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Induction of Cell-Mediated Immune Response to Peptides Produced by Enzymatic Digestion of Elastin from Human Lung Parenchyma[1] (40997)

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Abstract. Guinea pigs were sensitized with elastin peptides dissolved in complete Freund's adjuvant, and with complete Freund's adjuvant alone. Four weeks after sensitization, the guinea pigs were challenged intradermally with elastin peptides. Only guinea pigs sensitized and challenged with elastin peptides produced erythema and induration. Histological studies of the indurated skin showed heavy infiltration of mononuclear cells. Migration of peritoneal exudate cells of those guinea pigs was strongly inhibited in the presence of elastin peptides, whereas other proteins such as serum albumin did not inhibit the migration. These observations suggest that the reaction is specific for elastin peptides, although a slight but significant effect was shown by peptides derived from other connective tissue proteins of human lung, i.e., microfibrils and collagen. Cell-mediated immunity could also be induced in normal guinea pigs by the transfer of spleen cells obtained from guinea pigs sensitized and challenged with elastin peptides.

Delayed-type hypersensitivity (cell-mediated immunity) is an earlier manifestation of the immune response to antigenic challenge than the production of circulating antibodies. This was first suggested by Dienes and Schoenheit (1) and further substantiated by studies of Mote and Jones (2) and Simon and Rackeman (3), who found that none of a large number of human subjects exhibiting delayed hypersensitivity had developed serum antibodies at that time. Similarly Salvin (4) showed that in guinea pigs delayed-type hypersensitivity could be demonstrated long before the appearance of circulating antibodies. Chase (5) has shown that cells of the lymphoid tissue and spleen involved in delayed hypersensitivity are also involved in antibody production. These observations suggest that the same antigen which produces antibody may also elicit the delayed-type hypersensitivity. The weak response to elastin peptides in the production of antibodies (6–8) and the reported occurrence of cell-mediated immunity to soluble connective tissue extracts derived from lung elastin and collagen (9) prompted us to investigate whether cell-mediated immunity in animals can be induced to human lung elastin peptides.

Materials and methods. Eagle's minimum essential medium (MEM), Hanks' balanced salt solution, fetal calf serum, and penicillin/streptomycin were obtained from Grand Island Biological Co., Grand Island, New York (Gibco). Human serum albumin was purchased from Worthington Biochemical Corporation, Freehold, New Jersey, and complete Freund's adjuvant (CFA) from Difco, Detroit, Michigan. Guinea pigs, Hartley strain, male, weighing between 300–350 g, were obtained from CAMM Research Institute, Inc., Wayne, New Jersey.

The human lung elastin peptides used in this study were prepared as described by Darnule *et al.* (8). These peptides elicited circulating antibodies in rabbits (8) and, more recently, were found to produce higher-titer antibodies in guinea pigs (10).

Sensitization of guinea pigs. Eight healthy guinea pigs were sensitized with human lung elastin peptides. Ten milligrams of elastin peptides was dissolved in 1 ml of 1 *M* NaCl and emulsified in an equal

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volume of complete Freund's adjuvant (CFA). One milliliter of the emulsion containing 5 mg of elastin peptides was injected at multiple sites in the 4 foot pads of each guinea pig. As controls four guinea pigs of the same strain and weight received injections of 1 ml emulsion of CFA and 1 *M* NaCl and four additional guinea pigs were injected with 1 *M* NaCl only.

Skin testing of sensitized guinea pigs. Four weeks after sensitization the guinea pigs were subjected to skin tests. The backs of the guinea pigs were shaved and residual hairs removed by applying a hair remover (Neet, Whitehall Labs., N.Y.). Two hours later both groups were challenged by intradermal injection in the back of 0.1 and 0.05 ml of a solution of 20 mg elastin peptides/ml of 1 *M* NaCl. Each guinea pig was challenged at four sites, at two sites with 0.1 ml and at the other two sites with 0.05 ml. The development of a reaction was followed by measuring the diameter at the challenge site at 6, 24, 30, and 48 hr using a Susceptibility Zone Size Measuring Scale (Burroughs Wellcome Co., Research Triangle Park, N.C.).

Histological studies. At 24 hr skin biopsies were taken of the challenged sites from representative guinea pigs sensitized with elastin peptides and from control guinea pigs (CFA sensitized). The tissues were fixed in buffered formaldehyde, dehydrated with increasing ethanol gradient, treated with xylol, and mounted in paraffin. Sections were cut at 5 μm thickness and stained for light microscopical observations with hematoxylin and eosin as described by Lillie and Fulmer (11).

Testing for delayed hypersensitivity by glass capillary tube cell migration assay. The capillary migration assay described by David *et al.* (12) was used to determine the extent of cell mediated immunity *in vitro*. Sterile conditions and siliconized glassware were used throughout the experiment. The eight sensitized guinea pigs were injected intraperitoneally with 30 ml of mineral oil each. Three days later the guinea pigs were anesthetized and bled to death. Sixty milliliters of cold Hanks' solution was then injected into the peritoneal cavity of each guinea pig. The abdomen was gently massaged and the mixture of oil and Hanks' solution was aspirated carefully with a syringe and transferred into separate conical flasks. The mixture was shaken gently and centrifuged at 121*g* for 10 min at 4°. Both the oily and the aqueous layers were removed and cells were washed by centrifuging three times for 5 min with Hanks' balanced salt solution and once with minimum essential medium (MEM) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Peritoneal exudate cells (macrophages, neutrophils, and lymphocytes) were suspended in MEM, viability was assayed using trypan blue, and the cell suspension was adjusted to 1×10^{10} cells/ml. Small capillary tubes (1.1–1.2 mm i.d. and 75 mm length) were filled up to one-quarter of the length with the cell suspension by capillary action and the other end was sealed by melting the glass in a flame. The capillary tubes were centrifuged at 200*g* for 5 min at room temperature, cut at the cell fluid junction, and the portion containing the cells was placed on the bottom coverslip of Sykes–Moore type chambers of 1-ml capacity and secured with silicone grease. Two glass capillary tubes were located in each chamber. The top cover slip was put in place and the closed chambers were filled through side holes. Cells from each guinea pig were introduced into (a) two chambers filled with MEM, (b) two chambers filled with human serum albumin, (c) duplicate chambers filled with two concentrations (4 and 0.4 mg/ml) of elastin peptides, microfibrillar peptides, and collagen peptides. All chambers were placed in a horizontal position in an incubator at 37° for 24 hr. The pattern of migration of the cells was visualized using a Durst analyzer M 301 at a fixed magnification and traced. The area of migration was measured with a planimeter. The migration index (MI) was calculated from the average readings of each set of tubes using the following formula:

$$\text{percentage of migration in the presence of antigen} = \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}} \times 100.$$

Collection of spleen cells. The spleens from the sensitized guinea pigs were removed and placed in MEM. Fat and blood vessels were mechanically removed by dissection and discarded. The spleens were placed in a petri dish containing 10 ml of MEM and teased into small pieces with forceps. The cell suspension was passed through a gauze to remove fibrous stroma. The cells were then centrifuged at 200*g* for 10 min. Ten milliliter of 0.35% NaCl was added to the packed cells and the cells left suspended for 1 min to lyse the erythrocytes. After centrifugation at 200*g* for 1 min and removal of the supernatant, the cells were resuspended in 0.15 *M* NaCl to restore physiologic tonicity. Finally the cells were suspended in MEM.

Passive transfer of immunity. Passive transfer of immunity was achieved by injecting into normal guinea pigs spleen cells obtained from the guinea pigs which had been sensitized with elastin peptides. Each guinea pig was injected ip with 1×10^{11} cells in 1 ml. Four days after the injection, the guinea pigs were subjected to skin tests as described above. Each guinea pig was challenged id with 0.1 ml and 0.05-ml solutions of 20 mg elastin peptides/ml saline and the extent of the reaction was measured after 6, 24, 30, and 48 hr.

Results. Determination of cell mediated immunity *in vivo* by skin tests produced reactions as listed in Table I. The guinea pigs sensitized and challenged with elastin peptides showed no reaction or minimal reaction during the first 6 hr as expected for delayed hypersensitivity. By 24 hr, inflammation and induration of the challenge site accompanied by thickening of the skin were observed. The reaction reached a maximum at 24 to 30 hr and was decreased at 48 hr (Table I). The control guinea pigs did not show any reaction. Histological studies of biopsies from the experimental and control guinea pigs also showed significant differences. Hematoxylin- and eosin-stained sections obtained from experimental guinea pigs demonstrated a massive infiltration of mononuclear cells (Fig. 1) which was absent in sections obtained from control guinea pigs.

The migration of peritoneal exudate cells obtained from experimental guinea pigs in

TABLE I. SKIN REACTION OF GUINEA PIGS ACTIVELY SENSITIZED WITH HUMAN LUNG ELASTIN PEPTIDES

Experimental group	No. of guinea pigs	Elastin peptides (20 mg/ml) Challenge in ml	Skin test diameter (mm induration)			
			6 hr[a]	24 hr	30 hr	48 hr
Elastin peptides sensitized	8	0.1	<3	21.69 ± 4.27[b]	21.69 ± 4.27	12.94 ± 3.68
		0.05	<3	13.44 ± 3.23	13.44 ± 3.23	7.87 ± 1.77
Control I CFA + 0.15 *M* NaCl sensitized	4	0.1	<3	4.5 ± 1.0	4.5 ± 1.0	<3
Control II 0.15 *M* NaCl sensitized	4	0.1	<3	3.75 ± 0.5	3.75 ± 0.5	<3

[a] Time after challenge.
[b] Mean ± SD.

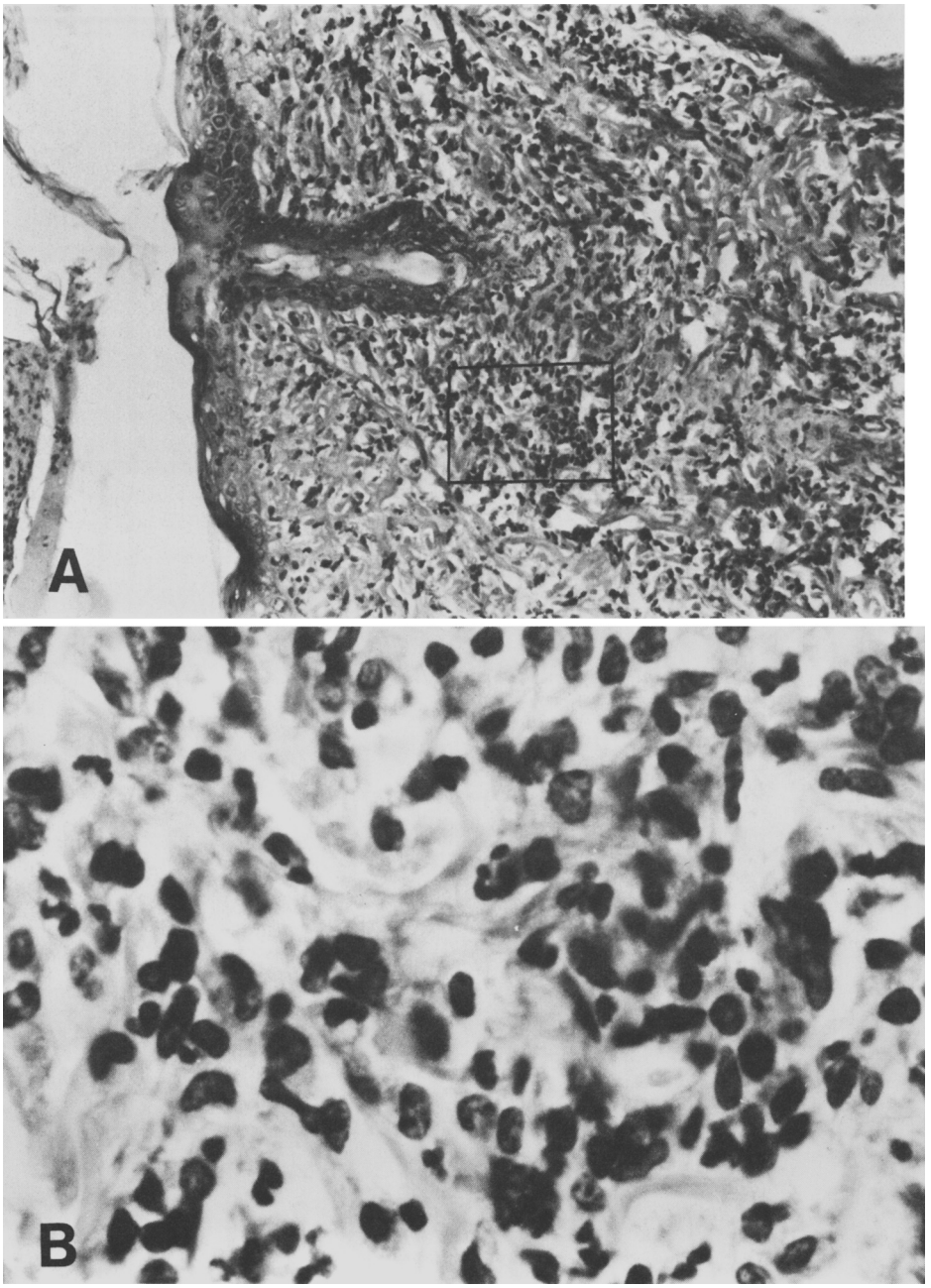

FIG. 1. (A) Hematoxylin- and eosin-stained section of 24-hr skin biopsy of a guinea pig actively sensitized with elastin peptides. Heavy infiltration of mononuclear cells and thickening of the epidermis is apparent (×200). (B) An enlargement of the area outlined in (A), showing the presence of mononuclear cells (×950).

the presence of MEM containing 4 mg human serum albumin/1 ml did not differ from that obtained in the presence of MEM only. The migration of peritoneal exudate cells was significantly inhibited in the presence of the nondialyzable elastin peptides

with which the animals had been sensitized (Fig. 2). Nondialyzable peptides derived from the microfibrillar elastin fiber component or lung collagen had some inhibitory effect which could have been caused by contamination with elastin peptides. No inhibition of migration of peritoneal exudate cells obtained from control guinea pigs was observed in the presence of elastin peptides, microfibrillar peptides, or collagen peptides.

Both the guinea pigs passively sensitized with spleen cells showed positive skin reactions when challenged intradermally with elastin peptides (Table II).

Discussion. Cell-mediated immunity to human lung elastin peptides was demonstrated by both *in vivo* and *in vitro* procedures. The skin reaction observed in guinea pigs sensitized with elastin peptides conforms with the criteria generally accepted as governing delayed hypersensitivity reactions. Delayed hypersensitivity is characterized by an initial latent period of about 6 hr, usually reaches a maximum at 24–48 hr, and the reaction diminishes in intensity by 72 hr. The guinea pigs sensitized with elastin peptides showed no reaction or minimal reaction up to 6 hr postchallenge, ruling out the possibility of an immediate-type hypersensitivity (Arthus reaction). The skin reaction reached a maximum at 24 hr and subsided at 48 hr. These observations are in agreement with those of other investigators (13–17), who found a maximum skin reaction when collagen was used as a sensitizing and challenging agent. In the present study histological evaluation of skin biopsies obtained at 24 hr postchallenge with elastin peptides showed massive infiltration of mononuclear cells in the sensitized guinea pigs (Fig. 1). The skin reaction could not be due to cutaneous–basophil hypersensitivity since it was

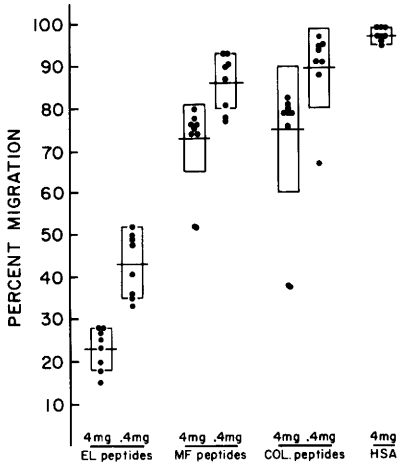

FIG. 2. Migration indices of peritoneal exudate cells of guinea pigs sensitized with elastin peptides in the presence of elastin peptides, microfibril peptides, collagen peptides, and human serum albumin. Each point represents the average reading from one animal. The bars represent the mean and the rectangles the SD.

TABLE II. SKIN REACTION OF GUINEA PIGS PASSIVELY SENSITIZED WITH SPLEEN CELLS FROM GUINEA PIGS SENSITIZED WITH ELASTIN PEPTIDES

Experimental group	Number of guinea pigs	Elastin peptides (20 mg/ml) Challenge in ml	Skin test diameter (mm induration) 6 hr[a]	24 hr	30 hr	48 hr
Sensitized with spleen cells obtained from guinea pigs sensitized with elastin peptides	2	0.1	<3	14.0 ± 2.67[b]	14.0 ± 2.67	5
		0.05	<3	7.5 ± 0.57	7.5 ± 0.57	3
Control (sensitized with spleen cells obtained from guinea pigs sensitized with CFA + 0.15 *M* NaCl)	1	0.1	<3	<3	<3	<3

[a] Time after challenge.
[b] Mean ± SD.

performed 4 weeks after sensitization and cutaneous basophil hypersensitivity is a transient reaction elicited approximately 1 week after sensitization (18). No basophilic infiltration was detectable by Wright stain.

The specificity of cell-mediated immunity was determined by comparison with the effects of human serum albumin as well as peptides cleaved by enzymatic digestion of other connective tissue proteins. Guinea pigs sensitized with elastin peptides did not give positive skin reactions when challenged with human serum albumin or microfibrillar peptides but nondialyzable collagen peptides showed some reaction. However, these nondialyzable collagen peptides also produced positive skin reactions in guinea pigs sensitized with CFA and saline only, suggesting that this reaction is not due to cross-reaction with elastin peptides but is a characteristic reaction of the collagen peptides. It should be noted that the nondialyzable fraction of the collagen peptides obtained by *Clostridium histolyticum* collagenase digestion represents a minor portion. It is not the characteristic helical collagen protein which is digested to dialyzable peptides. Alternatively elastin peptide contamination cannot be entirely ruled out.

Additional evidence for the production of cell-mediated immunity to elastin peptides was obtained from studies of passive transfer of immunity by injecting 1×10^{11} spleen cells from the guinea pigs sensitized and challenged with elastin peptides into normal guinea pigs. These findings are in agreement with those of Gentner and Adelmann (14), who successfully transferred the cell-mediated immunity of collagen from actively sensitized guinea pigs into normal guinea pigs by injection of 2×10^8 cells. Similarly, Skog (19) with 0.5×10^9 cells and Dunn and Patnode (20) with 1×10^{11} cells were able to transfer cell-mediated immunity of tuberculin into normal guinea pigs. The successful passive transfer of immunity depends upon a number of factors, such as the donor-recipient system, cell types, and number of cells used in the transfer.

A variety of tests for *in vitro* detection of cell-mediated immunity through the use of cell suspensions have been devised. One of the most interesting and most thoroughly investigated is the inhibition of lymphoid cell migration. In this system, two cell types are involved. The nonsensitive migrating cell capable of being inhibited in the presence of sensitizing antigen, is the macrophage or macrophage-like cell. The other cell which possesses the migration inhibition "information" is the lymphocyte. Peritoneal exudate cells (PEC) contain about 70% mononuclear cells, i.e., lymphocytes and macrophages and 30% polymorphonuclear cells (21). The inhibition of PEC in the presence of sensitizing antigen, i.e., elastin peptides, was much greater than the inhibition in the presence of other antigens, e.g., peptides derived from collagen or microfibrils. Human serum albumin (HSA) which did not produce any detectable inhibition served as negative control in comparisons with other antigens.

Although the inhibition of migration cannot be directly correlated with the diameter of the skin reaction, there is general agreement in the order of magnitude. Migration inhibition is most commonly observed with PEC collected from guinea pigs in which a strong delayed hypersensitivity is observed, i.e., in animals with skin reactions averaging 15 mm or more in diameter (12) and skin tests done 3 weeks after sensitization (22). The present study of migration inhibition was performed using PEC of guinea pigs sensitized with elastin emulsified in CFA, and in those guinea pigs which showed skin reaction 15 mm or more in diameter when tested at 4 weeks it may be considered to reflect cell-mediated immunity. Characterization of the immune responses of human elastin peptides may be of value in defining degradative processes of human lung elastin *in vivo* in the early stages of diseases such as pulmonary emphysema.

Immunological destruction of host tissue could result from either cell-mediated or humoral-immune mechanisms. Dill *et al.* (23) have demonstrated cell-mediated immunity to normal lung antigens in chronic pulmonary diseases including chronic bronchitis and Hennes *et al.* (24) have shown that lung antigens resistant to tryptic digestion may be of importance in the pathogenesis of pulmonary emphysema.

Cellular-immune mechanisms to normal host antigens have also been implicated in nonpulmonary diseases (23). Our observations suggest a possible pathogenic role for cellular immunity to connective tissue proteins such as elastin.

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