JOURNAL OF PATHOLOGY, VOL. 178: 215–220 (1996)

THE TRYPSIN-INDUCED LEUCOSTASIS WHICH LEADS TO EMPHYSEMA IN THE HAMSTER IS NOT DUE TO CONTAMINATING ENDOTOXINS

E. REICHART*, P. BOERKMANN*, F. PLENAT†, B. MARIE†, F. ROGERIEUX‡ AND C. LAMBRE‡

Unité de l'INSERM, Physiopathologie Respiratoire, †Laboratoire d'Anatomie Pathologique, Faculté de Médecine, Université H. Poincaré, 54500 Vandoeuvre-lès-Nancy, and ‡Département de Toxicologie et Ecotoxicologie de l'INERIS, 60550 Verneuil-en-Halatte, France

SUMMARY

Intravenous injection of trypsin in the rat induces early lung leucostasis and emphysema of delayed onset. This report confirms that this emphysema is not rat-specific and that the leucostasis is not related to the presence of contaminating endotoxin in the trypsin. In hamsters ($n=37$), leucostasis did not occur when they were injected with heat-treated trypsin, but numerous granulocytes were sequestered in the vessels of hamsters receiving a fresh solution of trypsin. In these hamsters, the number of granulocytes harvested by lavage increased significantly ($1{\cdot}87 \times 10^6$ per ml, $P<0{\cdot}001$) compared with hamsters injected with either heat-denatured trypsin (0·89) or saline (0·86), or compared with controls (0·86). Emphysema was inconstantly observed in hamsters 6 or 12 weeks after injection with trypsin for 1 h. It was frequently (17/20) present and always (20/20) well developed (intercept+180 per cent) in the 2-h perfused hamsters whose lungs were abnormally heterogeneous (index+100 per cent) relative to the seven controls and to the nine saline-injected hamsters. The efficiency of trypsin in triggering emphysema (percentage of hamsters having abnormal values of intercept) was dependent on the time of perfusion. This form of experimental emphysema is thus considered to be due to an endotoxin-independent leucostasis.

KEY WORDS—**emphysema; leucostasis; lung; polymorphonuclear leucocytes; trypsin; hamster**

INTRODUCTION

Local protease–antiprotease imbalance plays a key role in lung degradative processes.[1–3] In order to set up an animal model for emphysema, both intratracheal and intravenous injections of protease have been carried out, using mostly the rat and the hamster. In the case of intratracheal injection, elastase is commonly used, as it may act directly on the lung structure through local protease overloading. In the case of intravenous inoculation, trypsin seems more relevant, as it is supposed to decrease plasma and, as a consequence, pulmonary antiprotease defences. We have shown that a 2-h intravenous injection of trypsin in the rat resulted, 8 weeks later, in the appearance of emphysema.[4–7] Both histologically and morphometrically, the characteristics were similar to emphysema brought about in this species by intratracheal instillation of elastase.[4–7] As the elastase-instilled hamster is the most commonly used animal model of emphysema,[8,9] we decided to test the susceptibility of hamster lung to intravenously injected trypsin. Plasma protease inhibitors differ between mammals, with the rat having a typical pattern of plasma antiprotease.[10–13]

As described in sheep by other authors,[14] we found in rats that 4 h after the end of perfusion, trypsin induces an early circulatory lung disturbance, with granulocyte sequestration within the lung microvessels.[4,15] We thus propose that the release of proteases and/or oxidizing agents by granulocytes (polymorphonuclears, PMN) may be implicated in tissue destruction, since trypsin itself, having no elastolytic activity, cannot directly produce emphysema. In the present study, we looked for leucostasis within the lungs of hamsters receiving trypsin intravenously.

Endotoxins are known inducers of experimental leucostasis[16] and emphysema.[17] As the trypsin that we used was not guaranteed to be free of endotoxins, we verified that lung leucostasis did not occur when the hamsters were injected with trypsin that had been heated previously. This treatment will inactivate trypsin without altering endotoxin activity.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters (*Mesocricetus auratus*), weighing $112{\cdot}6 \pm 18{\cdot}9$ g at the beginning of the experiment, were distributed into three sets according to the intended purposes:

First set=early injuries. Fourteen hamsters were used to assess the early effects of the infusion of trypsin. Six hamsters were injected with 4·5 mg/kg of body weight per h over 2 h and compared with four controls and four saline-treated hamsters. The animals were killed 4 h after the end of the perfusion by an anaesthetic overdose.

Second set=delayed alterations. Twenty hamsters were divided into several groups as described in Table I to study the delayed pulmonary consequences of trypsin when injected venously at the same dose that we previously used in rats ('TIV dose'). They were matched with seven untreated controls and nine saline-treated hamsters.

Addressee for correspondence: E. Reichart, Unité 14 INSERM, CO No. 10, 54511 Vandoeuvre-lès-Nancy Cedex, France.

CCC 0022–3417/96/020215–06

Received 30 January 1995
Accepted 26 June 1995

Table I—Experimental procedures for the study of long-term effects of trypsin infusion

Group identity	No. of hamsters	Trypsin (mg/kg of BW per h)	Duration of infusion (h)	Period of observation (weeks)
Dose	4	4·5	2	12
Half-dose 2 h	4	2·25	2	12
Half-dose 1 h	5	4·5	1	12
Half-time observation	7	4·5	1	6

BW = Body weight.

Third set = test for endotoxin effect. In order to discriminate the early tissue alterations due to trypsin from those of possible contaminating endotoxins, 13 hamsters treated with heat-denatured trypsin were compared with 15 hamsters treated with unheated trypsin. The trypsin action (4·5 mg trypsin/kg body weight per h for 2 h) was demonstrated by referring to seven controls and two saline-perfused hamsters. PMN were counted in lavage fluid from isolated lungs 150 min after the end of the perfusion.

Injection procedures

Injections were carried out under general anaesthesia with intraperitoneal sodium pentobarbital (38 mg/kg of body weight). Isotonic saline (37°C) or bovine pancreatic trypsin (Sigma T 8253 type III, 10 U/mg of protein) dissolved in sterile saline (1 mg/ml) was perfused through a microcatheter into the left jugular vein using a pump. The solutions were renewed after the first hour of perfusion to avoid trypsin autodigestion. The hamsters used for long-term experiments were kept warm until awake; the others were kept warm under anaesthesia until killed. The untreated controls were kept under anaesthesia for the same time as the corresponding treated hamsters.

Trypsin denaturation

The solution of trypsin was inactivated in a water bath (75°C) for 1 h. This treatment does not alter endotoxin activity. This solution was then rapidly brought to 37°C and infused.

Lung lavage

To avoid gravity differences between the different lobes, the excised lungs (third set of hamsters) were placed on a Petri dish. They were then washed with 30 ml of warmed (37°C) phosphate-buffered saline at pH 7·2. Six 5 ml syringes were used successively, each being pushed and pulled five times. A push–pull lasted 30 s. The lavage fluids were pooled and centrifuged at 250 *g* for 15 min at 4°C; the pellets were resuspended in phosphate solution for further counting.

Histology

The techniques for the fixation of lungs differ with the histological purpose:

(i) when pulmonary leucostasis was the main morphological feature to be observed (first set of hamsters), the lungs were not fixed by tracheal instillation, to avoid the fixative fluid displacing the exudate present within the terminal air spaces. Samples of each lung lobe were immersed in Bouin's fluid for 24 h;

(ii) The lungs of the hamsters for observation of delayed alterations were fixed *in situ* under general anaesthesia according to Weibel[18] with 2·5 per cent glutaraldehyde dissolved in 0·1 M, pH 7·2 cacodylate buffer at a constant pressure of 32 cm H_2O. Five-millimetre-thick slices were cut from the diaphragmatic lobes, post-fixed in glutaraldehyde for 24 h, and washed in phosphate buffered saline.

Five-micrometre-thick sections of paraffin-embedded blocks (first and second sets) were dewaxed and brought to water through several washes to toluene and graded ethanols for 24 h. They were stained with haematoxylin and eosin and observed under a light microscope.

Polymorphonuclear evaluation

PMN density within the microcirculatory network as well as in the terminal air spaces was qualitatively assessed in sections of every pulmonary lobe and scored from 0 to 3. Three randomly chosen microscopic fields were studied per lobe and as the PMN density appeared evenly distributed throughout the lung, a mean value was considered representative of the whole lung.

PMN in lung lavages were counted in the phosphate buffer-resuspended pellets by the Thoma method for haemocytometry.

Alveolar morphometry

Alveolar linear intercepts were measured optically with 30 standard lines per section.[19] These measurements were made twice at a 1-month interval by an observer unaware of the histological results. The following were calculated:

(i) the mean linear intercept (MLI) of each animal ('individual intercept');

(ii) the average and range of MLI values within each group; and

(iii) the heterogeneity index for each lung (H% = SD/MLI), which represents the diversity of alveolar size in each lung.

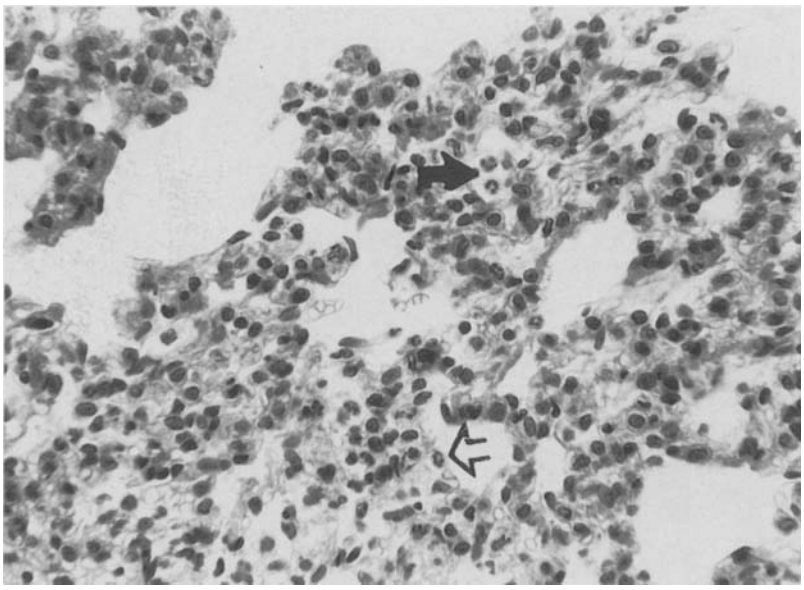

Fig. 1—In this section of a control hamster, occasional granulocytes are focally visible in the lumina of lung capillaries (⬅) and within the terminal air spaces (⇦). Granulocyte density in controls is much lower than in the trypsin-treated hamsters

A statistical test (*t*-test) was used to detect pathological changes. Using the value established in the untreated animals, the upper limit of normal MLI or normal H% values (mean+2SD) was defined. Each hamster characterized by an individual value outside this limit was considered as abnormal.

RESULTS

Anatomical observations and lavage counts

Early injuries—Morphological changes were found in every hamster killed at an early time point after the intravascular injection of trypsin. There was a focal collapse of terminal air spaces, congestion, interstitial and alveolar oedema, and an accumulation of neutrophils in great numbers within the pulmonary capillaries. In every case, a few PMN were observed within the terminal air spaces. These alterations were evenly distributed throughout the lungs. There was no detectable endothelial cell damage. The airways were always spared, without inflammation. No alterations were noticed in the lungs of three sterile saline-treated and three control hamsters. Occasional neutrophils were detected in the capillaries of one saline-treated and one control subject whose lungs were not oedematous.

Delayed alterations—Structural alterations in the lungs of the hamsters killed at the sixth or 12th week after trypsin treatment were variable from case to case. Compared with controls, the terminal air spaces of 17 of the 20 treated hamsters were increased beyond the normal size, with non-uniformity in the pattern of respiratory air space enlargement. In these cases, the tracheobronchial tree remained unaffected and there was no inflammatory exudate. No alterations could be detected in the lungs of the three hamsters which had been trypsin-treated for 1 h only.

Heat treatment of trypsin on PMN counting

PMN were scored in the lung microvessels from 0 to 2 in lung sections of the controls (mean value $m=0{\cdot}7$). PMN scoring was 1–3 when heated trypsin or fresh trypsin was given to hamsters ($m=2{\cdot}5$). PMN in the alveoli were scored from 0 to 2 in controls ($m=1{\cdot}1$). They were more numerous in the alveoli of hamsters treated with either fresh or heated trypsin ($m=2{\cdot}8$).

The PMN counts were significantly ($P<0{\cdot}001$) elevated in the lavage fluids of the hamsters which received unheated trypsin ($1{\cdot}87 \times 10^6$ per ml) compared with controls and saline-treated hamsters ($0{\cdot}86 \times 10^6$ per ml). The counts of PMN in lavage fluids of hamsters treated with heated trypsin ($0{\cdot}89 \times 10^6$ per ml) were comparable to those of saline-treated or control animals.

Mean linear intercept

No differences in MLI values were found between controls and sterile saline-treated hamsters; thus, all these animals were regarded as controls. As shown in Table II, the MLI in the lungs of trypsin-treated hamsters was always markedly greater than in control lungs. Indeed, when trypsin was injected for 2 h, the MLI increased (ΔMLI) by about 180 per cent and the alveoli of treated animals were three times as large as those of controls. The severity of emphysema did not depend on the dose of the enzyme but on the duration of the perfusion, the MLI being smaller in the group of hamsters injected for 1 h only. Finally, ΔMLI did not depend on the time elapsed since the perfusion (6 vs. 12 weeks). It must be noted that the range of MLI values within the group of hamsters which were given half the dose for 1 h was greater in the 12th week (54 per cent) than in the sixth week (22 per cent). The range of MLI values in the groups of the 2 h-infused hamsters was smaller (7·4 per cent at half-dose and 14·5 per cent at full dose).

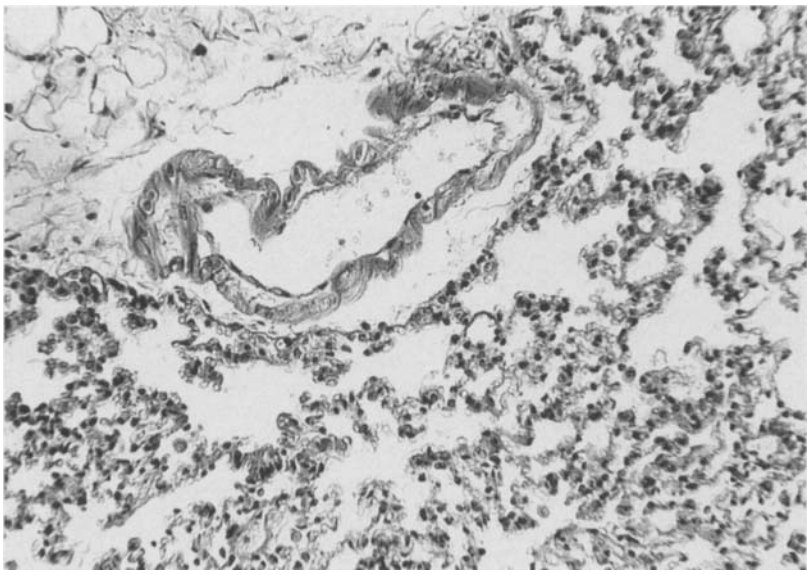

Fig. 2—Hamsters treated with a trypsin solution previously heated at 75°C for 1 h: No granulocytes are visible in the circulatory network

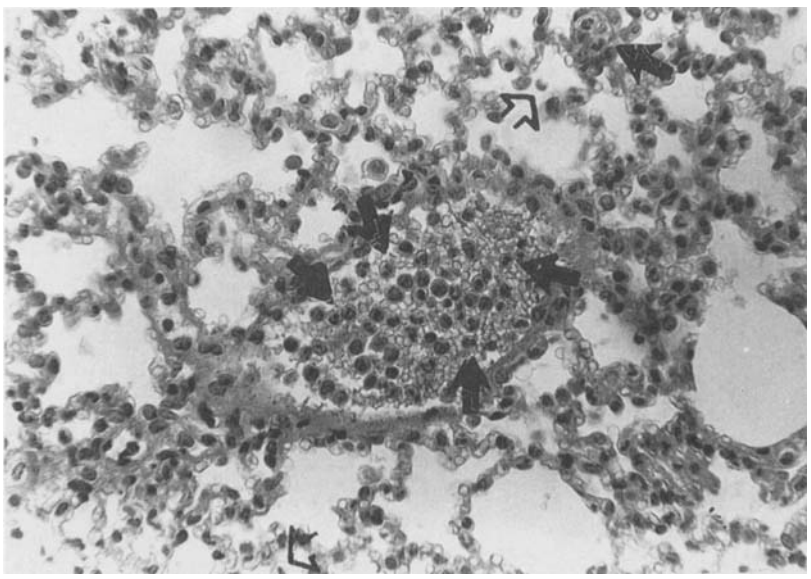

Fig. 3—Hamsters treated with unheated trypsin: granulocyte sequestration within the microcirculatory bed (⬅). Some granulocytes are visible in the alveoli (⇦)

Lung heterogeneity index

Whichever set of animals was studied, the indices of lung heterogeneity of the enzyme-treated hamsters were increased in comparison with controls (Table II). In hamsters given the dose for 2 h, these indices were twice as high as those of controls. This rise was smaller when the duration of perfusion was reduced to 1 h. The length of the period following the infusion appeared to be important, since heterogeneity indices were greater when time was longer.

Statistical establishment of pathology

As described above, the upper limit of normality was established in untreated controls matched with the groups of treated hamsters and killed at the same time: the percentage of abnormal hamsters found in each group is given in Table III. All the 2 h-treated hamsters had abnormal MLI and abnormal H% values. The percentage of hamsters having heterogeneous lungs was greater 12 weeks after a half-dose 1 h than after 6 weeks. Three responses were found in the former group (Table IV): normal lung, abnormal lung heterogeneity with normal-sized alveoli, and abnormal heterogeneity with oversized alveoli.

DISCUSSION

In hamsters injected intravenously with trypsin, the early leucostasis is not due to endotoxins and is indeed related to the action of the trypsin. Endotoxins are not

Table II—Mean linear intercept increase (ΔMLI) and lung heterogeneity increase (ΔH%) expressed as a percentage of the control mean value (the groups of hamsters are classified by increasing ΔMLI)

Group identity	ΔMLI	Range of MLI values (%)	ΔH%
Half-time observation	+72	22·0	+33
Half-dose 1 h	+76	54·0	+60
Half-dose 2 h	+187	7·4	+105
Doses 2 h	+183	14·5	+102

The increase of the MLI (ΔMLI) depends on the duration of the infusion (1 or 2 h) but not on the length of time (6 or 12 weeks). The lungs are highly heterogeneous when trypsin is injected over 2 h. The lung heterogeneity increases with observation time when trypsin is injected over 1 h only.

Table III—Trypsin efficiency: percentage of hamsters having abnormal MLI or H% values in each group

Group identity	MLI>control +2 SD	H%>control +2 SD
6 weeks		
Half-time observation	100%	10%
12 weeks		
Half-dose 1 h	40%	53%
Half-dose 2 h	100%	100%
Dose 2 h	100%	100%

All the 2 h-injected hamsters have very heterogeneous lungs with abnormally oversized alveoli. Curiously, 12 weeks after 1 h-injection only 40 per cent of the hamsters have an abnormal lung (abnormal MLI+abnormal H%) in contrast to their half-time observed counterparts. In this latter group, 90 per cent of the hamsters are characterized by oversized alveoli distributed in lungs that are normally heterogeneous; so it is deduced that the alveolar oversizing of these hamsters affects the whole lung.

implicated in the genesis of leucostasis, since few PMN are found in LBA when trypsin is heat-inactivated; their number is similar to controls or saline-perfused hamsters. Thus, trypsin does not contain heat-stable endotoxins which could induce lung leucostasis.[16,17] Conversely, the role of trypsin in triggering alveolar leucostasis (or 'granulocyte alveolitis') is confirmed, since PMN are significantly increased in LBA when a fresh solution of unheated trypsin is injected. The PMN recovered in lavage fluids are the cells present in airways and alveolar lumina. Alveolar PMN were also observed in histological slides prepared from washed lungs; we suppose that lavage does not recover all the PMN that are firmly adhered to alveolar walls or partially trapped in the septa. Numerous granulocytes are also sequestrated in the hamster's lung vessels; the same observation was reported by other authors in sheep[14] and by us in rat[4,15] after intravenous trypsin injection.

Using fresh trypsin solutions, we have shown here that emphysema is a delayed change in the hamster. Our results differ from previous reports, which showed that emphysema is only initiated when elastases are given via the air–lung interfaces and not by an intravascular route.[20,21] The hamster has been the most commonly used species for the elastase model of emphysema. The lung sensitivity to elastase of the European strain of hamsters used in this study and that of their American counterparts do not appear significantly different.[22] Beforehand, we demonstrated the ability of trypsin to trigger emphysema in the rat: intravenously injected trypsin, a non-elastolytic enzyme, induces emphysema.[4–7] This study confirms that trypsin-triggered emphysema is not rat-specific, despite some authors' claim that macroglobulin-like antiproteases of the rat have distinctive properties.[10,11,13] We have shown here that the hamster lung also reacts to an intravenous injection of trypsin, inducing emphysema, as defined,[23] by enlargement of terminal air spaces with some destruction of their walls. Emphysema is also quantitatively demonstrated by a marked increase in the size of the terminal air spaces. The rise of 180 per cent in alveolar

Table IV— Responses in the groups of hamsters receiving TIV 4·5 mg/kg for 1 h

Typing of MLI	ΔMLI (%)	% of hamsters having an abnormal MLI value	Typing of H%	ΔH%
6 weeks				
Abnormal	+45	100	Normal	0 to +1·7
12 weeks				
Normal	0	47	Normal	0
Normal	0	13	Abnormal	+47 to 55
Abnormal	+162 to 206	40	Abnormal	+65 to 230

Several reactions are found in the 1 h-injected hamsters. Normal values of MLI correspond to hamsters having either highly heterogeneous lungs or not (12 weeks, 13 and 47 per cent respectively of the hamsters in the group). Abnormal MLI values correspond either to abnormal lung heterogeneity (12 weeks) or not (6 weeks), suggesting the development of local disturbances within the lungs with time. The greatest value of MLI are found in the most heterogeneous lungs; so it is deduced that important alterations are irregularly distributed within the lung.

intercept cannot be explained by distension or overinflation alone and alveolar wall rupture must therefore have occurred.

This emphysema is linked to infusion modalities. As found previously in the rat,[7] the intensity of alterations induced in hamster lung does not depend on the dose of trypsin but on the duration of perfusion: the MLI increased markedly in hamsters perfused for 2 h relative to the 1 h group, indicating that trypsin activity must be present for a minimum time in the blood in order to trigger emphysema. The lung response to trypsin given for 1 h was inconstant and varied. Overall, the hamster appears to be less susceptible, but more sensitive to the development of emphysema than the rat.

In conclusion, lung leucostasis and the possible resulting emphysema are attributable to trypsin and not to contaminating endotoxins. Leucostasis in the hamster lung occurs particularly in microvessels and further experiments are needed to study the mechanism(s) by which the injected trypsin facilitates *in vivo* PMN migration from the vessels towards the airways. Proteases could allow PMN to traverse connective tissue barriers by local destruction controlled by antiproteases.[24,25] Emphysema may be due to the release by leucocytes of oxidizing agents and to the secretion of potent proteases. Further investigations will be necessary to determine the sensitivity of the lung to enzymes other than elastase, since the early leucocyte influx into the lung can be associated with a release of various granulocyte proteases.[3]

ACKNOWLEDGEMENT

We are grateful to Cristina Gremillet, Unité 14 de l'INSERM for her skilful technical assistance.

REFERENCES

1. Janoff A, Carp H. Proteases and antiproteases in the lung. In: Newball HH, ed. Immunopharmacology of the Lung. Vol. 19. Lung Biology in Health and Disease. New York: Marcel Dekker, 1993; 173–208.
2. Kimbell P. Proteolytic damage and emphysema pathogenesis. In: Petty TL, ed. Chronic Obstructive Pulmonary Disease. Vol. 28, 2nd edn. Lung Biology in Health and Disease. New York: Marcel Dekker, 1985; 105–127.
3. Tetley TD. Proteinases imbalance: its role in lung disease. *Thorax* 1993; **48:** 560–565.
4. Reichart E, Plenat F, Boerkmann P, Vignaud JM. Emphysème expérimental consécutif à une injection intraveineuse de trypsine. *CR Acad Sci Paris* 1990; **610**(Sér III): 619–624.
5. Reichart E, Plenat F, Boerkmann P, Vignaud JM. Experimental emphysema following one single intravenous injection of trypsin. *Eur Respir J* 1990; **3**(Suppl 10): p 1121.
6. Reichart E, Boerkmann P. Administration endopulmonaire d'élastase pancréatique chez un hamster (*Mesocricetus auratus*) élevé en Europe. *Sci Tech Anim Lab* 1991; **16:** 153–160.
7. Reichart E, Boerkmann P, Plenat F. Parenteral administration of trypsin triggers lung emphysema. *Eur Respir J* 1992; **5:** 810–814.
8. Janoff A. Elastases and emphysema. Current assessment of the protease–antiprotease hypothesis. *Am Rev Respir Dis* 1985; **132:** 417–433.
9. Snider GL, Lucey EC, Stone PJ. Animal models of emphysema. *Am Rev Respir Dis* 1986; **133:** 149–169.
10. Saito A, Sinohara HJ. Rat plasma murinoglobulin: isolation, characterization and comparison with rat alpha-1 and alpha-2 macroglobulins. *J Biochem* 1985; **98:** 501–516.
11. Saito A, Sinohara HJ. Murinoglobine, a novel protease inhibitor from murine plasma. Isolation, characterization and comparison with murine alpha-macroglobulin and human alpha-macroglobulin. *J Biol Chem* 1985; **260:** 775–781.
12. Yamamoto K, Tsujino Y, Saito A, Sinohara H. Concentrations of murinoglobin and alpha-macroglobulin in the mouse serum: variations with age, sex, strain and experimental inflammation. *Biochem Int* 1985; **10:** 463–469.
13. Amemiya S, Yamamoto K, Sinohara H. Purification, characterization and acute phase response of plasma alpha-1 antiproteinase in the hamster, *Mesocricetus auratus*. *Comp Biochem Physiol* 1991; **100B:** 293–296.
14. Tahamont MV, Barie PS, Blumenstock FA, Hussain MH, Malik A. Increased lung permeability after pancreatitis and trypsin infusion. *Am J Pathol* 1982; **109:** 15–26.
15. Reichart E, Boerkmann P, Vignaud JM, Plenat F. Experimental emphysema following one intravenous infusion of trypsin. *Exp Lung Res* 1992; **18:** 45–53.
16. Wittels EH, Coalson JJ, Welch MH, Guenter CA. Pulmonary intravascular leukocyte sequestration. A potential mechanism of lung injury. *Am Rev Respir Dis* 1974; **109:** 502–509.
17. Guenter CA, Coalson JJ, Jacques J. Emphysema associated with intravascular leukocyte sequestration: comparison with papain-induced emphysema. *Am Rev Respir Dis* 1981; **123:** 79–84.
18. Weibel ER. Principles and methods for morphometric study of the lung and other organs. *Lab Invest* 1963; **12:** 131–155.
19. Fawell JK, Newman AS. Automated method of quantitating experimental pulmonary emphysema. *Am Rev Respir Dis* 1972; **105:** 849–851.
20. Schuyler MR, Rynbrandt DJ, Kleinermann J. Physiologic and morphologic observations of the effects of intravenous elastase on the lung. *Am Rev Respir Dis* 1978; **117:** 97–102.
21. IP MPC, Kleinermann J, Sorensen J. The effect of elastase on pulmonary elastin and collagen: comparison of intravenous and intratracheal exposure. *Exp Lung Res* 1980; **1:** 181–189.
22. Reichart E, Boerkmann P, Plenat F. Intraperitoneal injections of trypsin: a trigger of emphysema in rats. *Eur Respir J* 1991; **4**(Suppl 14): p 1152.
23. American Thoracic Society. Chronic bronchitis, asthma and pulmonary emphysema. A statement by the Committee on diagnostic standards for non-tuberculosis respiratory disease. *Am Rev Respir Dis* 1962; **85:** 762–768.
24. Deby-Dupont G, Croisier JL, Camus G, *et al.* Inactivation of alpha-macroglobulin by activated human polymorphonuclear leukocytes. *Mediators Inflammation* 1994; **3:** 117–123.
25. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989; **320:** 365–376.