A Model of Decreased Functional α -1-Proteinase Inhibitor

PULMONARY PATHOLOGY OF DOGS EXPOSED TO CHLORAMINE T

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ABSTRACT The objective of this study was to develop an animal model representative of chronic human α -1-proteinase inhibitor deficiency. Eight dogs were treated with a mild oxidizing agent, chloramine T, with varying regimens for 3–27 wk. The capacity of the serum to inhibit both trypsin and elastase was examined and found to respond differently. Although immunologically determined levels of protease inhibitor did not change, the ability of serum to inhibit elastase in an in vitro assay decreased in direct response to chloramine T treatment. The trypsin inhibitory capacity was less affected. Emphysemalike alterations in lung morphology were observable when histologic sections were evaluated both subjectively and objectively by mean linear intercept measurements. The data suggest that this model parallels the emphysema associated with the genetic α -1-proteinase inhibitor deficiency in man.

INTRODUCTION

Many investigators have tried to develop animal models of protease-antiprotease imbalance in order to study the resulting lung disease (1–3). Although most of the investigations have added exogenous enzymes,

or cell subfractions (4–8), reducing α -1-proteinase inhibitor (PI)¹ activity would have marked advantages as a model because it would closely simulate the genetic form of the human disease, α -1-PI deficiency.

Kleinerman and Rynbrandt (1) have achieved some success at reducing the serum trypsin inhibitory capacity (TIC) of small animals by administration of galactosamine, but this reagent has not achieved long-term depression of the TIC, and no pulmonary changes have been reported. In addition, galactosamine is a liver toxin and probably acts by a general effect on protein metabolism in the liver (9). Chan (2) has tried, unsuccessfully (10), to select a genetically deficient mouse strain. Therefore, to our knowledge, no adequate model of α -1-PI deficiency has been previously described.

Recent in vitro studies demonstrated that chloramine T (CT) depressed the elastase inhibitory capacity (EIC) of serum and inactivated α -1-PI (11–14). These studies suggested that an oxidation reaction was a possible mechanism for the inactivation of the inhibitor. A common source for such oxidizing agents exists in cigarette smoke (13, 15, 16). These data suggest that a causal relationship between cigarette smoking and emphysema development may involve such a mechanism. Cohen demonstrated in a monkey that intravenous infusion of 1,200 mg CT (12) caused an acute, marked reduction in serum EIC. Since all previous studies suggested that CT could preferentially reduce the elastase inhibitory capacity, we administered CT to dogs for several weeks to develop a chronic animal model for functional α -1-PI deficiency.

Proteolytic enzymes and their inhibitors are normally found in the lung. A protease-antiprotease hypothesis has been formulated that suggests that the pathogenesis of emphysema involves both the release of endogenous proteases from polymorphonuclear leu-

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¹Abbreviations used in this paper: CT, chloramine-T; DNE, dog neutrophil elastase; EIC, elastase inhibitory capacity; IV, intravenous; MLI, mean linear intercept; PI, proteinase inhibitor; SAPNA, succinyltrialanyl-nitroanilide; SEM, scanning electron microscopy; TIC, trypsin inhibitory capacity.

kocytes, macrophages, or other (17–21) as yet unknown sources into the alveolar spaces or interstitium and the reduced regulation of the activity of these proteases by proteinase inhibitors originating both in the blood and airways (22–24). An excess of available proteases over proteinase inhibitor defenses would then lead to the lung damage seen in pulmonary emphysema. Patients with a genetic deficiency in their serum concentration of α -1-PI tend to develop emphysema (25) at an early age and without a concomitant history of cigarette smoking.

This report examines the chronic *in vivo* effect of treatment with CT by intravenous (IV), oral, or combined oral and IV routes on the EIC and TIC of dog serum. In addition, histologic changes in pulmonary architecture are presented that may be a consequence of reduced functional α -1-PI.

METHODS

Materials. Specific reagents were obtained as follows: CT, J. T. Baker Chemical Co., Phillipsburg, N. J.; porcine pancreatic elastase, Worthington Biochemical Corp., Freehold, N. J.; bovine trypsin, ICN Nutritional Biochemicals, Cleveland Ohio; hemoglobin, agarose, Type IV, Sigma Chemical Co., St. Louis, Mo.; succinyl-trialanyl-nitroanilide (SAPNA), Protein Research Foundation, Osaka, Japan; beagles, 10 kg, White Eagle Farms, Quakertown, Pa.; DE-52 cellulose, Whatman, Inc., Clifton, N. J.

Solutions were prepared with deionized water of maximum conductivity of 1–2 μ mho. Other reagents were of the highest quality available and used without further purification.

Experimental protocol. Eight male beagles were treated with CT. All dogs were ~10 kg in weight, appeared in good health, and were free of heartworms. Serum was taken before treatment and served as an internal control. Four dogs received no CT and served as controls.

CT treatment. The protocol for IV treatment allowed for increased CT dosage as treatments progressed. The criteria for increasing the CT dose were (a) the physical well-being of the animal and (b) the EIC level measured after exposure. Four dogs were routinely anesthetized with sodium pentobarbital (26 mg/kg body wt) and each infusion consisted of the sequential administration of 1 ml methylene blue (10% wt/vol), 5 ml 0.9% sodium chloride (saline), 300 mg of CT in 200 ml saline delivered slowly over 1 h, 5 ml saline, 1 ml of 10 mg/ml methylene blue intravenously, and, finally, 1 ml i.m. of 200 mg/ml DL- α tocopherol. Samples of serum were taken for analysis before each CT exposure. Treatment was continued at a rate of 3 infusions/wk for 5 wk for two dogs (A and B). Two animals were observed for 6 wk additional, and were anesthetized only for blood sampling. These animals were not intubated during the IV procedure.

Gelatin capsules, containing 300 mg CT, were given to two dogs (C and D) 10 min before morning and evening feedings. During the initial exposure period (1–2 wk), the dogs regurgitated their food at several feedings, but there were no further side effects from the exposure. In one animal the treatment continued for 4 mo (dog C). In dog D, after 4 mo, the dose was increased from 600 mg/d to 1,200 mg/d for 2 mo more. Samples of serum were obtained periodically throughout the treatment.

A local inflammatory reaction with fibrosis of the peripheral

veins made prolonged daily administration by the IV route impossible. Therefore, a third regimen was tried. Initially, 200 mg CT in 200 ml saline was given intravenously to dogs E and F three times per week. Dogs E and F were also treated orally with 400 mg CT on the other 4 d of the week. After 14 d the IV frequency was reduced to twice weekly with an increase in concentration of CT to 400 mg/200 ml. To depress EIC values further, the intravenous CT dose was subsequently increased to 600 and 800 mg/200 ml on days 51 and 94, respectively. The oral dose of 400 mg CT was increased to 600 mg/d after 14 d and to 1,200 mg/d after 90 d. The animals were sacrificed after 128 d.

Finally, two dogs, G and H, were treated only intravenously in a similar fashion to dogs A and B but for only 3 wk. Morphometric analysis was performed on lungs from these dogs but blood for EIC and TIC was not obtained.

Control animals. Two animals (I and J) were anesthetized as for the IV-treated animals and infused with saline, blood samples drawn, and the animals sacrificed after 4 wks. Two additional animals (K and L) had blood samples taken and were housed in the facility for a period of 5 wk.

Bronchial lavage. Dogs A through F were anesthetized and bronchial lavage performed at least twice during each experiment. The lavages consisted of six instillations of 20 ml sterile, pyrogen free, 0.9% NaCl through an Olympus fiberoptic bronchoscope wedged in a single lobe (Olympus Corp. of America, New Hyde Park, N. Y.). Total recovery averaged ~100 ml. The lavage was centrifuged at 200 g for 15 min to remove cells. The supernate was filtered through multiple layers of cheesecloth to remove mucus, lyophilized, resuspended in 5–10 ml saline, dialyzed overnight against 1 liter of saline at 4°C, rehydrated, and suspended with H₂O to a 2-ml final volume. The cellular pellet from the lavage was examined for the distribution of cell types and the concentrated supernate was assayed for both total protein and α -1-PI. EIC was determined in the lavages from an untreated dog and from dog F.

Preparation of antisera. α -1-PI from dog plasma was purified to homogeneity by previously published methods (26). Antiserum was prepared in rabbits with an initial injection of antigen homogenized in Freund's complete adjuvant and with subsequent monthly boosts of antigen plus Freund's incomplete adjuvant. The IgG fraction of the rabbit antiserum was isolated by 33% ammonium sulfate precipitation and ion-exchange chromatography on Whatman DE-52 cellulose (27). It should be noted that human and dog- α -1-PI do not crossreact (26), which makes the use of commercially available antisera impossible.

Rocket immunoelectrophoresis. The concentration of α -1-PI in serum or concentrated bronchial lavage fluid was measured immunologically by rocket immunoelectrophoresis in 1% agarose containing antibody to dog α -1-PI (28). Rocket heights obtained from six different standard concentrations of α -1-PI were fit with the equation for a straight line with a correlation coefficient > 0.98. Experimental values were calculated from the best-fit line.

Chemical assays. Liver function was monitored before and during CT treatment by analysis for cholesterol, glucose, uric acid, creatine phosphokinase, bilirubin, phosphate, calcium, total protein, albumin, alkaline phosphatase, serum glutamic-aspartic transaminase, and lactate dehydrogenase on freshly prepared dog serum by Temple University Hospital's Department of Clinical Chemistry. The total protein content of bronchial lavages was determined by the method of Lowry et al. (29), with bovine serum albumin as a standard.

EIC and TIC were measured as previously reported (11), with 0.25% hemoglobin, 1% agarose plates. The resulting clear area is proportional to the concentration of active protease.

Exogenous protease (porcine pancreatic elastase or bovine trypsin) is incubated in wells in the agarose plates for 20 h at 37°C. Inhibitory capacity is determined from the volume of serum required to completely inhibit a fixed concentration of the control protease. For lavage fluids the inhibitory capacity was the amount (in micrograms) of α -1-PI, determined immunologically, required for 100% inhibition of SAPNA hydrolysis by a fixed amount (1 μ g) of elastase. Each inhibitor capacity determination was based on eight individual lavage concentrations. The SAPNA hydrolysis was followed at 410 nm in a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) maintained at 25°C as previously reported (11).

Histological examination. At the termination of each experiment, the lungs were rapidly excised and fixed in the inflated state in phosphate-buffered 10% formalin under 25 cm H₂O pressure. Routinely, five tissue cores from each lobe were taken. The cores were dehydrated, embedded, and used for the preparation of 10- and 30- μ m-thick sections for histology. The slides were stained by routine hematoxylin and eosin and examined by light microscopy. Randomly selected areas were analyzed for their mean linear intercepts (MLI) by the method of Dunnill (30).

Scanning electron microscopy (SEM). 200- μ m-thick paraffin sections were cut adjacent to sections cut for light microscopy. These were transferred to a 1" $\times$ 3" microscope slide and allowed to dry. These sections were then deparaffinized by immersion of the slide in xylene for 30–60 min. The section was then postfixed in 1% OsO₄ in 0.1 M Sym-collidine buffer for 30 min. They were dehydrated in graded ethanol solutions, and treated with 50% ethanol-50% amyl acetate and then with concentrated amyl acetate. They were then critical-point dried in a Denton DCP-1 critical point drying apparatus (Denton Vacuum Inc., Cherry Hill, N. J.). The sections still attached to the slide were coated with 100–200 Å of gold in a Denton Desk-1 sputterer. SEM was performed with a JSM-50A SEM (JEOL, Peabody, Mass.) at 25 kV. The samples were scanned at low magnification ($\times$ 100).

RESULTS

The health of the dogs during treatment was good, as judged by their ability to eat, drink, and exercise in a normal manner. Gross autopsies of these animals showed only splenic capsular thickening on dogs E and F. Microscopically, liver sections from dogs E and F showed a moderate number of golden-brown pigment-laden Kuepffer cells. Liver function measurements, which included total protein, albumin, calcium, phosphate, lactate dehydrogenase, glucose, uric acid, cholesterol, creatine phosphokinase, alkaline phosphatase, serum glutamic-aspartic transaminase, and bilirubin, did not change from pretreatment values and were within normal ranges for male dogs (31). The combined hematocrits on dogs E and F went from 40.8 ± 1.0 SD to 32.3 ± 2.9 during the treatment periods, which compares with a normal range for beagles of 41.9–50.3 (31). Hemolytic complement activity (CH 50) was 11 and 42% above pretreatment control values in two dogs 5 min after the CT infusion. Two dogs (E and F) had lung function tests performed before treatment with CT and again 2 mo after treatment. Functional residual capacity, vital capacity,

forced vital capacity, maximal expiratory flow at 50 and 25% of the total lung capacity, and steady-state compliance at functional residual capacity were similar at both times in each dog.

The effect of CT on hydrolysis of insoluble elastin was examined by comparing the rates of ³H-elastin digestion by 50 nM dog neutrophil elastase (DNE) in a 1-h period at 25°C (32) in 0.05 M Tris (pH 8.0), 0.15 M NaCl, and 0.1% Triton X-100 at 10 different concentrations of CT, from 0 to 5.0 mg/ml with no statistically significant changes in the rate of digestion. This level was two times higher than the highest concentration used for IV treatment. Therefore, the rate of elastin digestion by DNE was unaffected by direct exposure to CT. It has previously been shown that CT has no effect on the catalytic rate of DNE (11).

Fig. 1 shows the variation in EIC (A) and TIC (B) for the control animals I, J, K, and L. There were no

FIGURE 1 The values for 56 determinations of EIC (○) (A) and 63 determinations of TIC (●) (B) are shown as a function of time for four control animals. The 100% values represent the mean of all the control values for EIC and TIC.

differences between dogs given saline IV (I and J) or those simply maintained in the facility (K and L). The results of Figs. 1 and 2 are expressed as the percentage of EIC or TIC of the mean of all the control values. The experimental 100% values of EIC and TIC are: TIC = 0.664 ± 0.09 and EIC = 0.465 ± 0.057 mg protease inhibited per milliliter of serum.

Fig. 2 shows that the CT treatment, regardless of route

of administration, affected both serum EIC and TIC. In all cases, however, the EIC was decreased to a greater extent than the TIC. The extent of depression of EIC and TIC depended upon the route of CT administration. For example, with dogs given IV treatment (Fig. 2A and B), the EIC was decreased 70% from control levels while the TIC was lowered 30%. With oral treatment (Fig. 2C and D), the depression of EIC

FIGURE 2 The profile of TIC (●) and EIC (○) for representative experimental animals during CT exposure. A and B represent the IV route; C and D the oral route, and E and F the combined IV/oral route of treatment. Details of the experimental protocols are given in Methods. In A and B, the arrow indicates the day treatment was stopped. The experimental 100% values of EIC and TIC are given in the text.

was gradual over a 2-wk period and decreased to an average of 50–60% of the control values. The TIC remained fairly constant at ~80%. Combined oral and IV treatment (Fig. 2E and F) showed a pattern similar to both IV and oral treatments. The TIC remained essentially unaffected, whereas the EIC was lowered appreciably with CT exposure. After termination of treatment (Fig. 2A and B) it took ~14 d for the EIC and TIC to return to 100% of control levels. The levels of α -1-PI in serum did not change significantly ($P > 0.1$) during the treatment (Table I), although functional activity as measured by EIC was decreased in all dogs (Fig. 2).

Bronchial lavages were performed during treatment of six of the dogs and the cell distribution of the lavage given in Fig. 3. The treated animals showed an increase in the mean number of lymphocytes from 11 to as much as 29% of the recovered lavaged cell population. Macrophages decreased in percentage, whereas granulocytes did not change significantly. The elevation in the percentage of lymphocytes in dog B persisted in a lung lavage performed 6 wk after treatment.

The amounts of α -1-proteinase inhibitor determined immunologically per milligram of total lavage protein are given in Table II. In lavages from dog F on days 114 and 128, the amount of elastase inhibited in an in vitro assay per microgram of bronchial lavage α -1-proteinase inhibitor were 0.123 and 0.330 μ g, respectively. Two lavages from a normal dog gave a value of 0.70 ± 0.02 μ g/ μ g α -1-PI in the lavage. When purified dog α -1-PI was used instead of lavage protein, 0.51 μ g elastase was inhibited per microgram dog α -1-PI. The inhibitory capacity of lavage protein was found to deteriorate over a 6-mo period even with storage at -20°C . Lavage protein, by itself, did not hydrolyze the substrate (SAPNA) utilized in these assays.

Histology (light microscopy and SEM). Stratified

FIGURE 3 Cell distribution of bronchial lavage. The hatched areas are counts from control lavages ($n = 13$). The open areas represent lavages from CT exposed animals ($n = 17$). Both the decrease in macrophages and the increase in lymphocytes are significant, with $P < 0.001$.

random samples were taken from the lungs of the CT-treated dogs. The pathologist was given coded slides from the eight treated and four control dogs, which were then grouped according to the presence of focal areas of dilated air spaces and alveolar wall destruction. There was no difficulty in separating the samples into the CT-treated and normal animals in the blind study. Sections of lungs of normal dogs as seen in Figs. 4a and 5a showed well-defined terminal airways, respiratory bronchioles, alveolar ducts, and alveoli. The septa of the ducts were well preserved with no evidence of distortion or effacement. Alveoli were reasonably uniform in size and shape with intact thin walls.

TABLE I
Serum α -1-PI Levels

Dog	α -1-PI	
	Before treatment	After treatment
	$\mu\text{g/ml}$	
A	—	$749.2 \pm 97.0^*$
B	—	660.0 ± 103.0
C	706.7	844.8 ± 140.2
D	813.3	1398.1 ± 260.1
E	779.65 ± 31.3	1009.2 ± 139.0
F	808.2 ± 23.1	888.6 ± 146.1

* Means $\pm$ SD are given for all α -1-PI concentrations after the start of therapy on the same serum samples used for measurements of EIC and TIC in Fig. 1.

TABLE II
Total α -1-PI Concentration of Bronchial Lavage

Dog	Time	α -1-PI
	<i>d</i>	$\mu\text{g/mg Protein}$
A	77	10.11
B	35	5.28
	77	3.44
C	120	4.94
D	180	4.92
E	114	5.46
	126	11.03
F	114	3.94
	128	4.51
Control		Mean $\pm$ SD
		5.96 $\pm$ 2.70
		Mean $\pm$ SD
		5.30 $\pm$ 1.07

FIGURE 4 Light micrographs of paraffin sections ($\times 50$). (a) Lobe from a normal untreated dog; (b) abnormal region from dog B, IV treated; (c) a partially normal region from dog C, oral treatment; (d) an abnormal region from dog C, oral treatment.

Neither the alveolar walls or alveoli were excessively dilated. Representative histological slides from the experimental animals are shown in Figs. 4b–d and 5b–d.

The dogs treated intravenously had more areas of focal abnormalities as seen in the light micrograph in Fig. 4b and the corresponding SEM micrograph in Fig. 5b than the orally treated dogs, although abnormal regions in the orally treated dog seen in Figs. 4d and 5d also exhibited similar dilatation of the alveoli, thickening of the alveolar walls and fenestrations and tears in the alveolar walls. The MLI of randomly selected areas of lung sections were similar for all experimental groups and were significantly different than the MLI of sections from control animals, as indicated in Table III.

None of the control or treated dogs had any evidence of pneumonia or vascular lesions of any kind.

DISCUSSION

In this report a method for lowering the functional proteinase inhibitory capacity in an animal model was established. CT was chosen as the oxidizing agent for the following reasons: The chemistry of CT interaction with thiols is well known, i.e., methionine reacts

FIGURE 5 SEM micrograph of comparative sections to Fig. 4 ($\times 100$).

to form methionine sulfoxide (33). CT affects the EIC in serum in a manner similar to its affect on pure α -1-PI (11, 12). When these data are taken together with

TABLE III
MLI of Randomly Selected Lung Sections

Dog	Treatment	MLI* μm
A	IV	125 $\pm$ 7
B	IV	126 $\pm$ 6
G	IV	120 $\pm$ 23
H	IV	128 $\pm$ 26
C	Oral Capsules	121 $\pm$ 11
D	Oral Capsules	111 $\pm$ 15
E	Oral/IV	114 $\pm$ 17
F	Oral/IV	147 $\pm$ 19
I	Saline IV	92 $\pm$ 16
J	Saline IV	94 $\pm$ 14
K	None	101 $\pm$ 16
L	None	80 $\pm$ 11

* The means of MLI of treated animals (A–F) are significantly greater than the means for control animals (I–L) when compared with Student's *t* test for unpaired samples ($P = 0.0005$). The means of the IV treated group (A, B, G, and H) were also significantly greater than the mean of the control group ($P = 0.004$).

the in vitro studies on human α -1-PI (12, 13, 34–36), it is likely that inactivation in dogs occurred because of the same chemical mechanism involving an essential methionine. Acute studies by Cohen (12) in a monkey had shown that an animal could survive a single IV treatment of CT.

Animals were treated with various combinations of IV and oral doses of CT. Pathologic effects on organs other than the lungs were minimal. Changes in hematocrits were small or nonexistent and autopsies of treated animals showed splenic capsular thickening, pigmentation of Kuepffer cells of the liver, and vascular occlusion at the site of infusion. The treatments also had very little effect on chemical tests of liver function or on serum protein functions. Serum protein functions such as lactate dehydrogenase, creatine phosphokinase, alkaline phosphatase, glutamic-aspartic transaminase, and hemolytic complement were normal or, in the case of complement, slightly elevated. However, the EIC of the serum was markedly reduced to an average of 50–60% from control level, and TIC was lowered 20–30% from control levels. These studies show that the CT treatment had relatively little effect on the chemical and histologic variables outside of the systems of central interest, the EIC and the lung architecture. Further studies in vitro showed that CT has no effect on the hydrolysis of elastin peptide bonds by dog neutrophil elastase.

The in vivo effects on the EIC of serum are similar to that seen when CT is added to isolated α -1-PI or serum in vitro (11, 12, 14). That is, there is a preferential decrease in the EIC compared with the TIC. In these six treated animals the TIC always remained greater than the EIC. α -1-PI measured immunologically during the course of treatment was unchanged, which indicates that the protein was inactivated, but that the inactive α -1-PI was cleared in a normal fashion. The EIC did not return to normal concentrations until 14 d after the termination of treatment. This observation is comparable with previous observations, which suggested that α -1-PI has a turnover rate in man and monkey of about 4 d (12, 36–39).

Changes in the α -1-PI concentration and EIC in bronchial lavage fluids of treated animals closely paralleled those seen in the serum. The α -1-PI concentrations of treated animals were similar to those in control animals and the ability of the lavage of treated dog E to inhibit elastase was lower than predicted for the amount of α -1-PI present. The EIC of lavage from a normal dog was greater than theoretically (26) predicted from the amount of α -1-PI measured, which suggests that other elastase inhibitors were present in the normal lavage fluid (40). Cellular changes that occurred in lung lavages of treated animals included a persistent lymphocytosis and a relative deficiency of alveolar macrophages.

Histologically, lungs from all eight treated animals showed dilatation of air spaces as determined by an increase in the MLI measured in randomly selected sections. The damage was pronounced in focal areas with concomitant alveolar wall destruction. No other lesions were seen in the lungs.

The protease balance hypothesis implies that the levels of both proteolytic enzymes and proteolytic enzyme inhibitors are closely controlled to prevent damage to the lung tissue. The mechanism of lesion production in response to CT cannot be unequivocally connected to the effect that CT has on the EIC. CT probably modifies other body chemicals in addition to α -1-PI. The direct action of CT on elastin or on connective tissues in general is unlikely because elastin exposed to CT is not digested at any faster rate by dog neutrophil elastase in vitro.

Chronic exposure to CT, however, decreased the EIC to a level that functionally resembled the human disease state of deficient α -1-PI. The experimental animals showed dilatation of the terminal airways with patchy destruction of the alveolar walls, as confirmed by MLI measurements, which was indicative of early emphysema. This report represents the first example of a chronic model of α -1-PI deficiency with associated changes in lung architecture. This model opens the possibility of studying modes of therapy and analysis of whether such mildly altered lungs show increased susceptibility to other inflammatory insults such as cigarette smoke or instilled elastase.

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