# Neutrophil lysosomal dysfunctions in mutant C57 Bl/6J mice: interstrain variations in content of lysosomal elastase, cathepsin G and their inhibitors

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In this paper we report the serum antiprotease screening and the biochemical and functional characteristics of neutrophils in a variety of mouse strains with different susceptibilities for developing a protease-mediated injury. C57 Bl/6J mice and their mutants tight-skin and pallid have a lower serum elastase inhibitory capacity (−30, −65 and −70% respectively) than other inbred strains (i.e. NMRI and Balb/c, which both have similar values). We demonstrate that these values are a consequence of a decreased concentration of the $\alpha_1$-protease inhibitor for elastase [PI(E)], which is the major serum inhibitor of elastase and cathepsin G. In addition, neutrophil lysosomal dysfunctions characterized by abnormally high contents of elastase and cathepsin G, or defective lysosomal secretion are observed in tight-skin and pallid mice respectively. Another C57 Bl/6J mutant with lysosomal abnormalities is the beige mouse. Negligible amounts of elastase and cathepsin G, as well as defective neutrophil degranulation, have been described previously in this strain. We found, however, discrete amounts of a latent form of neutrophil elastase that undergoes a spontaneous activation by a protease-dependent mechanism. We also report that neutrophil cathepsin G in this mouse is tightly bound to lysosomal membranes, but is released in near normal quantities during exocytosis. Cytosolic elastase and cathepsin G inhibitors, which were previously reported as being specific for the beige neutrophils, have also been detected in all the examined strains. Neutrophil functions, lysosomal enzyme content and serum antiprotease screening may represent key elements in the protease–antiprotease balance and may explain the different interstrain susceptibility to developing lesions in which an elastolytic activity has been implicated.

## INTRODUCTION

Neutrophils play an important role at the site of acute inflammation by releasing the serine proteinases, elastase and cathepsin G, and reactive-oxygen species with the ability to degrade a number of structural proteins [1,2]. The extracellular matrix is protected from degradation by neutrophil proteinases by a critical balance between these enzymes and their major serum inhibitor, $\alpha_1$-antiprotease [3]. The protease-pathogenesis theory assumes that under certain conditions the neutrophils degranulate, releasing proteases in quantities above the protective threshold of serum antiproteases. This results in destruction of the interstitial matrix. Indeed, it has been demonstrated that changes in certain tissues may result from an imbalance between protease and antiprotease activity [4].

The cumulative data obtained in several experimental models over the past 20 years indicate that elastase (and cathepsin G) may play a pivotal role in certain inflammatory diseases [5,6]. In these conditions, the preventative administration of broad-spectrum inhibitors of serine proteases, active towards both elastase and cathepsin G, prevents or limits tissue damage consequent to proteolytic degradation [7,9]. Actually, elastase and cathepsin G are thought of as two of the main causative factors of tissue damage occurring in mouse models of pulmonary emphysema [10–12], certain immunomediated forms of glomerulonephritis [13–15] and experimental arthritis [16].

It is well-recognized that, in the above-mentioned experimental conditions, a varied susceptibility exists in different strains for developing lesions in response to identical challenges [17]. It has been reported for immune-mediated glomerulonephritis that the variability of the immune response may determine the susceptibility as well as the morphology of the glomerular lesions in genetically different strains of mice [18]. Although the importance of the genes responsible for the immune response cannot be denied, other genetic factors may be of importance in such a condition and in other ones in which lysosomal proteinase(s) may represent the ultimate effector of tissue damage.

In our opinion, strain differences in the amount of neutrophil lysosomal proteinases present, in their releasability and/or in the antiproteinase screen may contribute to the varied susceptibility to induction and/or development of proteolytic lesions.

This hypothesis prompted us to investigate a number of factors influencing the proteases–antiproteases balance in a variety of inbred strains of mice which are currently considered to be either lowly or highly susceptible to the development of proteolytic-mediated injury. In particular C57 Bl/6J mice have been shown to be highly susceptible to developing experimentally induced lesions such as pulmonary emphysema, immune-mediated glomerulonephritis and arthritis [17–20]. On the contrary, other strains of mice (i.e. NMRI and Balb/c) show low susceptibility to the development of these diseases. Some sublines of C57 Bl/6J strain, namely tight-skin (*Tsk*) and pallid (*pa*) mice, spontaneously develop lung emphysema [12,21]. Despite a similar deficit in anti-elastase serum [11,12], the severity and evolution of the emphysema is completely different in these two strains [12,22]. Another C57 Bl/6J mutant, the beige mouse, has been

Abbreviations used: EIC, elastase inhibitory capacity; TIC, trypsin inhibitory capacity; $\alpha_1$-PI(E), $\alpha_1$-protease inhibitor for elastase; SAPNA, succinyltrialanine-*p*-nitroanilide; BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; Met-O-Suc-Ala-Ala-Pro-Val-pNA, methoxysuccinyl-alanyl-alanyl-prolyl-valine-*p*-nitroanilide; BPT, bovine pancreatic trypsin; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol 12-myristate 13-acetate; HCG, human cathepsin G; MLE, mouse leucocyte elastase; PMN, polymorphonuclear neutrophils; CHS, Chediak–Higashi syndrome; $\alpha_1$-PI(T), $\alpha_1$-protease inhibitor for trypsin; HBSS, Hanks' balanced salt solution.

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reported to have a defective degranulation of neutrophil lysosomes and a marked deficiency of neutrophil elastase and cathepsin G activities [23–26]. This strain has been used by many investigators to demonstrate or exclude the role of these neutral proteases in a variety of pathological conditions [19,20,27–29].

In this study, we examined (i) endogenous protease inhibitors, (ii) neutrophil lysosomal enzyme content and (iii) the ability of neutrophils to release their enzymes in the above-mentioned mouse strains.

The data obtained may be useful in elucidating the basis for a different interstrain susceptibility for developing a proteolytic-mediated lesion, and in explaining some apparent discrepancies sometimes observed between the '*in vivo*' and '*in vitro*' responses.

## MATERIALS AND METHODS

### Materials

Materials and their sources were as follows: succinyltrialanine-*p*-nitroanilide (SAPNA), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA), methoxysuccinyl-alanyl-alanyl-prolyl-valine-*p*-nitroanilide (Met-*O*-Suc-Ala-Ala-Pro-Val-pNA), bovine pancreatic trypsin (BPT), cytochalasin b, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), ferricytochrome *c*, β-glucuronidase, peroxidase–anti-peroxidase complex prepared from rabbit serum (Sigma); human cathepsin G (HCG) (from Inalco); 3,3′-diaminobenzidine tetrahydrochloride (from BDH); antipain dihydrochloride, aprotinin, chymostatin, leupeptin and pepstatin (from Boehringer–Mannheim).

### Animals

Male 3- to 6-week-old mice were used. Normal C57 Bl/6J (+/+), and mutant C57 Bl/6J ($bg^+/bg^+$) (beige) were obtained from Nossan Laboratory (Correzzana, Milan, Italy). Heterozygous tight-skin C57 Bl/6J ($Tsk^+/pa^+$) (*Tsk*) and homozygous pallid C57 Bl/6J ($pa^+/pa^+$) (*pa*) mice were from our colony. NMRI and Balb/c were obtained from Charles River (Como, Italy).

### Serum elastase and trypsin inhibitory capacity

Blood samples were drawn from the aorta of animals under light ether anesthesia by means of capillary tubes. Sera were tested for elastase inhibitory capacity (EIC) [30] and trypsin inhibitory capacity (TIC) [31] against homologous mouse leucocyte elastase (MLE) and BPT respectively. MLE was purified as previously described [32] and active-site titrated using *Z*-Ala-Ala-ProazaAla-ONp [33]. It was found to be 96% active. EIC (or TIC) was expressed as milligrams of enzyme inhibited per millilitre of serum. Preliminary experiments, carried out on C57 Bl/6J sera, showed that elastase activity decreases linearly with increasing amounts of serum, in the range from 1 to 20 μl/ml of assay mixture. An amount of 5.8 μl of serum/ml of mixture was used in subsequent experiments. Such an amount of serum gave about 70% inhibition of MLE on SAPNA. The percentage of inhibition was calculated as follows: [(uninhibited rate − inhibited rate)/uninhibited rate] × 100.

### Serum $\alpha_1$-protease inhibitor for elastase [$\alpha_1$-PI(E)] concentrations

Concentrations of serum $\alpha_1$-PI(E) were determined by single radial immunodiffusion [34]. The monospecific antiserum was prepared according to Minnich et al. [35] by two injections of 0.5 mg of purified mouse $\alpha_1$-PI(E) in 0.5 ml of 0.15 M NaCl solution emulsified with complete Freund adjuvant into a male New Zealand White rabbit.

### Induction of peritoneal exudates and purification of polymorphonuclear neutrophils (PMN)

Mouse peritoneal cells were elicited by a single intraperitoneal injection of 1 ml of calcium caseinate (0.4% w/v in a solution of 0.168 M NaCl, pH 7.2). Exudate cells were harvested 3 h after injection by lavaging the peritoneal cavity with 5 ml of ice-cold saline and by centrifugation at 600 *g* for 10 min at 4 °C. Supernatants were used for enzyme determinations. Neutrophils were purified from exudate cells according to Watt et al. [36]. Cell suspensions containing 90–93% of PMN and > 95% viable cells were used for the preparation of cell extracts, the degranulation assay and superoxide production. Exudates containing a large number of contaminating erythrocytes were discarded.

### Superoxide production

Superoxide anion ($O_2^{-\cdot}$) generation was assessed according to the procedure of Babior et al. [37]. Neutrophils were incubated exactly as described for the degranulation assay, with the following modification. Cytochalasin b (5 μg) and ferricytochrome *c* (1.25 mg of type III, horse heart) were added and incubated at 37 °C for 10 min. Addition of fMLP (0.2 μmol/l) to the cell mixture was followed by further incubation for 10 min at 37 °C. The reaction was stopped by placing all samples in an ice bath and centrifugation at 12000 *g* for 5 min. The supernatant was assayed spectrophotometrically at 550 nm. Results from cells pretreated with superoxide dismutase served as controls for ferricytochrome *c* reduction. The final volume of both the experimental mixture and blank was 1 ml. Results were measured as nmol of cytochrome *c* reduced/1 × $10^6$ neutrophils per 40 min.

### Cell extract preparation

Neutrophil lysis was obtained by freezing and thawing four times. Nuclei and cell debris were centrifuged off at 2000 *g* for 10 min. The resulting supernatant was again centrifuged at 40000 *g* for 45 min at 4 °C to separate the granular fraction (pellet) from the post-granular supernatant fraction (cytosol). The granular fraction was resuspended in 1 M NaCl in the presence of 0.05% Triton X-100, and stored overnight at 4 °C for protease extraction. The suspension was centrifuged at 40000 *g* for 45 min, and the supernatant, designated 'lysosomal extracts', was stored at −20 °C and thawed just before use. In some cases, the pellet was subjected to a further extraction for 24 h at 4 °C. In additional experiments, the granular fractions from the various animal strains were subjected to a prolonged extraction for 48 h and processed as reported above. Fractions were assayed for protein concentration according to Hartree [38].

### Enzyme assays

Elastase, cathepsin G, and β-glucuronidase activities were measured in PMN extracts and degranulation assay media. Elastase activity was assayed on the elastase-specific substrate Met-*O*-Suc-Ala-Ala-Pro-Val-pNA according to Nakajima et al. [39]. Cathepsin G activity was determined using Suc-Ala-Ala-Pro-Phe-pNA as reported by Barrett [40]. β-Glucuronidase activity was assayed according to Fishman et al. [41] using phenolphthalein glucuronic acid as substrate.

### Western blotting and gel electrophoresis

Fractions of cellular lysates were subjected to SDS/PAGE according to the method of Laemmli [42]. The electrophoresed proteins were then transferred to nitrocellulose in a Bio-Rad Transblot apparatus as reported by Burnette [43] using 25 mM Tris/192 mM glycine/20% (v/v) methanol, pH 8.3, as blotting buffer. After washing nitrocellulose membranes with 10 mM Tris/HCl/0.9% NaCl/0.1% (v/v) Tween 20, pH 7 (TBS–Tween), endogenous peroxidases were blocked with 3% $H_2O_2$ in distilled water. BSA (3%, w/v) in TBS–Tween was used to block non-specific protein adherence during antibody staining.

Nitrocellulose containing transferred proteins was incubated with the rabbit anti-MLE antibody followed by a treatment with a sheep anti-(rabbit IgG) antibody.

The staining was revealed by adding peroxidase–anti-peroxidase complex prepared from rabbit serum. Detection was accomplished by incubating in diaminobenzidine freshly dissolved in 0.03% $H_2O_2$ in 100 mM Tris/HCl, pH 7.4.

### Immunoelectron-microscopy

The immunogold method (post-embedding technique) was used to localize elastase in neutrophil lysosomal granules from various strains of mice. Rabbit anti-MLE antibodies were prepared as previously described in detail [10]. Peritoneal neutrophils were fixed for 2 h in 4% (v/v) paraformaldehyde/0.1% glutaraldehyde in PBS (pH 7.2), dehydrated in acetone, and embedded in epoxy resin (Araldite) without post-fixation in 1% (w/v) $OsO_4$. Ultrathin sections [about 60 nm (600 A) thick] were picked up on nickel grids and pretreated with 20 mM Tris/HCl (pH 7.2) containing 0.15 M NaCl, 1% BSA, 1% Tween 20 and 20 mM glycine for 10 min. The grids were then floated on a drop of the diluted anti-elastase antibodies (1:400) for 16 h at 4 °C; the grids were thoroughly rinsed for 10 min with a mild spray of Tris buffer and transferred into 25 $\mu$l drops of gold conjugated goat anti-(rabbit IgG) colloidal particles (20 nm) (Bio Cell) solution diluted 1:30 in Tris buffer. The sections were then washed, dried, stained with uranyl acetate–lead citrate, and examined in a Philips 300 electron microscope.

Additional control samples from all the examined strains were processed in similar ways, but the antibodies were substituted with pre-immune serum, rabbit anti-MLE pre-adsorbed with excess MLE or Tris buffer as previously reported [10]. In some experiments, non-immune goat serum or ovalbumin was used instead of BSA.

### Assay for inhibitors and demonstration of enzyme–inhibitor complexes

The cytosols of PMN from various strains were assayed for their inhibitory capacity towards MLE and HCG on Met-*O*-Suc-Ala-Ala-Pro-Val-pNA and Suc-Ala-Ala-Pro-Phe-pNA respectively. MLE (0.12 $\mu$g) was incubated with different amounts of cytosolic preparations in 100 $\mu$l of 0.1 M Tris/HCl, pH 8.2/0.01% Brij at 25 °C for 30 min before the addition of chromogenic substrate. Following the pre-incubation, the complete reaction mixture (1 ml) was assayed for elastase activity according to the method of Nakajima et al. [39].

The assay for inhibitors of HCG utilized 0.2 $\mu$g of pure HCG and 2 mM synthetic substrate. HCG and different amounts of cytosols were pre-incubated at 25 °C for 30 min in 0.10 M Hepes buffer, pH 7.5. After the pre-incubation, the complete reaction mixture (2 ml) was incubated at 50 °C and assayed for enzyme activity according to the method of Barrett [40].

The inhibitory capacity towards MLE (or HCG) was expressed as micrograms of enzyme inhibited/mg of cytosolic protein under the described conditions.

The inhibition assays described above were carried out on cytosolic preparations of PMN from C57 Bl/6J and beige mice suitably pooled to obtain prefixed amounts of contaminating lysosomal enzymes (assayed as $\beta$-glucuronidase). The latter samples were prepared immediately before the assays by mixing different cytosolic preparations showing various degrees of lysosomal contamination (10–44 m-units of $\beta$-glucuronidase/mg of cytosolic protein).

In additional experiments, the cytosols from beige PMN were deliberately contaminated, just before the assays, by adding increasing amounts of lysosomal extracts from control C57 Bl/6J mice to obtain a lysosomal contamination ranging from 10 to 60 m-units of $\beta$-glucuronidase/mg of cytosolic protein.

The direct demonstration of enzyme–inhibitor complexes in C57 Bl/6J cytosols has been ascertained by Western-blot analysis of samples of purified MLE incubated at 25 °C for 30 min with cytosolic preparations from C57 Bl/6J mice containing lysosomal contamination of $<10$ m-units of $\beta$-glucuronidase/mg of cytosolic protein.

### Degranulation assay

Neutrophils were tested for their ability to release enzymes in response to cytochalasin b/fMLP according to the procedure described by Stahl et al. [44]. Briefly, 500 $\mu$l of Hanks balanced salt solution (HBSS), without calcium or magnesium, was added to microcentrifuge tubes containing 200 $\mu$l of a suspension of $1 \times 10^7$ neutrophils/ml. After a 10 min incubation, both $CaCl_2$ (2 $\mu$mol/l) and cytochalasin b (5 $\mu$g) were added in a total volume of 200 $\mu$l. Exactly 10 min later, 100 $\mu$l of the agonist fMLP (0.2 $\mu$mol/l), or an equivalent volume of HBSS, was added to produce a final volume of 1 ml. After an additional incubation of 10 min, the reaction mixture was immediately placed in an ice bath for 5 min, centrifuged at 12000 *g* for 5 min, and the resultant supernatant assayed for enzyme activities.

## RESULTS

### EIC and TIC analysis

The results of serum EIC against MLE from the different strains of mice are shown in Table 1. The sera of NMRI, Balb/c and beige mice show the highest values. In these strains 1 ml of serum inhibited approx. 0.70–0.75 mg of homologous elastase. The sera of the C57 Bl/6J mice and the derived sublines *Tsk* and *pa* mice

**Table 1 Serum $\alpha_1$-PI(E) concentration, EIC and TIC in various strains of mice**

The results are given as means ± S.D. obtained from 10 animal sera. The EIC and TIC values are expressed as milligrams of enzyme inhibited by 1 ml of serum. Values with the same superscript are not significantly different ($P < 0.05$). Data in the square also appear in [12].

| Strain | $\alpha_1$-PI(E) concn. (mg/ml) | EIC | TIC |
|---|---|---|---|
| NMRI | 5.7 ± 0.5* | 0.73 ± 0.06* | 1.59 ± 0.21* |
| Balb/c | 5.9 ± 0.5* | 0.75 ± 0.05* | 1.55 ± 0.25* |
| C57 Bl/6J | 4.4 ± 0.3† | 0.56 ± 0.03† | 1.54 ± 0.17* |
| *pa* | 2.7 ± 0.4‡ | 0.24 ± 0.03‡ | 1.52 ± 0.15* |
| *Tsk* | 2.7 ± 0.3‡ | 0.25 ± 0.02‡ | 1.45 ± 0.25* |
| Beige | 5.6 ± 0.4* | 0.70 ± 0.05* | 1.50 ± 0.21* |

**Table 2 Activities of elastase, cathepsin G and β-glucuronidase in neutrophil lysosomal extracts of various strains of mice**

Enzymes were assayed with their specific substrates as described in the Materials and methods section. One unit of enzyme activity corresponds to the release of 1 μmol of product per minute. Specific activities are expressed as means ± S.D. of four preparations of lysosomal extracts from six animals each. Enzyme values obtained after a further salt extraction (24 h, 4 °C) of residual lysosomal pellets of beige mouse are reported in parentheses. *$P < 0.05$; n.d., not detectable.

| | Enzyme activity (units/mg of lysosomal proteins) | | |
|---|---|---|---|
| Strain | Elastase | Cathepsin G | β-Glucuronidase |
| NMRI | 21.4 ± 1.4 | 26.6 ± 1.6 | 82.3 ± 9.3 |
| Balb/c | 20.6 ± 1.8 | 26.7 ± 1.5 | 80.5 ± 7.7 |
| C57 Bl/6J | 19.7 ± 1.1 | 25.9 ± 1.4 | 73.6 ± 8.5 |
| *pa* | 21.5 ± 1.6 | 25.1 ± 1.6 | 76.2 ± 9.8 |
| *Tsk* | 40.7 ± 1.8* | 54.3 ± 3.2* | 81.9 ± 9.8 |
| Beige | 2.0 ± 0.6* (n.d.) | 1.9 ± 1.2* (25.8 ± 1.7) | 77.3 ± 8.7 (n.d.) |

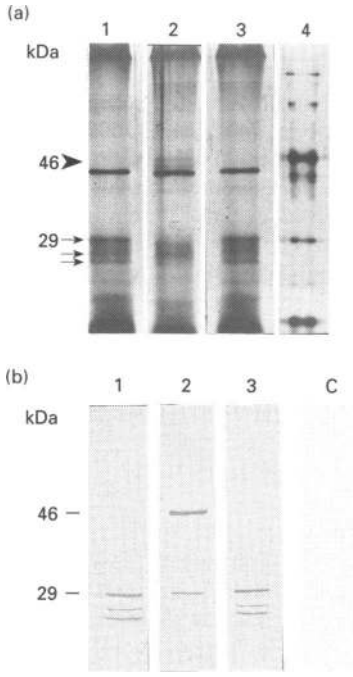


**Figure 1 (a) SDS/PAGE of lysosomal extracts from C57 Bl/6J (lane 1) and beige mice (lane 2) and (b) nitrocellulose sheet after immunostaining with rabbit anti-MLE IgG**

(**a**) In lane 3, beige lysosomal extracts after overnight incubation at 37 °C. An aliquot (2.5 μg) of each preparation was applied. In lane 4, there is a reference protein mixture including (top to bottom) phosphorylase B (92.5 kDa), BSA (66 kDa), chicken actin (45.5 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (21.5 kDa). Arrows indicate elastase isoforms, arrowhead points to the 46 kDa band present in lane 2. The slab gel (12% acrylamide) was silver stained. (**b**) Lanes 1–3 correspond to lanes 1–3 of (**a**). In lane C, there is a blot of lysosomal extracts (C57 Bl/6J) after immunostaining with control rabbit IgG.

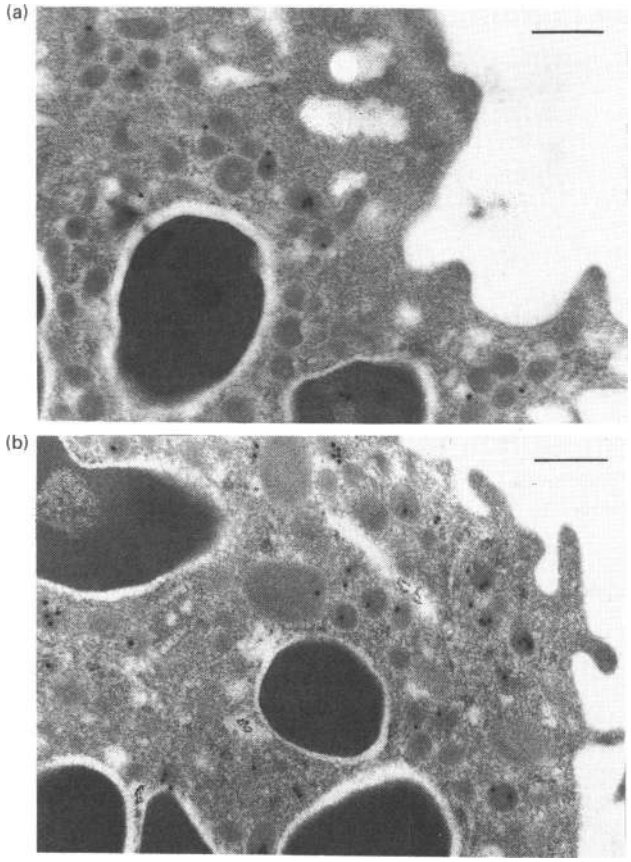


**Figure 2 Electron micrographs of neutrophils from a C57 Bl/6J mouse (a) and a beige mouse (b) after immunological staining with rabbit anti-MLE IgG**

Immunogold particles are present in lysosomes of the C57 Bl/6J mouse as well as in normal-sized and abnormal giant lysosomes of the beige mouse (Bars, 0.3 μm).

exhibit significantly lower values. The *Tsk* and *pa* mice share the lowest values of all (about 29–34% in respect to Balb/c, and about 40–50% in respect to C57 Bl/6J mice). In all strains of mice serum EIC levels did not change significantly during the course of their life (results not shown).

With regard to serum TIC, similar values have been obtained in all the different strains (Table 1). These values range from 1.45 to 1.59 mg of trypsin inhibited per ml of serum. The presence of two distinct inhibitors active towards elastase [$\alpha_1$-PI(E)] and trypsin [$\alpha_1$-PI(T)], explains why TIC levels do not correlate with EIC values [45].

### Serum $\alpha_1$-PI(E) concentrations

Serum $\alpha_1$-PI(E) concentration was determined in the different strains of mice by radial immunodiffusion. The values, expressed in mg/ml (mean ± S.D.), are shown in Table 1. Individual levels of serum $\alpha_1$-PI(E) and the mean levels for each animal group correlate with their respective serum EIC values ($r = 0.98$; $P < 0.01$).

### Superoxide production

Superoxide production levels in PMN from various strains of mice were measured in response to fMLP stimulation. No difference in this parameter was found among the various strains of mice. These values range from 11.2 ± 2.0 to 12.1 ± 2.3 nmol of cytochrome *c* reduced/$10^6$ PMN per 40 min.

### Elastase and cathepsin G activities in lysosomal neutrophil extracts

The serine proteinases elastase and cathepsin G were assayed in the lysosomal preparations, and extracted for 16 h at 4 °C on the specific substrates Me*O*-Suc-Ala-Ala-Pro-Val-pNA and Suc-Ala-Ala-Pro-Phe-pNA respectively. The values obtained are shown in Table 2.

Similar levels of elastase specific activity were found in neutrophils from NMRI, Balb/c, C57 Bl/6J and *pa* mice. On the contrary, this activity was markedly reduced in lysosomal neutrophils from beige mice and significantly increased in lysosomal extracts from *Tsk* mice.

NMRI, Balb/c, C57/6J and *pa* mice have the same level of activity of cathepsin G. Cathepsin G specific activity in *Tsk* mice is about twice that of C57 Bl/6J. In the beige extracts this activity is about 13-fold lower than that of C57 Bl/6J neutrophils.

No substantial differences are observed among the various strains in the specific activity of the lysosomal enzyme β-glucuronidase.

The residual lysosomal pellets from the first extraction were subjected to a further salt extraction for 24 h at 4 °C. This additional extraction did not result in any further recovery of elastase, cathepsin G or β-glucuronidase activities in all the examined strains, apart from the beige mouse (Table 2, values in parentheses). Surprisingly, in this animal, an almost normal amount of cathepsin G activity was found in the absence of any detectable amounts of elastase and β-glucuronidase. Given the above, the lysosomal preparations from various animal strains were subjected to a prolonged salt extraction for 48 h at 4 °C. This procedure resulted in no changes of enzyme recovery in all strains of mice except the beige mouse. In this animal, the levels of cathepsin G obtained after this prolonged lysosomal extraction were similar to those detected in other strains after a shorter (16 h) extraction time. With regard to elastase, a progressive recovery of this enzyme activity (up to 7.80 units/mg of lysosomal proteins) was observed by incubating at 37 °C beige lysosomal preparations obtained after a prolonged salt extraction (48 h). This recovery was not observed in beige lysosomal preparations (lacking cathepsin G) extracted for 16 h at 4 °C. However, in this latter case, a partial recovery of elastase activity (up to 6.7 units/mg of lysosomal proteins) was observed by incubating these extracts with 2 μM cathepsin G for 20 min at 37 °C before assaying.

### Electrophoretic studies

Lysosomal extracts obtained from various animal strains after 48 h salt extraction were subjected to SDS/PAGE analysis. Figure 1(a) shows the electrophoretic profiles of lysosomal extracts from C57 Bl/6J (lane 1) and beige (lane 2) mice. As can be seen, differences between control and beige mice are evident in the protein pattern over the range 45–70 kDa, and 24–31 kDa (where mouse elastase isoforms are located).

Lysosomal proteins separated by SDS/PAGE were subjected to immunoblotting to localize MLE (Figure 1b). The immunoreaction with the antibody raised to purified MLE showed a marked band (approx. 29 kDa) and two minor ones (approx. 27 and 26 kDa) in control mouse lysosomal extracts (lane 1). These bands correspond to the previously described elastase isoforms [32]. On the other hand, in extracts from beige mice only the 29 kDa band is weakly visible after immunoreaction, whereas a more evident additional band of approx. 46 kDa is present (lane 2). Figures 1(a) and 1(b, lane 3) showed the electrophoretic pattern of beige extracts after overnight incubation at 37 °C. In this case the band at 46 kDa disappears while the bands corresponding to elastase isoforms become evident.

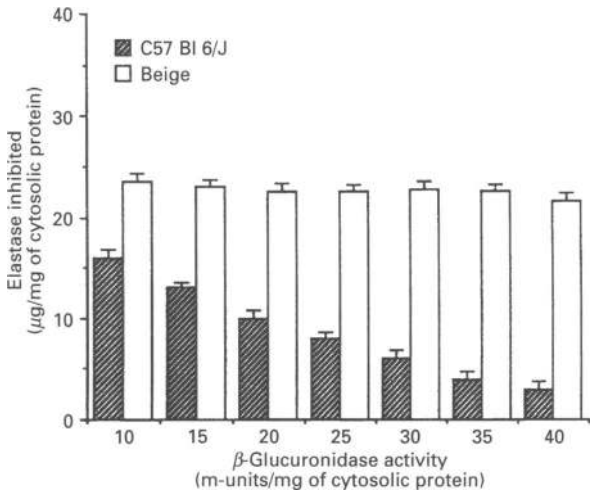


**Figure 3 Cytosolic inhibitory activity against mouse leucocyte elastase in C57 Bl/6J and beige mice**

Results are given as mean ± S.D. obtained from cytosolic preparations from six animals each. The values are expressed as micrograms of elastase inhibited by 1 mg of cytosolic protein. β-Glucuronidase activity is reported as m-units/mg of cytosolic protein. One unit of enzyme activity corresponds to release of 1 μmol of product per minute.

### Elastase immunolocalization

MLE has been localized in peritoneal PMN from various strains of mice using electron microscopy and an immunogold-labelling technique with rabbit anti-MLE IgG. As can be sen in Figure 2(a), elastase-labelled gold particles were localized in azurophil granules of PMN of normal inbred strains. The presence of an elastase-positive reaction was also seen in normal-sized and abnormal giant lysosomes of neutrophils from the beige mouse (Figure 2b). No apparent difference in lysosome gold-particle density was observed between beige mice and other strains.

A negative reaction was observed under the control conditions reported in the Materials and methods section.

### Inhibitory assays on cytosolic PMN fractions

Various preparations of cytosolic PMN fractions from the different strains of mice were analysed for their ability to inhibit MLE or HCG activities. In order to obtain cytosolic samples containing prefixed amounts of contaminating lysosomal enzymes, different cytosolic preparations having various degrees of lysosomal contamination were pooled just before the inhibitory assays.

In the different experiments we carried out, a consistent cytosolic inhibitory activity against MLE (or HCG) was detected only in preparations with very low lysosomal contamination (< 10 m-units of β-glucuronidase/mg of cytosolic protein). This feature has not been observed in preparations from beige mice, in which inhibitory activity towards MLE (or HCG) was always detectable even in the presence of higher amounts of β-glucuronidase activity (up to 40 m-units β-glucuronidase) (Figure 3). In the other strains, the degree of inhibitory activity towards MLE (and HCG) was inversely correlated ($r = -0.90$) with the amount of β-glucuronidase activity present in the different preparations, with a contamination ranging between 10 and

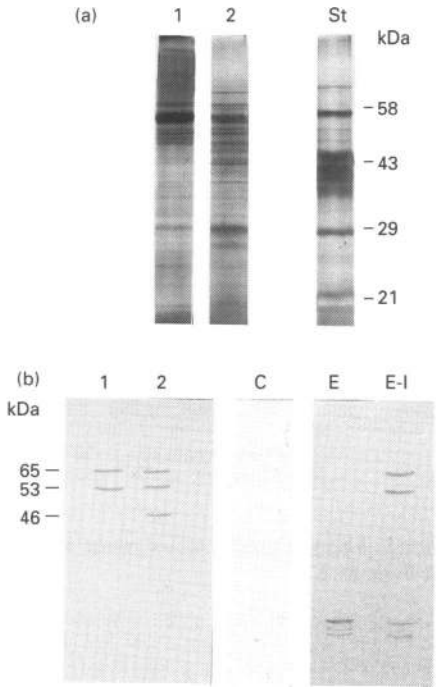


**Figure 4 (a) SDS/PAGE of cytosolic preparations from C57 Bl/6J (lane 1) and beige (lane 2) mice and (b) Western blotting of gels after immunostaining with rabbit anti-MLE IgG**

(**a**) An aliquot (3.5 $\mu$g) of each preparation was applied. St is a reference protein mixture. Arrows indicate 65 and 53 kDa regions; arrowhead the 46 kDa band. The slab gel (12% acrylarride) was silver stained. (**b**) Lanes 1 and 2 correspond to lanes 1 and 2 of (**a**). In lane C, there is a blot of beige cytosolic preparation after immunostaining with a control rabbit IgG. Lanes E and E–I are blots of purified MLE (0.025 $\mu$g) before (E) and after (E–I) incubation for 30 min at 25 °C with cytosolic preparation (4 $\mu$g of protein).

40 m-units of enzyme/mg of cytosolic protein. This means that progressive saturation of cytosolic inhibitors by elastase and cathepsin G occurred with increasing degree of lysosomal contamination.

This feature was also observed in beige mouse cytosols in which a progressive saturation of inhibitors has been attained by adding increasing amounts of lysosomal extracts from control mice. A complete depletion of EIC in beige cytosols has been observed when control lysosomal extracts were added to obtain contamination levels > 45 m-units of $\beta$-glucuronidase (results not shown).

The role played by elastase (and cathepsin G) in the saturation of endogenous inhibitors in the various cytosolic preparations emerged from additional studies carried out by electrophoretic and immunoblotting techniques employing rabbit anti-MLE IgG.

In Figures 4(a) and 4(b), SDS/polyacrylamide gel and Western-blot analyses of C57 Bl/6J and beige cytosolic preparations are presented. As can be seen in Figure 4(b), the presence of two inhibitor–MLE complexes at approx. 65 and 53 kDa is found after immunostaining in C57 Bl/6J preparations (lane 1) at relatively low levels of lysosomal contamination. Similar bands, and an additional band at approx. 46 kDa, can be detected in beige preparations with discrete amounts of lysosomal contamination (25–30 m-units of $\beta$-glucuronidase/mg) only after an overnight incubation at 37 °C (Figure 4b, lane 2). The same samples when incubated at 37 °C for a short period of time (30 min) show only an immunoreactive band at approx. 46 kDa (results not shown). This latter band corresponds to the immunoreactive protein found in the beige lysosomal extracts (Figure 1b, lane 2). The observed results are compatible with the presence in the cytosol of two endogenous inhibitors with molecular masses of approx. 36 and 24 kDa respectively [46]. When complete saturation of inhibitors occurred (i.e. fractions with a lysosomal contamination of > 40 m-units of $\beta$-glucuronidase/mg), three additional immunoreactive bands were appreciable in cytosolic preparations of normal inbred mouse strains (results not shown). These bands have the same molecular masses as the MLE isoforms found in lysosomal extracts. The presence of these bands is associated with an enzyme activity detectable on synthetic substrate for elastase. These bands were never present in cytosolic preparations from beige mice up to a lysosomal contamination of 60 m-units of $\beta$-glucuronidase/mg and even after a 37 °C incubation overnight. This means that a progressive binding of inhibitors to elastase occurs when the enzyme is gradually activated. An increase of the two inhibitor–MLE complexes of molecular mass approx. 65 and 53 kDa is appreciable in these preparations after immunostaining, and supports this conclusion.

The presence of inhibitors in C57 Bl/6J cytosols has been ascertained also by direct demonstration of the formation of enzyme–inhibitor complexes after addition of pure MLE (Figure 4b, lanes E and E–I). This was obtained by incubating (30 min at 37 °C) MLE with cytosolic preparations containing very low lysosomal contamination ( < 10 m-units of $\beta$-glucuronidase).

## Lysosomal enzyme release

Neutrophils from the different strains of mice were tested for their ability to release lysosomal enzymes after stimulation with fMLP/cytochalasin b. The data obtained are presented in Table 3. The levels of $\beta$-glucuronidase in the incubation medium have been taken as an estimate of the extent of degranulation, as similar levels of this enzyme have been found in lysosomes from most strains. Given this, no difference in the degranulation extent has been found among mice of all strains, except the *pa* mice. In this strain, only moderate amounts of $\beta$-glucuronidase, elastase and cathepsin G have been detected in the degranulation assay medium after stimulation with cytochalasin b/fMLP (Table 3) or PMA (results not shown). This suggests that a defective degranulation process is present in neutrophils of *pa* mice.

No difference in the various enzyme activities has been found among NMRI, Balb/c and C57 Bl/6J mice. The highest enzyme activities for elastase and cathepsin G have been found in the incubation medium of *Tsk* neutrophils in the presence of normal levels of $\beta$-glucuronidase. This feature may be accounted for by the high levels of elastase and cathepsin G detected in *Tsk* neutrophil lysosomes (Table 2).

On the other hand, very low levels of elastase activity (about 11% of those in C57 Bl/6J mice) have been found in neutrophils from the beige mouse in the presence of normal levels of $\beta$-glucuronidase and cathepsin G activities in the incubation medium. However, a partial recovery of elastase activity (about 3-fold in respect to initial values) was observed by incubating the medium from beige mouse neutrophils for 16 h at 37 °C. This recovery was more quickly found when degranulation media of beige mouse were pre-incubated with 2 $\mu$M human cathepsin G at 37 °C before the assay was performed (results not shown).

Neither pre-incubation with human cathepsin G, nor overnight incubation at 37 °C, produce a relative increase of elastase activity in incubation media of the other strains of mice.

**Table 3 Enzyme activities in incubation media from PMN stimulated with fMLP**

Enzymes were assayed with their specific substrates as described in the Materials and methods section. One unit of enzyme activity corresponds to the release of 1 $\mu$mol of product per minute. Specific activities are expressed as means ± S.D. of three different cellular suspensions from three animals each. *$P < 0.05$.

| | $10^{-2}$ × Enzyme activity (units/2 × $10^6$ cells) | | |
|---|---|---|---|
| Strain | $\beta$-Glucuronidase | Elastase | Cathepsin G |
| NMRI | 18.5 ± 1.1 | 4.81 ± 0.28 | 5.90 ± 0.34 |
| Balb/c | 19.1 ± 1.3 | 4.62 ± 0.42 | 6.00 ± 0.31 |
| C57 Bl/6J | 18.8 ± 1.0 | 4.40 ± 0.26 | 5.84 ± 0.28 |
| *pa* | 9.2 ± 0.9* | 2.30 ± 0.17* | 2.72 ± 0.21* |
| *Tsk* | 19.4 ± 1.5 | 9.37 ± 0.42* | 12.38 ± 1.21* |
| Beige | 18.9 ± 1.3 | 0.50 ± 0.16* | 5.84 ± 0.46 |

## DISCUSSION

In the present paper we report the results of a study on the serum antiprotease screening, as well as on the biochemical and functional characteristics, of PMN from a number of inbred strains of mice.

Some general conclusions can be drawn from the data reported in this paper: (a) in all the examined strains serum EIC levels assayed towards homologous leucocyte elastase correlate well with individual concentrations of immunoreactive serum $\alpha_1$-PI(E). This means that the decrease in EIC observed in C57 Bl/6J mice and in some of their mutants (*pa* and *Tsk*) cannot be ascribed to a functional deficiency of their $\alpha_1$-PI(E), but rather is a consequence of a decreased concentration of this serum protein. (b) Mutant strains derived from C57 Bl/6J mice are associated with neutrophil lysosomal dysfunctions. These include abnormal contents of enzymes (*Tsk*, beige) or defective lysosomal secretion (*pa*). Depressed lysosomal enzyme secretions and abnormal lysosomal enzyme concentrations have been reported previously as tissue-specific alterations for some organs and cells in certain mouse pigment mutants [47]. The neutrophil lysosomal abnormalities reported here could account for the interstrain susceptibility for development of proteolytic lesions or enhanced sensitivity to bacterial infections. (c) Intracellular proteinase inhibitors active towards elastase and cathepsin G have been detected in mature neutrophils from all the examined strains. They have been previously reported to be a peculiar feature of beige neutrophils [46]. The presence of cytosolic inhibitors in mouse PMN is not surprising, since similar inhibitors have been described in neutrophils from a variety of mammalian species [48–50]. In our opinion, the failure to demonstrate the presence of these inhibitors in the mouse [46] could be ascribed to methodological problems in obtaining pure cytosolic preparations.

Changes in protease–antiprotease balance may play an important role in the development of spontaneous or induced proteolytic lesions in the C57 Bl/6J strain and its mutants (*Tsk*, *pa* and beige mice). Based on the data reported in this paper, it is conceivable that neutrophil functions, lysosomal enzyme contents and serum antiproteinase content in these strains of mice represent key elements in their protease–antiprotease balance.

The low serum antielastase content in C57 Bl/6J mice (about 70% in respect to other inbred strains) may offer an explanation for the high susceptibility of this strain to develop a variety of lesions in which enhanced elastolytic activities have been implicated (i.e. pulmonary emphysema, immune-mediated glomerulonephritis, arthritis, etc.) [17–20]. In this context, it is not surprising that two different sublines of the C57 Bl/6J strain, namely *Tsk* and *pa* mice, with a still lower serum antielastase content, develop spontaneous lung emphysema [11,12,21].

It has been reported recently that the early development of severe emphysema in *Tsk* mice is accompanied by a marked lung elastinolysis coupled with increased alveolar elastolytic burden [10,51] and decreased EIC levels [11]. In the present paper we report evidence that *Tsk* mice have abnormally high amounts of neutrophil elastase and cathepsin G as well as low levels of serum $\alpha_1$-PI(E). These factors may be, at least in part, responsible for the rapid and dramatic development of the emphysematous lesions in *Tsk* mice [22]. Cathepsin G is generally thought to participate in the degradation of lung connective-tissue components, acting by itself [52] or in concert with leucocyte elastase [53,54]. In addition, a counterpart of human $\alpha_1$-antichymotrypsin (the major serum inhibitor of cathepsin G) is absent in mouse serum, where $\alpha_1$-PI(E) acts as an inhibitor of both elastase and cathepsin G [45].

The second animal model of spontaneous emphysema, the *pa* mouse, is characterized by a marked deficiency of serum EIC and a mild, slowly developing pulmonary lesion which occurs late in life [55]. Recently, we suggested that the low levels of serum $\alpha_1$-PI(E) in *pa* mouse may be a consequence of a defect of protein secretion by hepatocytes [12]. The results reported here demonstrate for the *pa* mouse a neutrophil lysosomal dysfunction characterized by defective degranulation ( < 50% in respect to other strains). Thus, the protease–antiprotease imbalance, which has been suggested as the major cause for the development of the pulmonary lesions in these mice, may be partially reduced by the defective secretion of neutrophilic lysosomes. This is probably why emphysema develops late in life and hardly progresses with time [12].

The third C57 Bl/6J mutant we studied is the beige mouse. This strain has been proposed as the mouse counterpart of human Chediak–Higashi syndrome (CHS) [56]. The major clinical symptoms of this syndrome, including increased susceptibility to infections, diluted pigmentation and giant lysosomes in many tissues, are also present in the beige mouse mutant [23]. Two other prominent dysfunctions of CHS patients and beige mice, which probably influence bacterial killing, are a low neutral protease activity and abnormal membrane fluidity of PMN lysosomes [24,25]. The increased susceptibility to infections in beige mice has been ascribed recently to a marked deficiency of neutrophil elastase and cathepsin G activities, and to a defective degranulation of neutrophil granules [23–26]. This latter condition, which characterizes human CHS, has not been confirmed by our data in the beige mouse.

With regard to the elastase and cathepsin G activities of beige mice, two different mechanisms have been put forward to explain the lowered enzyme activities detected in mature neutrophils of this strain. The first proposed mechanism implies an absence of enzymes in lysosomes (defect in enzyme synthesis) [24–27]. The second one associates the enzyme deficiency state with the presence of specific cytosolic inhibitors, which are supposed to be absent in mature neutrophils from other strains of mice [46].

The results presented here demonstrate that elastase and cathepsin G activities can be detected in mature PMN of beige mice, and that cytosolic inhibitors active towards elastase and cathepsin G, can be demonstrated in all of the examined strains. Cathepsin G seems to be tightly bound to lysosomal membranes of beige mice, so that the current methodology used for the extraction of neutrophil lysosomal enzymes is ineffective in this strain.

Changes of lysosomal membranes leading to abnormal membrane fluidity and giant granule formation have been reported in beige mice [25]. Although these changes may be responsible for the difficult cathepsin G extraction from lysosomes with the currently used methodologies, they do not seem to affect the enzyme secretion, at least under conditions *in vitro*. In fact, levels of cathepsin G activity similar to those reported for normal inbred strains were detected in degranulation media from fMLP-stimulated PMN. It is likely that the perturbations (i.e. focal destabilization of membrane bilayers, change in the net charges, etc.) after contact of the secretory vesicles and plasma membranes of cells undergoing exocytosis may be responsible for the release of this enzyme so tightly associated with lysosomal membranes.

With regard to neutrophil elastase, the results obtained are indicative of the presence in beige lysosomes of an activatable latent 46 kDa form of elastase that may undergo spontaneous activation by a proteolytic-mediated mechanism. The failure to obtain a complete recovery of elastase activity to levels seen in control mice may be accounted for, under our experimental conditions (37 °C overnight), by a progressive autolysis of the activated enzyme. Although the intracellular processes resulting in activation of the lysosomal proenzymes are not yet elucidated [57,58], it has been reported recently that activation of granule serine proteases results from the removal of terminal extensions by a proteolysis-mediated mechanism [57–60]. A similar mechanism may be involved in the activation of the beige latent form of elastase by human cathepsin G. Such an activation suggests a role for cathepsin G as an elastase activator in mouse lysosomes. However, further studies are needed before any conclusion can be reached as no information is available at the present time on the catalytic properties of the mouse counterpart of HCG. The inactive form of elastase detected in beige lysosomes may represent the result of defective proteolytic processing during azurophil granule packaging. The presence of an activatable latent from of elastase in beige lysosomes may explain why beige mice do develop (even if with a delay) a series of lesions that have been ascribed to the proteolytic activity of leucocyte elastase [19,20,28]. The data presented may also offer an explanation for the apparently conflicting results obtained *in vivo* and *in vitro* when this strain is used as a model of proteolysis-mediated lesions [19,20].

In the past years, the beige mouse has been used by many investigators as a model of neutrophil elastase- and cathepsin G-deficiency to demonstrate or exclude the role of these neutral proteases in a variety of pathological conditions [19,20,27–29]. In our opinion, many of the conclusions drawn from these studies should be reviewed in the light of the data reported in this paper.

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