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Antigenicity of Elastin: Characterization of Major Antigenic Determinants on Purified Insoluble Elastin†

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ABSTRACT: This investigation reports a systematic comparison of antibody responses to well-defined fragments of mature, insoluble elastin in order to identify antigenic regions of the molecule. Antiserum to insoluble elastin was used to screen by radioimmunoassay the serologic activity of elastin peptides purified from a thermolysin digestion of insoluble elastin. Fractions with positive antigenicity were characterized by amino acid analysis and protein sequence analysis. Our results indicate that antigenic determinants on elastin can be classified functionally into two categories: major determinants related to the cross-linking domain which show cross-reactivity between species and limited, species-specific determinants in non-cross-linked regions of the molecule.

Elastin is an important connective tissue macromolecule that imparts elasticity to elastic tissues. It is synthesized and secreted as a soluble, single-chain protein (tropoelastin) that undergoes numerous postribosomal modifications prior to organization of the elastic fiber in the extracellular space. Once secreted, tropoelastin molecules are joined covalently through chemical modification and cross-linking of specific lysyl residues to form mature, insoluble elastin.

Elastin possesses multiple, repeating structural domains which modulate its biological properties. One such domain is the cross-linking region, a polyalanine-enriched area of 30–40 amino acid residues that assumes a tight helical conformation (Gray et al., 1973; Foster et al., 1976). Since the proper helix configuration is an important determinant in correct cross-link formation, the amino acid sequence of this region is highly conserved, and cross-linking domains distributed throughout the molecule show high sequence homology. Interspersed among the cross-linking domains are regions of hydrophobic amino acids which form loose coils of β turns (Gray et al., 1973; Urry, 1974). These non-cross-linked or so-called "straight-chain" domains are thought to provide a molecular basis for the protein's elastic behavior. In contrast to the multiple cross-linking regions which exhibit compositional and structural homology, considerable sequence and conformational heterogeneity is found within the straight-chain regions.

Over the past several years, immunological techniques have become increasingly important in studies of elastin structure and biosynthesis. Antisera have been developed which are specific for elastin, but little is known concerning the antigenic determinants on the elastin molecule. This is due in part to the insoluble nature of elastin and its weak immunogenicity and because antigen–antibody complexes of elastin fragments often remain soluble (Jackson et al., 1966; Kucich et al., 1981; Mecham & Lange, 1981).

Recent studies have characterized at least two reacting antibody subpopulations in antisera to elastin: (a) antibodies which show species specificity and (b) antibodies that show

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broad species cross-reactivity (Houle & LaBella, 1977; Mecham & Lange, 1980; Darnule et al., 1980). From the structure of elastin, one might predict that shared or cross-reacting determinants are contained in structurally conserved cross-linking domains whereas species-specific determinants are most likely found in the more variable non-cross-linking regions of the molecule.

To investigate the antigenic structure of elastin, we examined the serologic activity of elastin peptides purified from a thermolysin digest of mature, insoluble bovine ligamentum nuchae elastin. By screening this defined peptide library against antisera to bovine ligament insoluble elastin in a competitive binding radioimmunoassay, we have identified the constitution and relative immunological activity of the major species-specific and cross-reacting determinants on the molecule.

Experimental Procedures

Preparation of Insoluble Elastin and α-Elastin. Insoluble bovine ligamentum nuchae and rabbit lung elastins were purified as previously described (Mecham & Lange, 1980) and solubilized by five successive 1-h extractions in hot 0.25 M oxalic acid (Partridge et al., 1955). Supernatants from each oxalic extraction were dialyzed extensively against water by using a Spectraphor III dialysis membrane (3500 molecular weight cutoff) and lyophilized. Supernatants from the fourth and fifth extractions of bovine elastin were resuspended in 0.01 M sodium acetate–0.1 M NaCl, adjusted to pH 5.5 with glacial acetic acid, combined, and heated at 37 °C. α-Elastin was isolated as a viscous coacervate which settled at the bottom of the tube after spinning at 5000*g* at 37 °C for 20 min. The fourth and fifth extracts of rabbit lung elastin were pooled and used directly.

Peptide Hydrolysis and Amino Acid Analyses. Samples were hydrolyzed in constant-boiling HCl at 105 °C for 48 h and dried by using a Speed Vac concentrator (Savant Instruments, New York). Amino acid compositions were determined by using a Beckman 119C amino acid analyzer with a modified program for resolving hydroxyproline and elastin cross-linking amino acids. Amino acid separation was achieved by using a 6 × 310 mm column packed with Beckman W-2 ion-exchange resin eluted at 51 °C with standard sodium citrate buffers: (1) pH 3.08, 0.20 M Na^+, 77 min; (2) pH 4.25, 0.20 M Na^+, 20 min; (3) pH 5.28, 0.35 M Na^+, 80 min; (4) pH 6.40, 1.00 M Na^+, 48 min. Buffer flow rate was 70 mL/h. Results were not corrected for losses due to hydrolysis.

Preparation of Antisera. Finely milled (>400 mesh) bovine ligament elastin was suspended at 1 mg/mL in 0.1 M sodium phosphate–0.15 M NaCl, pH 7.4, and emulsified in an equal volume of complete Freund's adjuvant. Four New Zealand white rabbits were immunized by multiple-site subcutaneous injections on the back, with each animal receiving a total of 2 mL in 10 divided doses. All animals were boosted monthly with 1.0 mg of insoluble elastin and bled 8–12 days after each immunization. Characterization of antiserum specificity for elastin has been previously described (Mecham & Lange, 1981).

Partial Reduction of Elastin Cross-Links and Fractionation of Elastin Peptides. Finely milled ligamentum nuchae elastin, partially reduced with sodium boro[^{3}H]hydride to stabilize and radiolabel reducible cross-linking amino acids, was digested with thermolysin as described previously (Mecham & Foster, 1978). The soluble digest was fractionated at 55 °C on a 0.9 × 60 cm column of Aminex 50W-X4 resin (20–30-μm particle size; Bio-Rad Laboratories, Richmond, CA) by using the pyridine–acetate gradient of Schroeder (1967). The column elution profile and amino acid analyses of isolated fractions have been given in a previous study (Mecham & Foster, 1978).[1]

Table I: Serological Activities of the First through Fifth Oxalic Acid Supernatants Obtained during α-Elastin Preparation[a]

ng tested	% inhibition[b] of labeled antigen binding for oxalic acid extracts				
	1st	2nd	3rd	4th	5th
20	15	15	27	70	67
50	21	41	47	72	71

[a] Each fraction was assayed by competitive protein binding radioimmunoassay with radiolabeled bovine ligamentum nuchae α-elastin as the specific probe. Shown is the percentage of inhibition of specific ligand binding by anti-insoluble elastin serum (1:3000 dilution). [b] Maximal inhibition by unlabeled α-elastin was approximately 70%.

Competitive Protein Binding Radioimmunoassay. Purified peptides or peptide hydrolysates were assayed for bovine-specific or interspecies cross-reacting determinants by competitive protein binding radioimmunoassay as described previously (Mecham & Lange, 1980). Bovine ligamentum nuchae α-elastin or solubilized rabbit lung elastin radiolabeled by using ^{125}I-labeled Bolton–Hunter reagent was used as antigenic probe. Duplicate aliquots from test samples at three dilutions were assayed in a final volume of 0.22 mL. A standard curve was determined by using stock solutions of unlabeled solubilized elastins. Isolated peptides in a volume of 120 μL were mixed with 100 μL of a 1:3000 dilution of rabbit antibovine ligament insoluble elastin in assay buffer [0.01 M sodium phosphate buffer, pH 7.4, 0.15 M NaCl, 0.5% (w/v) bovine serum albumin, and 0.03% (v/v) NP-40]. After incubation for 30 min at 37 °C, 0.1 mL of ^{125}I-labeled antigen in assay buffer (10 000–12 000 cpm) was added and the tubes were incubated at 4 °C overnight. Antigen–antibody complexes were precipitated with 0.04 mL of a 10% suspension of inactivated, formalin fixed *Staphylococcus aureus* cells (IgGSORB; The Enzyme Center, Boston, MA) by incubating for 60 min at 37 °C followed by centrifugation at 1500*g* for 20 min. Pellets were washed extensively by recentrifugation and counted for bound radioactivity by using a Beckman 7000 γ counter. Nonspecific binding was determined by using preimmune rabbit serum. Protein concentrations of digest fractions and α-elastin standards were determined by amino acid analysis.

Results

Composition and Immunological Reactivities of Oxalic Acid Extracts. For verification that no immunoreactive determinants were lost as small peptides during preparation of α-elastin, antiserum raised to insoluble elastin was tested for immunological activity against each of the five oxalic acid extractions obtained from preparation of α-elastin. Table I lists immunological reactivities of each extract as assessed by inhibition of antiserum binding of radiolabeled ligament α-elastin in a competitive protein binding radioimmunoassay.

[1] Peptide fraction identification codes enable cross-reference to an earlier paper (Mecham & Foster, 1978) describing amino acid compositions of many peptides described in this study. Codes are in three parts, letters denoting the protease used for digestion of elastin and chromatography columns used for peptide separations and numbers denoting the fraction number. For example, TA-12 signifies thermolysin digest, Aminex column, fraction 12. Codes for subsequent purification steps are added sequentially; TA-12, G25-III is fraction III when TA-12 was fractionated on Sephadex G-25.

Table II: Amino Acid Analyses of Insoluble Elastin, Purified α-Elastin, and the First through Fifth Oxalic Acid Supernatants Obtained during α-Elastin Preparation

	residues/1000 residues						
		oxalic acid supernatants					
	insoluble elastin	1st	2nd	3rd	4th	5th	α-elastin
Hyl	0	0	0	0	0	0	0
Lys	4.0	7.8	7.5	5.8	5.4	5.6	5.0
Arg	3.9	7.8	6.6	5.9	5.4	5.6	5.2
Hyp	9.0	17.0	14.9	13.8	10.7	8.6	5.7
Asp	5.6	18.1	13.6	9.1	6.9	5.1	3.3
Thr	6.0	11.5	10.1	8.9	8.3	8.9	7.4
Ser	5.2	12.7	11.7	9.8	9.4	10.3	7.3
Glu	13.7	23.6	20.9	18.2	17.8	18.5	18.0
Pro	117.2	112.5	111.1	107.3	110.0	112.5	110.5
Gly	327.7	324.6	311.0	309.6	302.0	301.3	316.6
Ala	241.4	166.8	204.7	229.4	252.4	260.1	261.3
Val	138.0	175.9	160.8	152.7	135.6	124.1	114.0
Ile	25.2	29.6	26.8	24.2	21.5	20.6	20.8
Leu	64.5	58.0	59.2	57.9	59.0	59.8	62.6
Tyr	4.3	3.8	2.6	2.8	3.6	3.3	3.5
Phe	31.7	29.2	36.2	41.0	47.2	50.5	54.0
Ides[a]	1.25	0.35	0.92	1.31	1.88	1.98	2.02
Des[a]	2.20	0.51	1.46	2.04	2.74	3.03	3.01

[a] Values are expressed as leucine equivalents divided by four.

The first, second, and third extracts did not inhibit binding significantly whereas the fourth, fifth, and unlabeled α-elastin competed equally well with the probe for antiserum. The antigenicity of later oxalic acid extracts was corroborated by direct binding studies: radiolabeled preparations of the first and third extracts bound poorly to antiserum while binding of labeled fifth extract was equal to binding of labeled α-elastin. These observations establish that α-elastin is a suitable soluble probe for characterizing antigenic determinants on insoluble elastin.

Localization of Antigenic Determinants on Elastin Peptides. Amino acid analyses of oxalic acid extracts (Table II) showed an enrichment in alanine and cross-linking amino acids in the highly immunogenic fractions, suggesting that cross-linking regions of the molecule contained a significant portion of the antigenic reactivity.

To localize more completely the antigenic determinants, we compared the serological activities of peptide fragments containing individual structural domains on elastin. Fragmentation of insoluble, reduced elastin was achieved by digestion with thermolysin, a metalloendopeptidase with activity directed toward the N-terminal peptide bonds of amino acids with hydrophobic side chains, such as leucine, isoleucine, valine, phenylalanine, and alanine. Incorporation of tritium into reducible cross-links by borotritide reduction provided a convenient marker for identifying cross-link-containing peptides during subsequent purification.

Approximately 80% of elastin peptides solubilized by thermolysin migrated at an apparent molecular weight of 7000 or less on sodium dodecyl sulfate–polyacrylamide gel electrophoresis or gel filtration chromatography (data not shown). Fractionation by ion-exchange chromatography (Aminex 50W-X4) conveniently resolved the digest into non-cross-linked (fractions 1–10) and cross-link-containing (fractions 11–18) peptides. Many peptides, mostly those containing cross-links, have been purified to homogeneity and their amino acid sequence has been determined (Mecham, 1977; Mecham & Foster, 1978). Column elution profiles and amino acid analyses of isolated column fractions have been published (Mecham & Foster, 1978).

Table III is a summary of serological activities of isolated digest fractions. Values are expressed as specific antigenic reactivity, the ratio of total immunoreactive protein per fraction (soluble elastin equivalents extrapolated from a standard α-elastin inhibition curve) to total protein per fraction measured by amino acid analysis. For each determination, the following criteria were adopted before accepting soluble elastin equivalents determined by radioimmunoassay: (1) the error between duplicate analyses of each sample was less than 10% and (2) the coefficient of determination for least-squares linear regression of values determined from three sample dilutions was greater than 0.90. Interspecies cross-reactivity was assessed for each fraction by substituting radiolabeled solubilized rabbit elastin for bovine α-elastin in the inhibition assay.

Table III: Serological Activity of Peptides Purified by Ion-Exchange Chromatography from a Thermolysin Digest of Ligamentum Nuchae Elastin[a]

		specific antigenic activity[b]	
	fraction no.	bovine probe	rabbit probe
no cross-links	TA3	0.20	0.00
	TA4, G25-I	1.05	0.00
	TA5	0.07	0.02
	TA6	0.02	0.02
	TA7, G25-II	0.85	0.13
	TA8	0.04	0.02
	TA9	0.04	0.01
allysine aldol cross-link	TA10	0.20	0.12
	TA11	1.29	0.61
	TA12	1.49	0.56
	TA13	1.28	0.34
	TA14	1.43	0.28
desmosine and lysinonorleucine cross-links	TA15	0.53	0.10
	TA16	0.35	0.09
	TA17	0.28	0.09
	TA17, G50-II	0.40	0.44
	TA18	0.42	0.10
	TA18, G50-I	0.73	0.24

[a] Fractions were assayed by competitive protein binding radioimmunoassay with radiolabeled bovine ligament α-elastin or radiolabeled rabbit lung solubilized elastin as antigenic probes. Antiserum was raised against purified bovine ligamentum nuchae insoluble elastin. [b] Expressed as the ratio of immunological α-elastin equivalents to total protein in each fraction.

A common feature of all antigenic fractions except TA-4, G25-I was a substantial enrichment in alanine and, except for fractions TA-4, G25-I and TA-7, G25-II, the presence of lysine-derived cross-links. Fraction TA-7, G25-II and fractions TA-11 through TA-17 possessed a significant capacity to inhibit binding of both radiolabeled bovine and solubilized rabbit elastin probes whereas fraction TA-4, G25-I inhibited binding of bovine but not nonbovine antigen. This result suggests the presence in TA-4 of a bovine specific determinant.

Amino acid analysis of TA-4 showed an enrichment in tyrosine, phenylalanine, and acidic amino acids, no cross-links, and a decrease in valine and leucine relative to whole elastin, suggesting that TA-4 originates from a non-cross-linked domain on the parent molecule. Also eluting from the ion exchange column as a non-cross-linked peptide was TA-7, G25-II, an alanine-rich peptide containing significant levels of radioactivity. Subsequent analysis, however, showed that bound radioactivity was associated with allysine, the reactive cross-link precursor resulting from the initial oxidation by lysyl oxidase of lysine ϵ-amino groups. The presence of allysine, together with an enrichment in alanine residues, suggests that fraction TA-7, G25-II contains a cross-linking region of elastin that has not formed covalent cross-links with another peptide chain.

Peptides having the greatest serological activity were found in fractions TA-11 through TA-14. TA-12, G25-II and TA-13, G50-II have been sequenced in our laboratory (unpublished results) and are both small peptides containing four to five alanine residues and the cross-linking amino acid allysine aldol. TA-12, G25-II was more effective than TA-13, G50-I at inhibiting binding of antigenic probe in the radioimmunoassay. Peptides containing desmosine, isodesmosine, an lysinonorleucine (fractions TA-14 through TA-18) reacted less well with antiserum than did peptides containing allysine aldol cross-linkages. The primary structure of desmosine-containing peptides has been previously described (Foster et al., 1974; Mecham & Foster, 1978).

Nature of the Cross-Linking Determinant. Synthetic peptides with amino acid sequences similar to the cross-linking domain of elastin were used as models to evaluate whether peptide chains without cross-links were recognized by elastin antiserum. Commercially available α-helical poly-L-alanine (10 000–25 000 molecular weight range) and a repeating polymer of $(Ala\text{-}Ala\text{-}Lys)_n$ were tested in the radioimmunoassay. Neither polymer proved to be antigenic. To test whether the cross-linking amino acid itself was a haptagenic antigen, we hydrolyzed aliquots from antigenic, cross-link-containing fractions by acid or base to component amino acids and reassayed them for antigenicity. In addition, purified desmosine or isodesmosine was tested in the radioimmunoassay. Neither the purified cross-linking amino acid at high concentrations nor hydrolysates of previously antigenic peptides competed with intact α-elastin for antibody.

Discussion

The purpose of this investigation was to identify antigenic regions of elastin. It should be noted, however, that the methods used in this study impose certain precautions on the interpretation of the results. The first precaution relates to the nature of the assay method. Because mature elastin is insoluble, the experimental design necessitated using a solubilized fraction of elastin as antigenic probe in the radioimmunoassay. We selected α-elastin because it has been well characterized (Partridge et al., 1955; Abatangelo et al., 1974), is easily prepared, and is likely to contain an adequate representation of antigenic determinants present on insoluble elastin. Although we have shown that antiserum to insoluble elastin reacts well with α-elastin, it should be kept in mind that some antigenic determinants initially on insoluble elastin might be removed or altered during α-elastin preparation [for example, see Kucich et al. (1981) and Houle & LaBella (1977)]. Accordingly, peptide fragments from insoluble elastin with unaltered determinants might remain undetected by not displacing labeled α-elastin in the radioimmunoassay.

The second precaution warns against assuming close congruence in antigenic reactivities of native and purified elastins. The conclusions of this study are based upon specific recognition of antigenic sites on peptide fragments of extracted elastin by antiserum raised to extracted elastin. We cannot exclude the possibility that extracted elastin acquires new antigenic properties during the necessarily harsh purification steps of autoclaving or treatment with hot NaOH. Thus, our antiserum to extracted elastin may be serologically dissimilar from antiserum to native elastin. Accordingly, antigenic determinants described in this study might be artifactual and may not accurately reflect intrinsic determinants on the native molecule. This possibility has been difficult to investigate because mature, undenatured "native" elastin has not been purified and direct comparison of antigenic cross-reactivity with "extracted" elastin has not been possible. Nevertheless, cross-reactivity of anti-insoluble elastin serum with tropoelastin (Mecham & Lange, 1981) strongly suggests antigenic similarities between extracted and native elastins.

The third precaution relates to using protease-generated fragments of elastin as defined antigens. Immunological analysis of proteins using peptide fragments has provided a penetrating approach to identifying the nature of antigenic determinants. However, this approach is limited since it is difficult to ascertain to what extent protease cleavage occurs within immunologically active regions of the molecule or if isolated peptides lose their antigenic conformation after separation from the parent protein. Hence, negative results in this study must not be viewed as conclusive evidence for peptide nonantigenicity.

Despite these limitations, results from this study demonstrated several interesting immunological features of elastin. One of the most striking findings was the universal antigenicity of peptides purified from the cross-linking domain. Of nine cross-link-containing fractions studied, all were antigenic. This was in contrast to peptides from non-cross-linked regions of elastin where only one peptide had significant antigenic activity. Additionally, cross-link-containing peptides from bovine elastin effectively displaced a nonbovine elastin probe in the radioimmunoassay, indicating that antigenic determinants in cross-linked peptides are similar in across-species barriers, even with the immunized host, the rabbit. This observation is consistent with primary sequence data supporting the evolutionary conservation of the cross-linking site on elastin, suggesting selective pressure against change in this region of the molecule.

It is doubtful that our distinction between shared and specific antigenic sites on elastin is due to tissue-specific rather than species-specific determinants. In an earlier study (Mecham & Lange, 1980), we showed that elastin purified from different tissues within the same animal species (bovine aorta and ligament) had identical reactivities in a competitive binding radioimmunoassay using antiserum to bovine ligament elastin. Homologous elastins purified from different animals (human and bovine aorta), however, were immunologically dissimilar. These observations are in agreement with biochemical data which show the amino acid compositions of

insoluble elastin purified from different tissues of a single animal to be remarkably similar, whereas amino acid heterogeneity is evident in interspecies comparisons (Starcher & Galione, 1976).

Cross-links in elastin are commonly one of three types: the aldol condensation product allysine aldol, lysinonorleucine formed through a Shiff base reaction, and desmosine or isodesmosine (Franzblau, 1971). One possible explanation for the immunoreactivity of the cross-linking region is that the cross-linking amino acid itself is acting as an antigenic hapten. This seems unlikely since (1) immunologically reactive peptides are no longer active in the radioimmunoassay following hydrolysis to free amino acids and (2) antiserum to whole elastin does not bind radiolabeled desmosine nor does antiserum to free desmosine react with α-elastin (King et al., 1980). Since ordered, non-cross-linked amino acid copolymers whose structures closely resemble the cross-linking site on individual chains also have no immunological activity, our results suggest that the antigenic determinant in the cross-linking domain represents a "conformational" determinant with antibodies directed to a spatially extended region comprising the cross-linking amino acid and an unknown number of amino acids on one or both linear chains immediately adjacent to the cross-link. A possible minimum size of the antigenic region is suggested by the immunogenicity of peptide TA-12, G25-II, which contains the cross-link allysine aldol plus four to five alanine residues. It is not possible, however, to state with absolute certainty that amino acids from both elastin chains or that cross-linking amino acids are necessary constituents of the antigenic determinant. Evidence to the contrary is the marked antigenicity of peptide TA-7, G25-II, a single-chain peptide enrichment in alanine, characteristic of the cross-linking site, and containing a non-cross-linked allysine residue, and by the reactivity of anti-insoluble elastin serum with soluble tropoelastin.

Differences in antigenic activity among cross-linked peptides raise interesting questions concerning properties of cross-linking determinants that affect their ability to induce antibodies. Although cross-linked peptides in elastin share extensive sequence homologies, we found that peptides containing allysine aldol were more antigenic than peptides joined by other cross-links, suggesting differences in accessibility of antigenic sites or that the degree of structural homology between cross-linking domains may be quite different. Indeed, our own structural studies indicate that the tertiary conformation of aldol cross-linking peptides differs appreciably from peptides joined by desmosine or isodesmosine (unpublished results).

The capacity of cross-link-containing peptides to stimulate an immune response has been demonstrated with proteins and peptides other than elastin. Gill et al. (1965) immunized animals with polypeptides cross-linked in various ways and showed that antibodies were formed to determinants in areas of linkage between two chains. Similarly, Chidlow et al. (1974) and Becker et al. (1975) have established the antigenicity of cross-linked peptides from rat tail and calf skin collagen. These studies have led to the conclusion that antibodies specific for the cross-linking site recognize a particular conformation. In the case of collagen and elastin, the conformation of the cross-linking determinant may be particularly rigid owing to the stability of the lysine-derived cross-linkage.

The studies described in this report were conducted with a single antiserum selected for high cross-reactivity with nonbovine elastins. Three other antisera to bovine insoluble elastin have been partially characterized by using our defined peptides, and in all cases, activity between species was localized to cross-link-containing peptides, suggesting a general antibody response to the determinants characterized in this report. It is notable, however, that the appearance of cross-reacting antibodies after immunization, and the degree of cross-reactivity, varied unpredictably between rabbits, even in antisera raised to the same batch of antigen. In general, our experience has shown that late sera have more cross-reactivity than early sera, with cross-reactivity appearing earlier and occurring at higher levels in antisera to insoluble rather than solubilized elastin (Mecham & Lange, 1981).

The conclusions of this study are that antigenic determinants on elastin can be classified functionally into two categories: determinants related to cross-linking domains which show species cross-reactivity and species-specific determinants in the non-cross-linked regions. The characterization of specific classes of antigenic determinants on elastin is an important first step in understanding the antigenic structure of this unique connective tissue macromolecule.

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