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Failure of Complete *in Vivo* Inhibition of Cathepsin D to Mitigate the Pulmonary Arthus Reaction (40758)

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The acid protease, cathepsin D, is an intracellular carboxyl endopeptidase, which has been characterized by Barrett (1, 2). It is inhibited by pepstatin which reacts stoichiometrically with cathepsin D forming a tight irreversible bond (3, 4). Cathepsin D is present in many tissues including polymorphonuclear leukocytes and the lung (5). Evidence has been presented that cathepsin D is important in mediating the Arthus reaction in skin, however the extent of this enzyme in mediating the Arthus reaction in lung is unknown (6, 7). This study was undertaken to investigate the role of cathepsin D in mediating the pulmonary Arthus reaction. As part of this investigation a schedule was developed for dosage and administration of pepstatin, a recognized inhibitor of cathepsin D, which completely inhibits cathepsin D in the rat lung extract (3, 4).

Materials and methods. Pulmonary Arthus reactions were induced by a modification of the method described by Johnson and Ward (8, 9). Male Long–Evans rats weighing approximately 125 g were used. Under ketamine and Cetacaine anesthesia each rat was injected intravenously with 10 mg bovine serum albumin (BSA) obtained from Miles Research Laboratories, Elkhart, Indiana. Rat serum albumin (RSA), also obtained from Miles Research Laboratories, was iodinated enzymatically by the method described by Marchaloni (10) and modified by Herrup (11). Each rat received approximately 500,000 cpm of ^{125}I-radiolabeled RSA intravenously. The rats were endotracheally intubated with a 22-gauge Intracath which was inserted until it met minimal resistance and then withdrawn slightly. Control rats were given 0.3 ml normal saline (NS) intratracheally (IT). Experimental rats were given rabbit anti BSA 0.3 ml IT. Rabbit antibody to BSA was prepared from hyperimmune rabbit serum. This was partially purified to a fraction rich in IgG by ammonium sulfate precipitation and ion-exchange chromatography with a Whatman DE 52 cellulose column at pH=8 and 0.1 M phosphate buffer. The protein was concentrated by lyophilization and used in a final concentration of 7 mg/ml of antibody protein as precipitating antibody.

Antibody protein was determined following heat inactivation of compliment by reacting the IgG fraction with an appropriate amount of BSA in cold saline. Protein concentration of the washed resolubilized precipitate was measured by the Lowry method. Antigen protein was subtracted from the total to give antibody protein (12, 13).

Six hours following the intubation, the animals were sacrificed with high-dose nembutal given intraperitoneally. The rat lungs and heart were removed from the thorax and perfused with 10 ml NS to remove the radioactivity associated with the serum in the pulmonary vasculature. The radioactivity of both lungs of each rat after perfusion was counted in a solid-crystal scintillation spectrometer and compared to the radioactivity in 1 ml of rat blood from the same animal. The ratio cpm in the lung to cpm in the blood is an index of the per-

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meability change in the pulmonary vasculature due to the Arthus reaction (8).

In three animals the presence of the pulmonary Arthus reaction was confirmed histologically. For this purpose the lungs were fixed under 12 cm of airway pressure with formalin and stained with hematoxalin and eosin.

The rat lung homogenate was prepared by removing and perfusing the heart and lungs, dissecting the airways and large blood vessels from the pulmonary parenchyma, and then blenderizing the pulmonary parenchyma in 0.1% Triton X in water. The blenderized lung was then homogenized in a two-stage hand homogenizer. The homogenate was centrifuged at 3000*g* for 10 min and the supernatant was saved while the pellet was discarded. Protein in the supernatant was measured by the Lowry method (13) and then diluted to 1.5 mg/ml.

Assay of lung cathepsin D was carried out by the procedure of Grayzel *et al.* (14). Tritiated denatured hemoglobin was used as the substrate and was kindly supplied by Dr. Victor Hatcher of Montefiore Hospital, Bronx, New York. The substrate had 0.38 mg/ml of protein and 1.7×10^7 dpm/ml of tritium. Fifty lambda volumes of each of the four reactant substances, which included substrate (tritiated Hb), buffer (0.2 M acetate, pH=4), enzyme (rat lung homogenate 1.5 mg/ml), and distilled water were incubated at 37°. The pH of the reaction mixture was checked with a microelectrode and found, in all instances, to be between 4.1 and 4.3. At intervals of 15, 30, and 60 min a 50-lambda aliquot was withdrawn and added to an equal volume of 3% hemoglobin. To stop the reaction, trichloracetate 6% (100 lambda) was added. The precipitated protein was then centrifuged. The 100 lambda of the resulting supernatant was counted in a liquid scintillation spectrometer. Specific activity of cathepsin D was calculated by plotting the increase in nonprecipitable disintegration per minute, of the supernatant, per minute of incubation time per milligram of protein. Thus specific enzyme activity is expressed in units dpm/min/mg.

Pepstatin (obtained as a gift from H. Umezawa, Institute Microbial Chemistry, Tokyo, Japan) was prepared as a 2 mg/ml suspension in normal saline (NS) and adjusted to pH 7. This was administered intraperitoneally at a dosage of 80 mg/kg per injection. There were two administration schedules for pepstatin. One group of rats received a single dose in the morning on each of 3 consecutive days with the animals being sacrificed 6 hr after the final injection. Another group of animals received a single dose in the morning on each of 2 consecutive days, with the animals being sacrificed 24 hr after the final injection.

Results. Figure 1 is a photomicrograph of a rat lung undergoing the pulmonary Arthus reaction following the procedures described. A neutrophil-rich hemorrhagic alveolitis is seen as was described by Johnson and Ward (8).

The results of the cathepsin D assay on pulmonary homogenates are given in Table I. The normal rat lung has a high endogenous level of cathepsin D, and averaged 480 dpm/min/mg SD 57. In a typical experiment (Fig. 2) the substrate is approximately 50% hydrolyzed in 30 min and almost completely hydrolyzed in 60 min. Pulmonary tissue undergoing an Arthus reaction also had high levels of cathepsin D which were not significantly different from normal rat lung.

A single intraperitoneal dose of pepstatin gave variable inhibition of cathepsin D in the lung homogenate. However, two doses, as indicated above, of pepstatin consistently inhibited 97% of acid protease activity from lung 24 hr following the last injection. A similar degree of inhibition was achieved with the three-dose regimen 6 hr following the last injection. *In vitro* addition of excess pepstatin to the assay mixture in amounts 10,000 times that necessary to achieve 95% inhibition of acid protease did not increase the amount of inhibition of hydrolysis of the substrate above that observed with either two or three doses of pepstatin. In the presence of this level of excess of pepstatin, the amount of inhibition of pepstatin-inhibitable enzyme, was interpreted to be 100%. Therefore, both two or three doses of pepstatin inhibit 100% of cathepsin D in the rat lung homogenate.

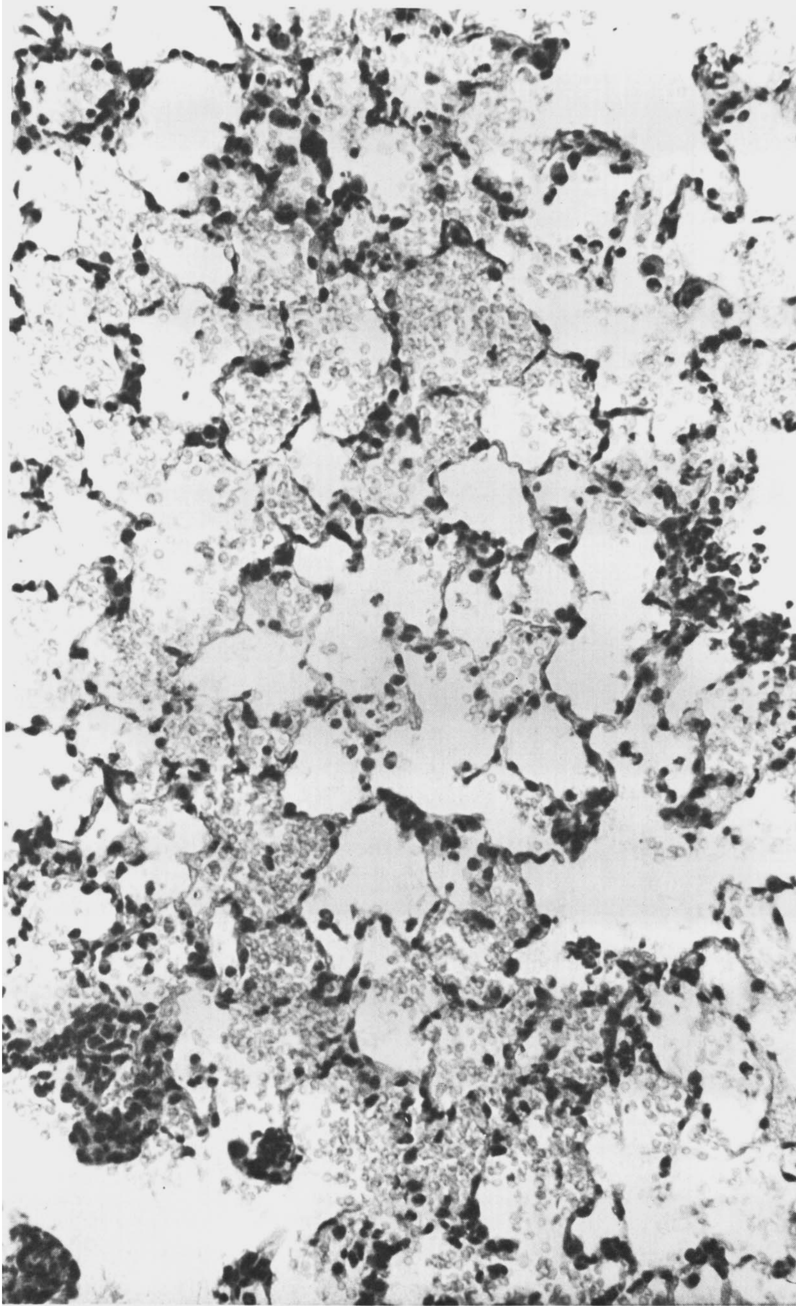

FIG. 1. Histology of the pulmonary Arthus reaction—There is a neutrophil-rich hemorrhagic alveolitis. Hematoxylin and eosin.

TABLE I. ACID PROTEASE ACTIVITY IN RAT LUNG HOMOGENATES

Rat lung homogenate	Acid protease activity dpm/min/mg	Percentage normal rat lung acid protease activity	Number of animals
Normal control (intratracheal saline)	480 ± 57	100	6
Pulmonary Arthus reaction without pepstatin	460 ± 62	96	7
Pulmonary Arthus reaction with pepstatin (three doses)	24 ± 5	5	7
Normal rats with two doses of pepstatin	14	3	3
Excess pepstatin added during assay *in vitro*	24	5	—

The fact that there was some hydrolysis (approximately 5%) that could not be inhibited suggests: (a) that there are acid hydrolases in the lung other than cathepsin D, (b) that neutral proteases may have some activity in the pH range of 4.1 to 4.3, or (c) that there is nonenzymatic hydrolysis of substrate under the conditions of the assay.

Table II gives the results of the pulmonary Arthus reaction in normal and pepstatin-treated rats. Animals which received antibody and antigen had twice as much permeability change in the lung as

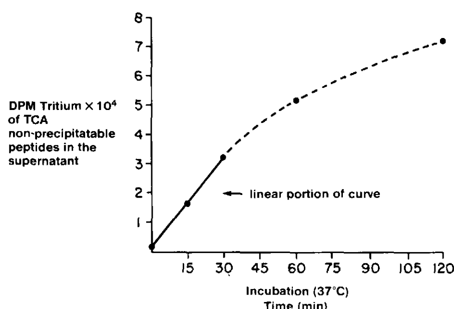

FIG. 2. A typical experiment assaying the cathepsin D activity in the normal rat lung homogenate. Cathepsin D activity is the slope of the linear portion of the curve.

control animals and this difference was significant by the Student t test for unpaired data, $P < 0.01$. Animals which received a dose of pepstatin capable of inhibiting the pulmonary cathepsin D also had pulmonary hemorrhage significantly greated than control rats, $P < 0.01$. Animals pretreated with pepstatin did not have a significantly different amount of pulmonary hemorrhage from animals which did not receive pepstatin. The results indicate pepstatin is unable to inhibit the pulmonary Arthus reaction.

Discussion. The Arthus reaction first described by Arthus in 1903 was recently reviewed in great detail in a monograph by Cochrane and Janoff (6). Antibody–antigen complexes activate complement which attracts PMN leukocytes. The leukocytes are believed to participate in the reaction by releasing agents which cause an injury to the vasculature allowing hemorrhage into the affected tissue. Precisely which agents mediate this injury is unknown.

Movat demonstrated that antibody–antigen complexes trigger degranulation of PMN leukocytes. The material released from the PMN leucocytes was phlogistic, causing increased vascular permeability and gaps between adjacent endothelial

TABLE II. MEASUREMENT OF PULMONARY INJURY IN RAT LUNG ARTHUS REACTION WITH AND WITHOUT PEPSTATIN

	Control (antigen + saline)	Arthus reaction (antigen + antibody)	Arthus + pepstatin (antigen + antibody + pepstatin)
Number of animals	6	7	7
Permeability change (ml blood equivalents)	0.19 ± 0.07	0.38 ± 0.10[a]	0.32 ± 0.08[a]

[a] Mean difference from control, $P < 0.01$.

cells. This material was characterized as high molecular weight approximately equal to 50,000 and having a negative charge at neutral pH (15). This description fits cathepsin D which has a molecular weight of 42,000 and an isoelectric point of 5.5–6.5 (1, 2).

Cochrane demonstrated with electron microscopic studies that the structural barrier which was disrupted in the Arthus reaction was the vascular basement membrane. He found that the polymorphonuclear leukocyte (PMN) lysates which were capable of degrading vascular basement membrane were acid proteases specifically cathepsin D and cathepsin E. There was no fraction of the PMN lysate which when injected intradermally caused an Arthus-like reaction or destruction of the vascular basement membrane equal to that seen in the Arthus reaction. This was interpreted to be due to the lack of an appropriate acid environment in the skin under the artificial condition of having acid protease in the absence of PMN leukocytes. Cochrane concluded that the Arthus reaction was mediated by acid protease acting in an acid environment as a consequence of anaerobic neutrophil metabolism (7). Recently, Davies found that golmerular basement membrane is not hydrolyzed by cathepsin D but is hydrolyzed by cathepsin B at pH=4 (16). The skin Arthus reaction may also be mediated by cathepsin B. The results of the present study on lung are at variance with previous studies of the skin Arthus reaction which implicate cathepsin D as the primary mediator of the Arthus reaction (7).

A case has been made for primary direct cathepsin D envolvement in the skin Arthus reaction (7, 14). Our study provides strong evidence that cathepsin D is not the primary direct mediator of the pulmonary Arthus reaction. We do not, however, interpret our data as evidence for excluding cathepsin D from any indirect role in the Arthus reaction. For example, cathepsin D may act in concert with other proteases which are not pepstatin inhibitable and cathepsin D may act intracellularly in the generation of kinin (17). An alternate interpretation of these results is that the dermal Arthus reaction differs from the pulmonary Arthus reaction. Oxygen tensions are undoubtedly higher in the pulmonary interstitium than the dermal interstitium and this may not allow for the necessary anaerobic metabolism to create an acidic environment.

Previous studies on the pharmacological fate of pepstatin indicate that serum levels of 5–10 μg/ml are reached 6–24 hr after i.p. administration; and urinary excretion occurs up to 72 hr after a single i.p. injection (18). We are unaware of any published study which measured tissue levels or distribution of pepstatin. This is the first study in which total tissue pepstatin levels were inhibitory to total tissue cathepsin D.

The results of this study indicate that in control lung homogenates, cathepsin D activity was easily detectable in all experiments. However, after *in vivo* administration of pepstatin cathepsin D activity was no longer demonstrable in any lung homogenates similarly prepared. This provides strong evidence of complete inactivation of cathepsin D under these experimental conditions. Conceivably, certain intracellular locations in lung cells could contain cathepsin D activity which was not inhibited initially by pepstatin *in vivo,* since there is no definitive information on the movement of pepstatin to intracellular sites. However, if such cathepsin D were released from intracellular loci to the interstitium to participate in an Arthus reaction, inhibition by pepstatin would occur.

The location of cathepsin D in the lung is uncertain, however, alveolar macrophages definitely contain this enzyme (19). In our preparation lung homogenates probably represent the cytoplasm of all pulmonary cells mixed with cytoplasmic organelles including lysosomes (20). Nuclei connective tissue fragments were probably removed by centrifugation.

The duration of cathepsin D inhibition in our model was not determined, however, since both the two- and three-dose regimen inhibited completely, we believe that there was complete inhibition for the duration of the Arthus reaction. The two-dose regimen represented the state of the rat at the time of initiation of the Arthus reaction and the

three-dose regimen represented the state of the rat at the time of termination of the Arthus reaction.

The usefulness of our model of cathepsin D inhibition in the pulmonary interstitium could be challenged if cathepsin D were to act entirely intracellularly. Poole has demonstrated extracellular localization of cathepsin D in cartilage by histochemical staining (21). Lin demonstrated that the local Schwartzman phenomenon may be partially inhibited by systemic or local administration of pepstatin (22). Thus, there is evidence for both interstitial localization and action of cathepsin D and our model should be useful to clarify this in future studies.

Summary. This is the first demonstration that pepstatin levels following i.p. injection may be reached which are totally inhibitory to cathepsin D contained in the lung. Thus an animal model has been created, which is devoid of cathepsin D as demonstrated by measurement of an extract of the lung. When this model was challenged with the pulmonary Arthus reaction no inhibition of the Arthus reaction was observed. We conclude that cathepsin D is not the primary direct mediator of the pulmonary Arthus reaction.

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