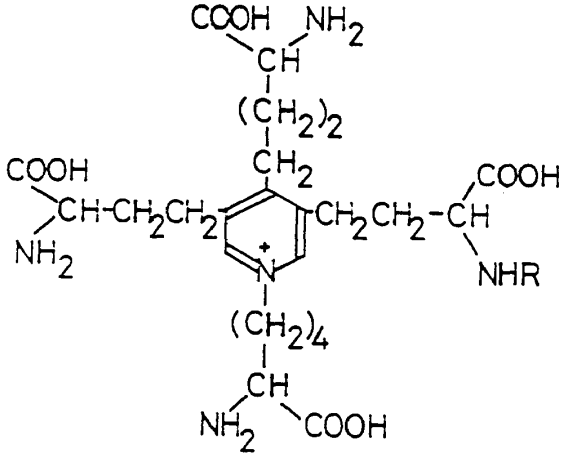
Preparation of desmosine-protein conjugates. Two conjugates were prepared. The conjugate to be used as immunogen was made with BGG and the coating conjugate with KLH. The immunogen was synthesized using a heterobifunctional coupling reagent. The coating conjugate was synthesized by the carbodiimide method.

(a) Preparation of SPDP-monoacylated desmosine: Desmosine acetate (38 mg, 65 μmol) was dissolved in 25 ml of 0.05 M phosphate buffer (pH 5.9) containing 0.1 M NaCl. SPDP (21 mg, 68 μmol) dissolved in 3 ml of ethanol was then added dropwise to the stirred desmosine solution. The reaction mixture was stirred for 3 h at room temperature and the resulting mixture was evaporated. The dry residue was then dissolved in 5 ml of 0.1 M acetate buffer (pH 4.5) and the solution was applied to a column (1.5×90 cm) of Sephadex G-25 superfine equlibrated with the acetate buffer at room temperature. The elution was performed with the buffer at a flow rate of 0.1 ml/min (Fig. 1). Eluant fractions (3 ml/tube) were monitored by uv-absorption and HPLC. These groups of fractions gave characteristic uv-avsorption spectra (N. 26-29), λ269 nm ; No. 33-35, λ277 nm ; No. 38-40, λ277 nm). The first group of fractions (No. 26-29) contained free desmosine. Two desmosine derivatives were found in the second group (No. 33-35) and in the third group (No. 37-40). When 0.1 ml of 50 mM aqueous dithiothreitol (DTT) was added to small aliquots of these fractions, fractions in the latter two groups showed a new uv-absorption at 343 nm by released pyridine-2-thione (PT). By comparing with the uv-spectra before this reaction, these derivatives were shown to be SPDP-monoacylated desmosine and di-SPDP-acylated desmosine. The fractions in the second group containing only SPDP-monoacylated desmosine were combined and desalted by passing through a Bio-Gel P-2 column (2.5×91 cm). Elution was performed with 0.1 M acetic acid and eluant fractions were monitored by uv-absorption and HPLC. The fractions containing the desmosine derivative were again combined and the acetic acid was removed by evaporation. The residue was redissolved

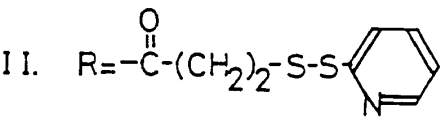
Table 1. Comparison of SPDP-monoacylated desmosine (II) and desmosine (I) in NMR chemical shifts.

I	II	Assignments
1.37 (m, 1H) 1.48 (m, 1H) 1.58 (m, 1H) 1.67 (m, 1H)	1.40 (m, 1H) 1.47 (m, 1H) 1.59 (m, 1H) 1.67 (m, 1H)	aliphatic CH_2
1.98 (s, 3H)	2.06 (s, 3H)	$CH_3CO_2^-$
1.89 (m, 2H) 2.00–2.10 (m, 4H) 2.17 (m, 4H)	1.89 (m, 2H) 1.96–2.11 (m, 4H) 2.17 (m, 4H)	aliphatic CH_2
	2.78 (m, 2H)	S_2-CH_2 or NCO-CH_2
2.92 (m, 4H) 3.04 (m, 2H)	2.90 (m, 4H) 3.02 (m, 2H)	benzyl type CH_2
	3.12 (t, 2H)	S_2-CH_2 or NCO-CH_2
3.73 (t, 1H) 3.80 (m, 1H)	3.73 (t, 1H) 3.79 (m, 1H)	$CH(NO_3^+)CO_2^-$
3.88 (t, 2H)	3.88 (m, 2H)	$CH(ND)CO_2^-$ and $CH(ND_3^+)CO_2^-$
4.51 (t, 2H)	4.48 (t, 2H)	CH_2 attached to N^+
	7.30 (m, 1H) 7.84 (m, 2H) 8.39 (m, 1H)	pyridyl protons
8.54 (s, 2H)	8.46 (s, 1H) 8.52 (s, 1H)	pyridinium protons

NMR spectra were recorded on a BRUKER AM-500 (500MHz) and are given in parts per million (ppm) downfield shift from the internal standard, sodium 2, 2-dimethyl-2-silapentane-5-sulfonate, in deuterium oxide. Assignment as shown above were allowed by comparison with values given in other works (11). Abbreviation: s, singlet; t, triplet; m, multiplet.



I. R=H

II. R= 

in distilled water and lyophilized to give SPDP-monoacylated desmosine acetate as a yellowish powder (15. 6 mg, 20 μmol).

The number of 2-pyridyl disulfide groups in the desmosine derivative was determined by measuring the molecular mass of the desmosine derivative and the amount of PT released by the reaction with DTT. The molecular mass ($M^+=723$) was obtained by SIMS. The desmosine derivative (2 mg, 2. 6 μmol) was then dissolved in 3 ml of 50 mM aqueous DTT and stirred at room temperature. After 2 h, when the reaction was almost complete, the uv-absorption at 343 nm was measured. The amount of PT released was calculated to be 2. 6 μmol from the uv-absorption and the molar absorptivity of PT (8. 08×1, 000 at 343 nm) (13). The structure of SPDP-monoacylated desmosine acetate was identified by ^{1}H-NMR spectroscopy (500 MHz) (Table 1).

(b) Preparation of desmosine-BGG conjugate : BGG (100 mg) was dissolved in 5 ml of 6 M quanidine hydrochloride containing 0. 5 M tris-buffer (pH 8. 1) and 0. 02 M EDTA, and the solution was kept at 50°C for 30 min under N_2 gas flow. After 103 mg of DTT had been added, this solution was stirred for 4 h again under N_2 gas flow. The volume of the solution was restored to 5 ml with distilled water and then 5 ml of 10% trichloroacetic acid (TCA) was added. After being centrifuged (3, 000 rpm, 10 min), thiolated BGG was obtained as a white precipitate and lyophilized. This protein (20 mg) was dissolved in 10 ml of 6 M urea containing 0. 1 M NaCl and 0. 1 M phosphate buffer (pH 7. 5), and 4. 67 mg of the SPDP-monoacylated desmosine acetate was added. The solution was stirred continuously for 45 min at room temperature after which 15 ml of 10% TCA was added. A white precipitate was obtained after centrifuging as before. After washing with 1 ml of distilled water, the precipitate was lyophilized to give the conjugate as a white powder (11 mg). This time, after addition of 10% TCA to the supernatant, precipitation did not occur. This conjugate was used for immunization.

The number of desmosine residues introduced per molecule of BGG was calculated from the uv-absorption at 343 nm of released PT. If the molecular weight of the thiolated BGG was taken to be 150, 000, the number of introduced desmosine residues would become 10 per molecule of BGG. The homogeneity of this desmosine-BGG conjugate was examined by passing through a Sephadex G-100 column (1. 5×90 cm). This conjugate was eluted as a single peak using 0. 1 M phosphate buffer (pH 7. 5) containing 0. 1 M NaCl.

(c) Preparation of desmosine-KLH conjugate ; KLH (60 mg) was added to 6 ml of 0. 015 M NaCl and undissolved material was removed by a centrifuge. A solution of desmosine (11. 3 mg) dissolved in 3. 0 ml of 0. 01 M phosphate buffer (pH 7. 0) was mixed with the KLH solution and a further 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride. After stirring for 20 h at room temperature, the solution was dialysed against water (12 liters) for 50 h. The supernatant obtained using a centrifuge was lyophilized to give the desmosine-KLH conjugate as a white powder (25. 5 mg).

Antisera to be the desmosine-BGG conjugate. Two rabbits (male New-Zealand White) were immunized against the desmosine-BGG conjugate. The conjugate (1. 5 mg) was dissolved in 0. 3 ml of 8 M urea, mixed with 0. 5 ml of 0. 9% aqueous NaCl and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected, in six 0. 15 ml portions, into the rear foot pads. Nine days later, desmosine-BGG conjugate (1. 5 mg) emulsified with

Freund's incomplete adjuvant was injected subcutaneously, in ten 0.1 ml portions, into the back. The animals were then boosted for 3 months at two-week intervals with intravenous injections of desmosine-BGG alone (0.1 mg each). Ten days after the last injection, they were bled. The serum was collected, and stored at 4°C in 0.1% sodium azide until ready for use.

Buffers for ELISA. (A) Coating conjugate dilution buffer: 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.1% sodium azide; (B) coating buffer: 0.05 M carbonate/bicarbonate, pH 0.6, containing 0.1% sodium azide; (C) rinse buffer: phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20 and 0.1% sodium azide; (D) sample dilution buffer: 0.1 M phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% sodium azide; (E) antiserum dilution buffer: 0.1 M phosphate buffer, pH 7.4, containing 0.05% KLH, 0.1 M NaCl and 0.1% sodium azide; (F) labeled IgG dilution buffer: 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% sodium azide; (G) substrate buffer: 10% diethanolamine buffer, pH 9.8, containing 0.2% sodium azide.

Enzyme-linked immunosorbent assay. Desmosine-KLH was dissolved in dilution buffer (A) at 100 μg/ml. After being centrifuged to remove traces of undissolved material, the solution was stored at 4°C until ready for use. This solution was diluted in coating buffer (B) at 2 μg/ml immediately prior to use and 300 μl was dispensed into each of the 60 inner wells of microtitration plates. The plates were then incubated at 4°C overnight. This solution was decanted from the wells, and each plate was washed with 4×400 μl rinse buffer (C) and blotted. Serial dilutions of desmosine and unknowns were made in sample dilution buffer (D). Duplicate or triplicate aliquots of 100 μl were placed into adjacent wells, a standard curve of desmosine and blanks of 100 μl sample dilution buffer being included on each plate. One hundred micro liters of antiserum diluted 1:15,000 in antiserum dilution buffer (E) were added to each well except for a "no antibody" blank and 100 μl dilution buffer alone added into the blank. After 18 h incubation in a humid box at 4°C, the wells were emptied and the plates were washed and blotted as before. A solution (200 μl) of alkaline phosphatase labeled goat anti-rabbit IgG diluted 1:500 in F buffer was added to each well. The plates were incubated at 37°C for 3 h, emptied, wahsed and blotted as before. In addition the plates were washed with 3×400 μl double-distilled water and blotted. A fresh solution of 4 mM p-nitrophenylphosphate was prepared in a prewarmed substrate buffer (G) and 200 μl dispensed to each well. After the solution had been incubated for 90 min at 37°C, the color reaction was terminated by the addition of 50 μl of 3 M NaOH and then the absorbance at 405 nm was taken. A typical standard curve for desmosine is shown in Fig. 5 together with those of isodesmosine and lysine.

Subjects. The subjects were 25 patients with COLD and 8 healthy controls. The patient group contained 15 males and 10 females, ranging in age from 22 to 76 years old. The diagnosis of COLD was based on the definition of COLD described by American Thoracic Society (16). Fourteen among 25 cases were smokers before realizing the disease. On the other hand, all of the healthy controls were males, ranging in age from 23 to 39 years old and six were smokers.

Sample preparation. Urine was collected over a period of 24 h and, after mixing, 2 ml samples were subjected to hydrolysis in 6 M hydrochloric acid (final concentration) at 110°C for 72 h. The hydrolyzed samples were evaporated to dryness and redissolved in 15 ml of double-

distilled water. These solutions were then adjusted to pH 7. 0 with 1 M NaOH and lyophilized. After that, desmosines in the hydrolyzed samples were partially purified using Skinner's method (17). A slurry was prepared by mixing CF11 cellulose (10 g) with the mobile phase, n-butanol-acetic acid-water (4:1:1) 200 ml. Columns (0. 7×20 cm) were then packed to about 5 cm height by pipetting 5 ml of the slurry. A further 5 ml of mobile phase was added to each column. After each lyophilized sample was redissolved in 2 ml of water, 0. 5 ml of the solution was mixed, in order of addition, with acetic acid (0. 5 ml), cellulose slurry (0. 5 ml) and n-butanol (2 ml) in a vial. The slurry was transferred onto a prepared column and the vial washed with 2 ml mobile phase which was also transferred to the column. The column was then eluted with a further 15 ml mobile phase. The desmosines were eluted from the column with water (10 ml) inot a vial. After these aqueous fractions containing the desmosines were evaporated to dryness and redissolved in 1 or 2 ml of D buffer, aliquots of 0. 1 ml wwere used in the ELISA for determination of desmosine.

Measurement of creatinine content. Creatinine content was measured on a second aliquot from the same speciemn by automatic chemical analysis (18).

RESULTS

Preparation of desmosine immunogen.

Since desmosine is not immunogenic in itself because of its low molecular weight, it has to be coupled to larger molecules in order to make it suitable for immunization of animals. According to other reports (4-6), desmosine and isodesmosine were coupled to carrier proteins using the carbodiimide method. This coupling procedure is very simple and widely used. However, since desmosine contains four alpha-amino groups and four alpha-carboxyl groups, the coupling may be achieved by the formation of peptide bonds between carboxyl groups (and/or amino groups) of desmosine and amino groups (and/or carboxyl groups) of a protein, and the resulting conjugates contain a lot of products such as polymers of the reacting components and polyconjugates. In addition, the desired conjugates are difficult to purify for use. This may be why high-titer antibodies have not been obtained using this method. In order to obtain a high-titer antibody with good specificity, it is desireable to perform the coupling exclusively at only one of the eight functional groups in desmosine.

Selective coupling of SPDP onto an amino group of desmosine was performed under a mild aqueous condition (pH 5. 9) with a limited amount of SPDP. The resultant mixture was chromatographed on a column of Sephadex G-25 with 0. 1 M acetate buffer (pH 4. 5). The elution profile is shown in Fig. 1. The first group of fractions (No. 26-29) contained free desmosine, SPDP-monoacylated desmosine was found in the second group (No. 33-35) and di-SPDP-acylated desmosine was in the third group (No. 37-40). These were judged to be present from the uv-spectra and the PT contained in the tubes.

The second group of fractions containing SPDP-monoacylated desmosine were combined and desalted by passing through a Bio-Gel P-2 column. The desired compound after elution with 0. 1 M acetic acid was lyophilized to give a yellowish powder in a yield of 31%. The preparation was examined by HPLC which gave a single peak indicating its pruity. The molecular mass of the desmosine derivative was measured together with the amount of PT released in the

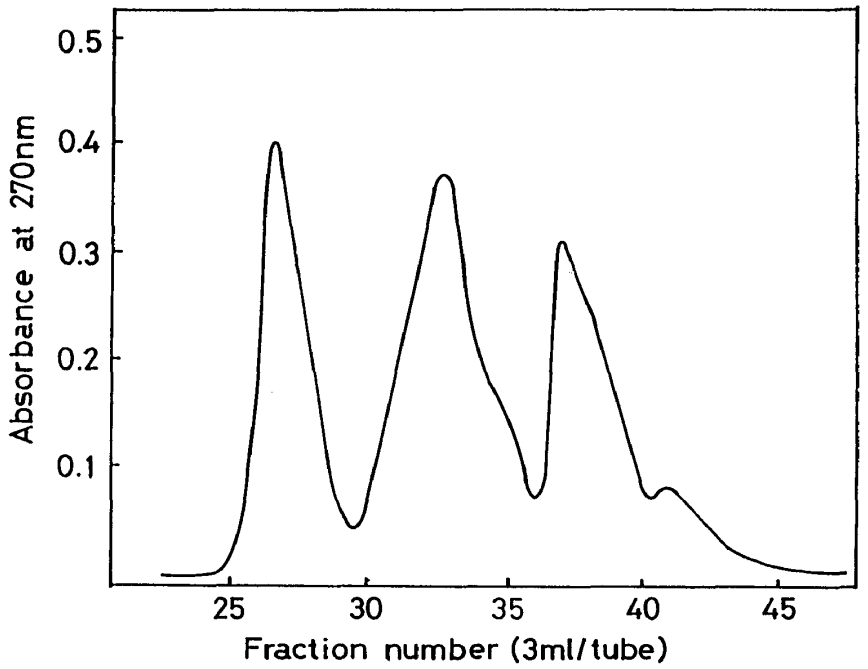


Fig. 1. Chromatogram of SPDP-acylated desmosine on Sephadex G-25 column. The uv-absorption spectrum was determined for every fraction.

reaction with DTT. The amount of PT released is proportional to the number of 2-pyridyl disulfide groups in the derivative. Thus, from these two results, the number of 2-pyridyl disulfide groups in the derivative could be checked. SIMS gave the molecular mass (M^+=723) equivalent to the molecular weight of SPDP-monoacylated desmosine. Furthermore, the amount of PT released was equivalent to that of the desmosine derivative used in the reaction with DTT. Thus the desmosine derivative was confirmed as SPDP-monoacylated desmosine. The structure was identified by ^{1}H-NMR spectroscopy (500 MHz) and NMR signals of the protons of SPDP-monoacylated desmosine acetate are summarized in Table 1 and compared with those of desmosine acetate.

SPDP-monoacylated desmosine acetate was coupled to BGG thiolated by dithiothreitol as described in MATERIALS AND METHODS via thiol-disulfide exchange in order to form disulfide-linkage. The desired product, desmosine-BGG conjugate, was obtained as a white powder in good yield. The desmosine-BGG was fairly homogeneous because this was eluted as a single peak when passed through a Sephadex G-100 column. The number of desmosine residues introduced per molecule of BGG was calculated to be 10 from the amount of PT released as a result of the thiol-disulfide exchange reaction. Injection of the conjugate into rabbits gave a satisfactory antiserum to desmosine.

Assay conditions.

The sensitivity of an ELISA is dependent on both the dilution of antiserum and the concentration of hapten-protein conjugate used to coat the wells of the microtitration plate. While a precise optimization of both these parameters is not necessary, a suitable combination of the two parameters for routine analysis needs to be established.

Optimal antiserum dilution for the desmosine ELISA was established by testing serial dilutions of the antiserum on plates coated with desmosine-KLH conjugate dissolved in the

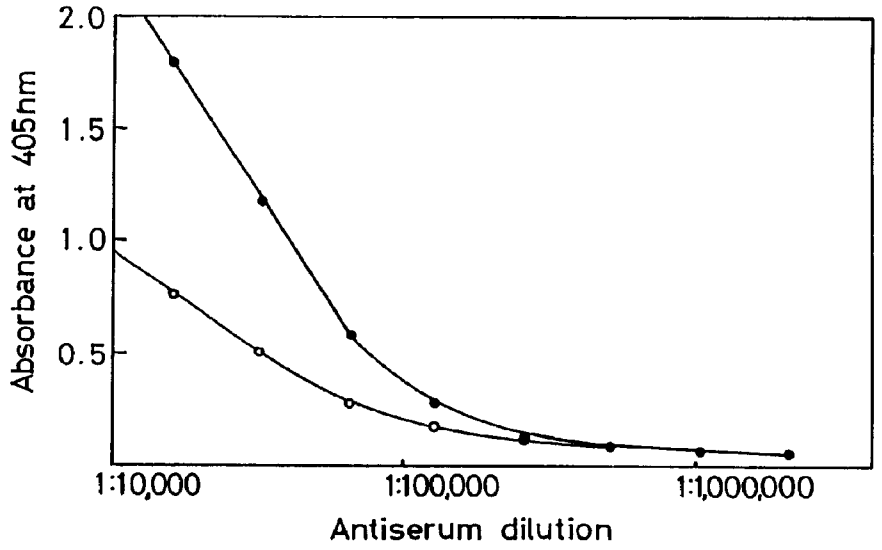


Fig. 2. Antiserum dilution curves. Either 1 μg/ml (○) or 5 μg/ml (●) of desmosine-KLH was coated in the wells. Serial dilutions of the rabbit antiserum were added to the wells. The amount of bound antibody was determined by incubating with alkaline phosphatase labled IgG diluted 1:1000 and 2.7 mM p-nitrophenylphosphate. The enzyme activity was measured by the absorbance at 405 nm.

coating buffer at concentrations of 1 and 5 μg/ml (Fig. 2). The amount of bound antibody was determined by incubating with alkaline phosphatase labeled IgG diluted 1:1,000 and 2.7 mM p-nitrophenylphosphate as the substrate. As the antiserum dilution was increased to more than 1:15,000, the absorption at 405 nm by the enzyme reaction was observed within the absorbance range of the microplatephotometer routinely used.

Then, a number of plates were coated with serially diluted desmosine-KLH conjugate from

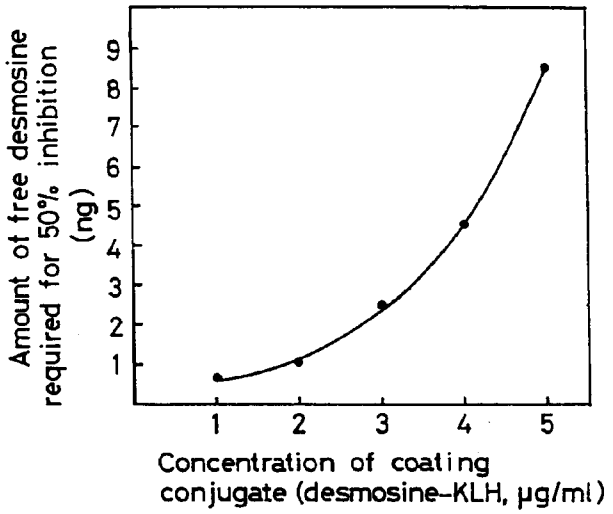


Fig. 3. The effect of concentration of coating conjugate on the amount of free desmosine which is required for 50% inhibition of the reaction of the bound desmosine and the antibody. Using the plates coated with serially diluted desmosine-KLH conjugate from 5 to 1 μg/ml, standard curves of desmosine were prepared from 0.02 ng to 100 ng with the usual blanks. The standard curves were developed with antiserum diluted at 1:15,000. The enzyme activities were measured by the absorbance at 405 nm after incubating with alkaline phosphatase labeled IgG diluted 1:1000 and 2.7 mM p-nitrophenylphosphate.

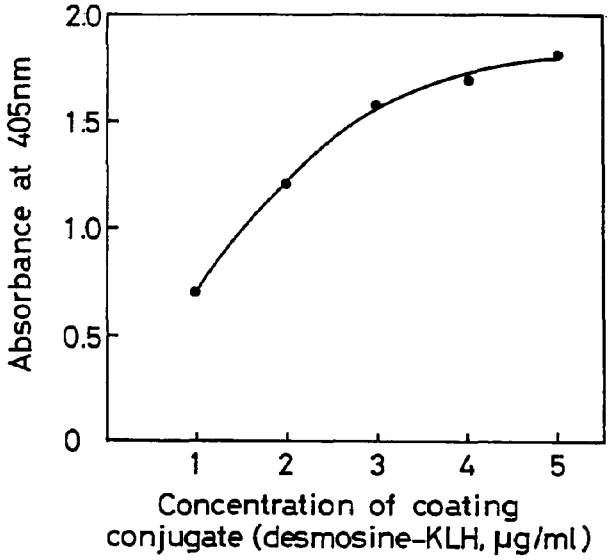


Fig. 4. The concentration of coating conjugate and the absorbance of blank solution. Using the plates coated with serially diluted desmosine-KLH conjugate from 5 to 1 μg/ml, the enzyme activities in the blank solution were determined as in Fig. 3. Antiserum diluted at 1:15,000 was used.

5 to 1 μg/ml and a standard curve of desmosine was prepared on each plate from 0.02 ng to 100 ng with the usual blanks. The standard curve was developed on each plate with antiserum diluted at 1:15,000. Fig. 3 shows the effect of the concentration of coating conjugate on the amount of free desmosine which is required for 50% inhibition of the reaction of the bound desmosine and the antibody. With less concentrated coating conjugate, the free desmosine required for 50% inhibition was decreased. It is likely that the amount of desmosine required for 50% inhibition is constant when the coating conjugate concentration is reduced to 1 μg/ml or less. However, the absorbance of blank solution decreased with the decrease of the coating conjugate concentration (Fig. 4). The absorbance of blank solution obtained from plates coated at 1 μg/ml was too low for routine use. For this reason, 2 μg/ml of desmosine-KLH conjugate solution was selected as a suitable concentration of coating conjugate.

The optimum incubation time, dilution of alkaline phosphatase labeled IgG and concentration of substrate were determined to obtain good response over the low concentration range of desmosine within the absorbance range up to 2. The absorbance increased linearly with the incubation time up to 2 h. However prolonged incubation was inclined to lead to a high background absorbance relative to the maximal absorbance and to a loss of sensitivity. Therefore a incubation time of 90 min was selected for routine use. Three plates were coated at 2 μg/ml. On each plate three standard curves were developed with alkaline phosphatase labeled IgG diluted at 1:1,000, 1:667 and 1:500, respectively. In addition, standard curves on the three plates were developed with substrates of 2.7, 4.0 and 5.4 mM, respectively. The increased concentratio of alkaline phosphatase labeled IgG was quite effective in obtaining a better response. But the effect of the concentration of the substrate on the color development was negligible over this substrate concentration range. Therefore, a dilution of 1:500 for alkaline phosphatase antibody and 4 mM for substrate concentration were selected for routine use.

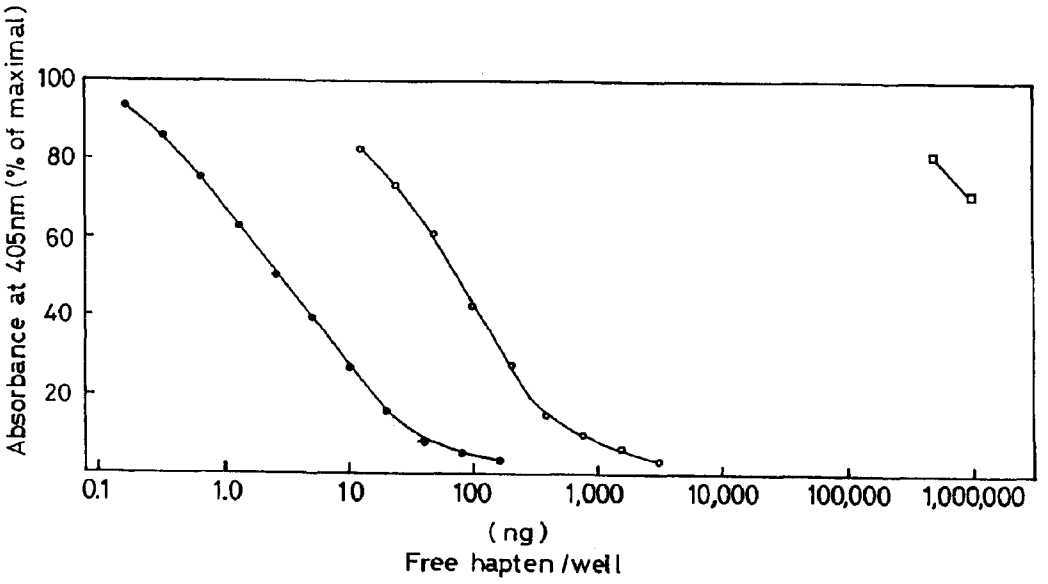


Fig. 5. The standard curve for desmosine ELISA. The absorbance at 405 nm is expressed as the percentage of the maximum. The horizontal line shows the amount of test substance incubated with the antiserum in the wells (plotted logarithmically). Linearity of inhibition between 0.4 ng and 25 ng was obtained when desmosine (●) was used as test substance. Isodesmosine (○) gave 4% cross reaction and lysine (□) gave <0.0003% cross-reaction.

Finally, the relation between the concentration of coating conjugate and the dilution of antiserum was examined. One half of a plate was coated at 2 μg/ml and the other at 1 μg/ml. The standard curve of desmosine on the former was developed with antiserum diluted at 1:15,000 and the latter with antiserum diluted at 1:7,500. The results showed that, with 2 μg/ml of coating conjugate and 15,000 dilution of antiserum, a good response was obtained over the concentration range 0.4 ng to 25 ng. Therefore, the combination of 2 μg/ml of conjugate and 15,000 dilution of antiserum was selected for routine use. A typical standard curve is shown in Fig. 5.

Specificity.

The cross-reactivity of the antiserum with various amino acids was determined. Appreciable cross-reactivity was found only with isodesmosine (4%) (Fig. 5). However, since this compound is also specific to elastin, this slight cross-reaction did not decrease the usefulness of the method in measuring elastin breakdown products. By contrast, an amino acid that is not unique to elastin did not show any cross-reactivity with the antiserum.

Clinical application.

We measured urine desmosine/creatinine (D/C) ratios to evaluate the usefulness of this assay in clinical applications. The results are expressed as micrograms of desmosine per gram of creatinine in urine samples collected over 24 h. Fig. 6 shows the results obtained from a group of normal controls and 25 patients with COLD. The average values and standard deviations for D/C in normal controls and patients with lung disease were 48 ± 18 and 216 ± 149, respectively. The difference between the average values is statistically significant ($p < 0.001$). The average 24 h desmosine excretion value in these patients was 168 ± 118 μg, whereas the average value in the controls was 63 ± 31 μg. The difference between the average values is statistically signi-

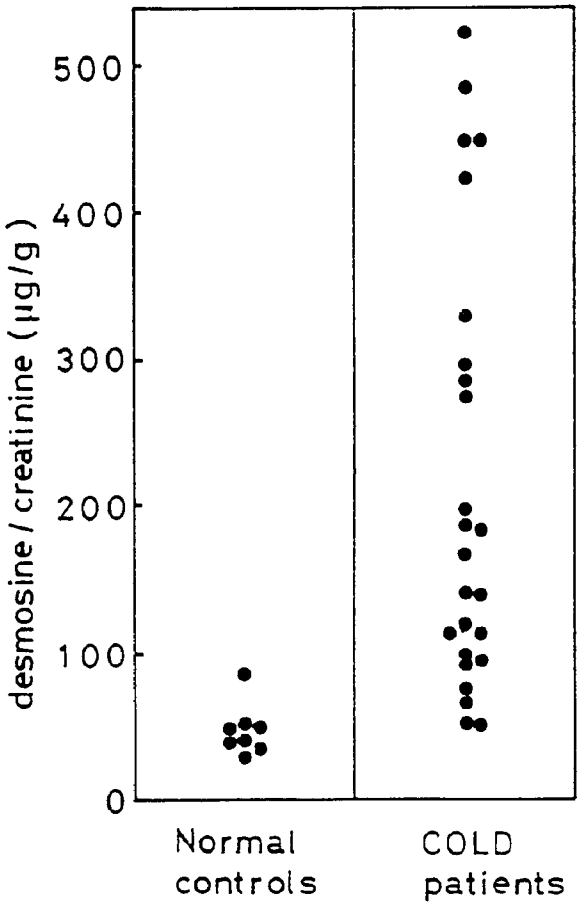


Fig. 6. Desmosine/creatinine ratios in 24 h urine of normal controls and patients with COLD. Samples were assyed by the ELISA and autoanalyzer described in the text.

ficant ($p<0.01$). This ELISA can detect as little as 0.4 ng of desmosine and, with slight modifications, could probably be extended to even lower values. In triplicate determinations the assay is reproducible to within 3%. These results show that this sensitive and specific ELISA for desmosine could be used to monitor lung elastolysis in humans.

DISCUSSION

Proteolysis of the elastic fibers of the lung is considered to be the primary cause of pulmonary emphysema (19). Degradation of elastin in the human lungs may be monitored by examining the level of urinary desmosine, an elastin-specific crosslinking amino acid. Harel et al. (5) measured the amount of desmosine excreted in the human urine by radioimmunoassay, and found that the daily excretion is increased in patients with chronic obstructive lung disease (COLD) compared to healthy individuals. Urinary desmosine has also been reported to be increased in smokers and pneumonia patients. Davies et al. (20), however, did no observe significant differences in urinary desmosine excretion between smokers and non-smokers, and ascribed their results to insufficiency of the sensitivity of the measuring technique for evaluation of elastolysis.

For radioimmunoassay, Harel et al. obtained anti-desmosine antibodies by sensitizing rabbits using a conjugate of desmosine and bovine serum albumin (BSA) prepared in the

presence of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (ECDI). By this method, however, the antigenicity of the conjugate is so weak that King et al. (21) reported that the antisera were obtained from only 2 of the 4 inoculated rabbits. The rate of successful sensitization was also less than 50% in our study. We, therefore, first reacted desmosine with SPDP as a coupling agent, and SPDP-monoacylated desmosine was selectively conjugated with BGG uing a Sephadex G-25 column. By this procedure, the binding linkage between BGG and desmosine became longer, and since desmosine was sufficiently exposed on the molecular surface, a high-activity homogeneous antigen could be obtained. As compared to 40 desmosine molecules bound to 1 mole of BSA when desmosine is conjugated with BSA using ECDI, 10 desmosine molecules are bound to 1 mole of BGG by our method.

The measurement was made by the inhibition technique of enzyme-linked immunosorbent assay (ELISA) using no radioisotopes so that the procedure can be carried out routinely in clinical laboratories. To eliminate non-specific inhibition due to the reaction of contaminants such as amino acids contained in the samples, the samples were applied prior to the measurement to a CF11 cellulose mini-column for partial purification of desmosine. This manipulation sufficiently decreased the background level.

In this study, the daily urinary desmosine excretion was higher in most of the COLD patients than in healthy individuals. Our results, which were consistent with those of Harel et al. (5), are considered to indicate increased elastolysis in the lung. However, a considerable variation was observed in desmosine excretion among COLD patients, the cause of which is as yet unknown.

We are planning to investigate the possible relationships between urinary desmosine excretion and clinical features in patients with pulmonary disorders other then COLD as well as cardiovascular diseases. Our ELISA method is considered to be suited for such studies.

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