

ELASTASE AND CATHEPSIN G ACTIVITIES ARE PRESENT  
IN IMMATURE BONE MARROW NEUTROPHILS AND  
ABSENT IN LATE MARROW AND CIRCULATING  
NEUTROPHILS OF BEIGE (CHEDIAK-HIGASHI) MICE

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The Chediak-Higashi Syndrome (CHS)<sup>1</sup> is a genetic disease that occurs in the human (1), cow (2), cat (3), mink (4), and mouse (5). In the mouse, the recessive beige gene is located on the proximal end of chromosome 13 (5). Homozygous beige mice exhibit most of the phenotypic characteristics of human Chediak-Higashi patients (5).

One of the more striking quantitative observations of CHS is the very large decrease in neutral proteinase activity of circulating leukocytes and peritoneal neutrophils. In humans, Vassalli et al. (6) found a virtual absence of neutral proteinase activity in circulating leukocytes of three CHS patients. We found that the similar neutral proteinase deficiency in peritoneal neutrophils of the beige mouse is due to a profound decrease of both elastase and cathepsin G (7). This is true for peritoneal neutrophils of four independent beige mutants and could explain the increased susceptibility to infection of beige mice and Chediak-Higashi patients (8, 9).

As is well known, neutrophils undergo progressive development and differentiation in bone marrow from the primitive myeloblast before becoming mature neutrophils (10, 11). Mature neutrophils are stored in bone marrow before release into the circulation (10). Lysosomal enzyme(s), presumably including lysosomal elastase and cathepsin G, are synthesized relatively early, that is, in the promyelocyte or early myelocyte stage (10, 12).

We present evidence that precursor neutrophils of bone marrow of beige mice have considerable neutral proteinase (elastase and cathepsin G) activity. Mature marrow neutrophils, in contrast, have greatly decreased neutral proteinase activity. Also, blood neutrophils of beige mice have a virtual absence of elastase and cathepsin G activities. This result indicates that the major mechanism for the lowered neutrophil elastase and cathepsin G activities in mature neutrophils of beige mice is not due to an absence of enzyme synthesis, but rather, it is

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<sup>1</sup> *Abbreviations used in this paper:* AMC, amino methyl coumarin; CHS, Chediak-Higashi Syndrome; MCA, 4-methylcoumarinyl-7-amide; PMN, polymorphonuclear leukocyte.

caused by a novel loss of the activities of these enzymes during neutrophil maturation in bone marrow.

### Materials and Methods

The following materials were obtained from the indicated sources: Hanks' Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free balanced saline solution (HBSS; Gibco Laboratories, Grand Island, NY); Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden); methoxysuccinyl-alanyl-alanyl-prolyl-valine-4-methylcoumarinyl-7-amide (MeO-Suc-Ala-Ala-Pro-Val-MCA), *t*-Boc-valyl-glycyl-arginine-4-methylcoumarinyl-7-amide (*t*-Boc-Val-Gly-Arg-MCA), and amino methyl coumarin (AMC) were purchased from Vega Biotechnologies, Inc., Tucson, AZ; Z-glycyl-leucyl-phenylalanine chloromethyl ketone (Z-Gly-Leu-Phe-CH<sub>2</sub>Cl), and methoxysuccinyl-alanyl-alanyl-prolyl-valine chloromethyl ketone (MeO-Suc-Ala-Ala-Pro-Val-CH<sub>2</sub>Cl) from Enzyme Systems Products, Livermore, CA; succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-*p*NA), succinyl-glycyl-prolyl-leucyl-glycyl-proline-4-methylcoumarinyl-7-amide (Suc-Gly-Pro-Leu-Gly-Pro-MCA), PMSF, *N*-ethylmaleimide, DMSO, *p*-chloromercuribenzoate, soybean trypsin inhibitor,  $\alpha_1$ -antitrypsin, aprotinin, elastin-orcein, naphthol AS-D chloroacetate, and fast garnet GBC from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

**Animals.** C57BL/6J (+/+) and C57BL/6J beige (*bg*<sup>J</sup>/*bg*<sup>J</sup>) male and female mice were originally purchased from The Jackson Laboratories (Bar Harbor, ME) and were subsequently bred at the animal facilities of Roswell Park Memorial Institute.

**Preparation of Bone Marrow Cells.** Bone marrow was prepared according to the method of Watt et al. (13) with the following modification. Femoral shafts were removed from hind legs and marrow plugs flushed with 1–2 ml of HBSS containing 5 mM EDTA (HBSS-EDTA) using a 25-gauge needle. The cells were gently suspended, centrifuged, and washed once in HBSS-EDTA at 4°C. Red cells were lysed by suspending the cell pellet in 10 ml of 0.168 M NH<sub>4</sub>Cl for 5 min at room temperature. The cells were washed three times with HBSS-EDTA. Differential counts were performed on an aliquot of the final washed cells by light microscopy after Stat-staining (VWR Scientific Inc., San Francisco, CA). The remaining cells were resuspended with cold 0.2% Triton X-100, 0.2 M sucrose, 0.15 M NaCl, 5 mM EDTA in 20 mM imidazole-HCl, pH 7.4 and extracted according to the methods described elsewhere (7). The homogenate was centrifuged at 13,000 *g* for 10 min at 4°C, and the supernatant was used as a source of elastase and/or cathepsin G.

**Isolation of Mature Neutrophils From Peripheral Blood.** Blood was drawn by cardiac puncture with a 22-gauge needle attached to a 1-ml plastic syringe that contained 0.1 ml of 3.8% sodium-citrate (14). Each milliliter of blood was mixed with 5 ml of HBSS-EDTA containing dextran to make a 2% (wt/vol) final dextran concentration. The mixture was gently shaken for 10 s and left for 45 min at room temperature.

The leukocyte-rich supernatant and buffy coat were carefully harvested, and platelets were separated with the supernatant after centrifugation at 200 *g* for 5 min at 15°C. The pellet was suspended in HBSS-EDTA and underlaid with Ficoll-Paque for further purification of neutrophils (7). Lymphocytes, monocytes, and platelets from the interface were withdrawn and discarded, whereas neutrophils from the pellet were collected. The pellet was washed once with HBSS-EDTA and the contaminating red cells were removed by a 0.168 M NH<sub>4</sub>Cl. The cells were washed three times with HBSS-EDTA. An aliquot of final washed cells was examined by light microscopy after Stat staining. Extracts of cells were prepared as described previously (7).

**Induction and Preparation of Elicited Peritoneal Neutrophils.** Peritoneal neutrophils were elicited by the copper-rod implantation technique originally described by McGarry (15). Harvest and purification of peritoneal neutrophils were described previously (7).

**Enzyme Assays.** Elastase activity was measured with the fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-MCA as described by Takeuchi et al. (7). 1 U of elastase activity is defined as the formation of 1 nmol of AMC per minute. The protein substrate, elastin-orcein, was also applied to examine elastase hydrolytic activity (16). 6 mg/ml elastin-orcein was incubated with extracts at 37°C. The change of absorbance at 570 nm was determined.

TABLE I  
*Activities of Elastase and Cathepsin G in Bone Marrow Extracts of Normal (+/+),  
 Heterozygous (+/bg<sup>J</sup>), and Beige (bg<sup>J</sup>/bg<sup>J</sup>) Mice*

Genotype	n	Total cells per Femur	Neutrophils	Elastase		Cathepsin G	
				Units per neutrophil			
		$\times 10^{-6}$	%	$\times 10^7$		$\times 10^7$	
C57BL/6J (+/+)	5	14.03 $\pm$ 1.84	49.3 $\pm$ 2.3	4.35 $\pm$ 0.17 (5)	5.01 $\pm$ 0.18 (5)		
C57BL/6J (+/bg <sup>J</sup> )	4	12.29 $\pm$ 3.95	51.7 $\pm$ 3.1	5.0 $\pm$ 0.11 (4)	5.19 $\pm$ 0.15 (3)		
C57BL/6J (bg <sup>J</sup> /bg <sup>J</sup> )	6	13.78 $\pm$ 0.74	51.8 $\pm$ 1.8	2.54 $\pm$ 0.14 (6)*	2.90 $\pm$ 0.31 (4) <sup>‡</sup>		

Bone marrow cells were extracted as described in Materials and Methods. Elastase was assayed with the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA. 1 U of elastase activity corresponds to the release of 1 nmol of product per minute. Cathepsin G activity is expressed as  $\Delta E/\text{min}$  at 410 nm ( $\times 10^3$ ). Specific activities are expressed as mean  $\pm$  SEM of assays of number of individual mice in parentheses. No significant difference in enzyme activity was found between male and female mice.

\*  $p \leq 0.001$

<sup>‡</sup>  $p \leq 0.005$

Cathepsin G was assayed using Suc-Ala-Ala-Pro-Phe-pNA as substrate as described previously (7). Cathepsin G activity is expressed as  $\Delta E/\text{min}$  at 410 nm ( $\times 10^3$ ) at 50°C. Other enzyme assays and inhibition studies were performed as described by Takeuchi et al. (7).

*Enzyme Histochemistry of Bone Marrow Neutrophils.* The combined elastase and cathepsin G activities in neutrophils were examined histochemically using naphthol AS-D chloroacetate as substrate (17). Bone marrow smears were air dried and fixed in 10% formalin/90% MeOH for 30 s at 0°C. They were subsequently rinsed with distilled water for 5 min and air dried. The fixed smears were stained for enzyme activity for 30 min according to the method of Moloney et al. (17). The number of neutrophils of each of five precursor classes (myeloblasts, promyelocytes, myelocytes, metamyelocytes, and PMNs) were enumerated and intensity of enzyme stain was independently estimated for each cell on a 0 to 4+ scale by two observers. Some smears were also preincubated with 0.2 mM MeO-Suc-Ala-Ala-Pro-Val-CH<sub>2</sub>Cl (neutrophil elastase inhibitor) or 0.2 mM Z-Gly-Leu-Phe-CH<sub>2</sub>Cl (cathepsin G inhibitor) or both inhibitors, before examination.

## Results

*Bone Marrow Elastase and Cathepsin G in Normal and Beige Mice.* To investigate whether elastase and cathepsin G in neutrophils from beige mice are missing at all levels of neutrophil maturation, neutrophil elastase activity, assayed with the specific and sensitive fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-MCA (18–20), and cathepsin G, assayed with Suc-Ala-Ala-Pro-Phe-pNA, were determined in cell suspensions of bone marrow from normal and beige mice (Table I).

Unlike in beige mice of elastase and cathepsin G in peritoneal neutrophils, bone marrow maintained >58% of normal bone marrow elastase activity per neutrophil (Table I). Similarly, beige marrow had >57% normal cathepsin G levels. Heterozygotes produced from a cross between normal and beige mice had normal levels of elastase and cathepsin G activities (Table I). These combined results indicate that two copies of the beige gene are necessary for production of the lowered enzyme levels. No significant differences were found among the three types of mice in total marrow cellularity or total marrow neutrophils.

The bone marrow and elicited peritoneal neutrophil extracts of normal and

TABLE II  
*Activity of Elastase in Bone Marrow and Elicited Peritoneal  
 Neutrophils as Measured by Hydrolysis of Elastin*

Genotype	Bone marrow	Peritoneal neutrophil
C57BL/6J (+/+)	0.181 ± 0.003 (3)	0.194 ± 0.008 (3)
C57BL/6J ( <i>bg</i> <sup>1</sup> / <i>bg</i> <sup>1</sup> )	0.126 ± 0.005 (3)*	0.038 ± 0.007 (3)*

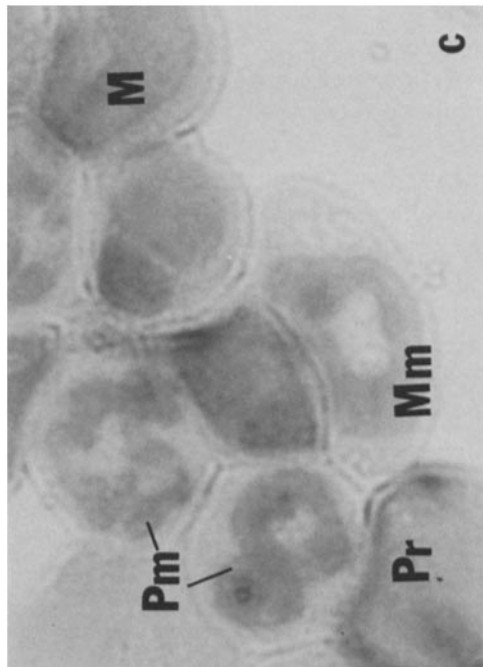
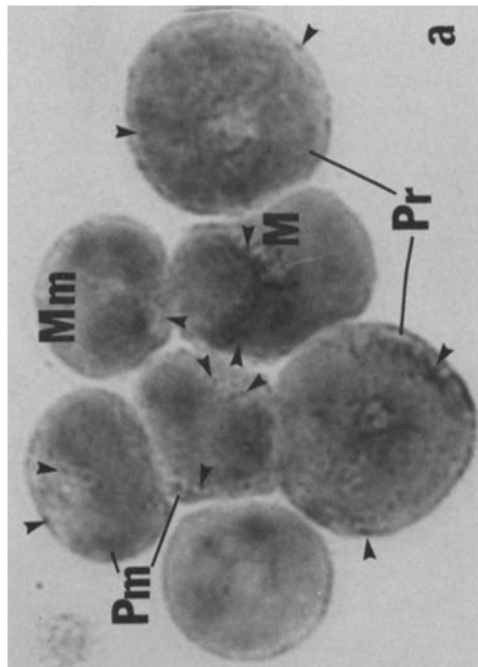
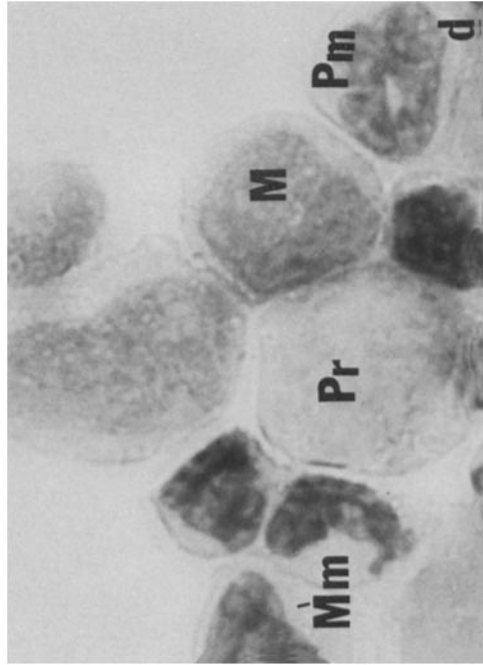
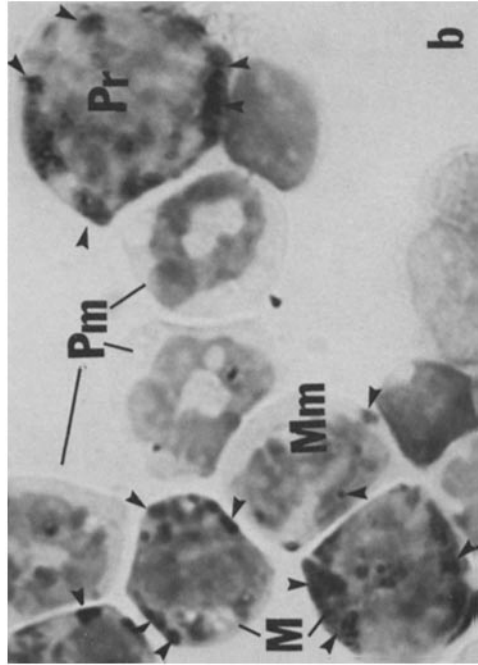
Bone marrow cells were extracted as described in Methods. Peritoneal neutrophils were elicited by a copper-rod implantation described previously (7). Elastase was assayed with the substrate elastin-orcein. The elastolytic activity is expressed as  $\Delta E/h/mg$  protein at 570 nm. Specific activity is expressed as mean  $\pm$  SEM of assays of the number of individual mice in parentheses.

\*  $p \leq 0.001$

beige mice were also examined for their elastolytic activity against a second elastase substrate, elastin-orcein. Beige bone marrow had nearly 70% of normal bone marrow elastolytic activity (Table II). Extracts of elicited peritoneal neutrophils of beige mice, however, had <20% normal elastolytic activity (Table II). Thus the two elastase assays gave similar results although the residual elastase activity in beige peritoneal neutrophils is lower (7) with the synthetic substrate, MeO-Suc-Ala-Ala-Pro-Val-MCA.

*Evidence that Bone Marrow Elastase Is Found in Neutrophils.* Since bone marrow is composed of a heterogeneous cell population, it was uncertain if the measured elastase activity was predominantly in neutrophils and neutrophil precursor cells. A direct test would be to analyze purified bone marrow neutrophils. However, it is presently not possible to isolate pure neutrophils from marrow. We have therefore used a histochemical test plus two indirect tests to determine the cellular location of marrow elastase.

In the first test, the localization of elastase in bone marrow was examined by an enzyme histochemical technique using naphthol AS-D chloroacetate as substrate (17). The naphthol AS-D chloroacetate esterase reaction is performed as an azo-coupling sequence with fast garnet GBC. As seen in Fig. 1 *a*, normal neutrophils exhibit a strong hydrolytic activity toward this substrate. The activity evident as reddish brown aggregates was found only in neutrophils or neutrophil precursors (Fig. 1, *a* and *b*). Eosinophils and basophils (not shown), along with monocytes, lymphocytes, and megakaryocytes (Fig. 2), did not demonstrate enzyme activity with this substrate in agreement with the original report of Moloney et al. (17). A very noticeable difference in staining patterns was apparent between beige neutrophils in which reaction product was deposited in giant granules (Fig. 1 *b*) and normal neutrophils in which reaction product was dispersed in very small granules (Fig. 1 *a*). The enzyme activity in normal and beige bone marrow was characterized by its elimination with the specific inhibitors MeO-Suc-Ala-Ala-Pro-Val-CH<sub>2</sub>Cl for neutrophil elastase and Z-Gly-Leu-Phe-CH<sub>2</sub>Cl for cathepsin G. Pretreatment of bone marrow smears with either chloromethyl ketone inhibitor caused a greatly, though not completely, diminished activity in neutrophils of normal and beige marrows. However, bone marrow smears pretreated with both inhibitors had no activity (Fig. 1, *c* and *d*). These



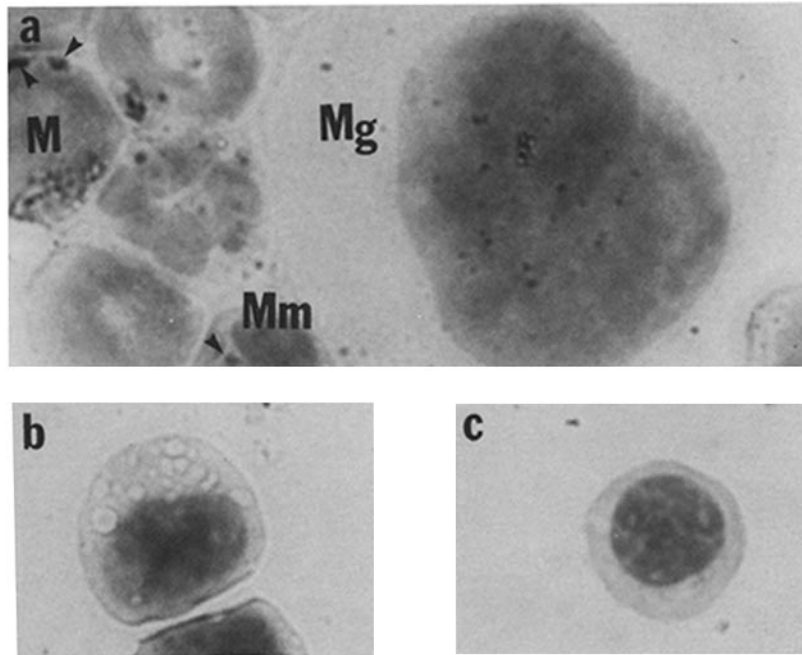


FIGURE 2. Neutral proteinase activity staining is specific for neutrophils. Beige marrow cells were histochemically stained for neutral proteinase as described in Fig. 1. (a) Megakaryocyte (*Mg*) has no detectable activity. In the original slides it is apparent that the black "dots" apparent within the megakaryocyte nucleus are not the true reddish-brown reaction product of the neutral proteinase substrate. Adjacent myelocytes (*M*) and metamyelocytes (*Mm*) have obvious reaction product in giant lysosomes (arrows). Lymphocytes (*b*) and monocytes (*c*) have no visible reaction products.  $\times 2,000$ .

results indicate that the histochemical enzyme activity represents a combination of both elastase and cathepsin G activities in bone marrow neutrophils.

In the second test, the proportion of neutrophils in bone marrow was perturbed and then marrow elastase-specific activity was tested for a corresponding change. Implantation of a copper-rod into the mouse peritoneal cavity resulted in a significant increase in neutrophils of bone marrow (Table III). Corresponding to the increase in the number of mature neutrophils was an increase in the specific activity of elastase (U/mg protein) in bone marrow of normal mice 3 and 6 d after copper-rod implantation (Table III). In beige bone marrow, on the

FIGURE 1. Neutral proteinase activity of normal and beige bone marrow. (a) Normal bone marrow. Marrow cells were stained for neutral proteinase activity with naphthol AS-D chloroacetate as substrate as described in Materials and Methods. All stages of neutrophil maturation (*Pr*, promyelocyte; *M*, myelocyte; *Mm*, metamyelocyte; *Pm*, PMN) demonstrate evenly dispersed enzyme activity in very small granules (arrows). This dispersed activity is more evident when compared with inhibitor-treated (zero activity) cells (*c*). (b) Beige bone marrow. Immature neutrophils (promyelocytes [*Pr*] and myelocytes [*M*]) have intense reaction products in giant granules (arrows). Later stages of neutrophil maturation (metamyelocytes [*Mm*] and PMNs [*Pm*]), however, have completely negative or very weak activity. The metamyelocyte of this field has very weak activity, most metamyelocytes have substantial activity (see Table V). (c and d) Either normal marrow (*c*) or beige marrow (*d*) were pretreated with MeO-Suc-Ala-Ala-Pro-Val-CH<sub>2</sub>Cl (neutrophil elastase inhibitor) plus Z-Gly-Leu-Phe-CH<sub>2</sub>Cl (cathepsin G inhibitor) before staining for activity. These treatments resulted in no detectable activity staining in neutrophils of either normal marrow or beige marrow.  $\times 2,000$ .

TABLE III  
*Percentage of Immature and Mature Neutrophils and Neutrophil Elastase in Bone Marrow of Normal (+/+) and Beige (bg<sup>J</sup>/bg<sup>J</sup>) Mice at Various Times After Peritoneal Implantation of a Copper-rod*

Exp.	Marrow	n	Day	Neutrophils		Elastase	
				Immature	Mature	U/mg protein	Units per neutrophil × 10 <sup>7</sup>
%							
A	Normal	5	0	8.8 ± 0.9	40.5 ± 1.8	5.25 ± 0.23	4.35 ± 0.17
		5	3	16.3 ± 0.8*	62.2 ± 1.3 <sup>‡</sup>	8.05 ± 0.71 <sup>§</sup>	4.20 ± 0.32
		4	6	7.7 ± 2.2	74.1 ± 2.8 <sup>‡</sup>	13.00 ± 0.78 <sup>‡</sup>	6.28 ± 0.53 <sup>§</sup>
B	Beige	6	0	6.3 ± 1.2	45.6 ± 2.7	2.57 ± 0.29	2.54 ± 0.14
		5	3	18.6 ± 1.3 <sup>‡</sup>	60.5 ± 0.7*	3.29 ± 0.25	2.28 ± 0.27
		5	6	3.9 ± 0.5	78.4 ± 1.0 <sup>‡</sup>	1.84 ± 0.07	1.03 ± 0.05 <sup>‡</sup>

Percent immature neutrophils = 100 × (immature neutrophils/total marrow cells). Percent mature neutrophils = 100 × (mature neutrophils/total marrow cells). Immature (promyelocyte, myelocyte) and mature (metamyelocyte, band, PMN) neutrophils were differentiated under the light microscope after Stat staining. Total bone marrow extracts were assayed for elastase activity using the fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-MCA on the indicated days after implantation of a copper-rod (see Materials and Methods for detail). Total cells per femur were not different on the 3 d. Values represent the mean ± SEM.

\*  $p \leq 0.005$

<sup>‡</sup>  $p \leq 0.001$

<sup>§</sup>  $p \leq 0.05$

other hand, there was little or no change in elastase-specific activity (U/mg protein) during this time, despite the fact that the number of mature neutrophils was increased in parallel to normal marrow. On day 6, when mature neutrophils were >10 times greater than immature neutrophils, the elastase activity was actually reduced in beige mice (Table III).

A third method of testing whether the measured elastase in bone marrow is bona fide neutrophil elastase is to use inhibitors of elastase activity that are cell specific. Neutrophils, macrophages and platelets contain elastases of different substrate and inhibitor specificity. Neutrophil elastase is a serine proteinase (6, 7, 20). Elastase derived from macrophages (21, 22) is a metal-requiring enzyme and is susceptible to the chelating agent, EDTA, but totally insensitive to serine proteinase inhibitors such as PMSF. The elastolytic activity in platelets, unlike neutrophil elastase, is not inhibited by  $\alpha_1$ -antitrypsin (23).

When bone marrow extracts were pretreated with a series of typical proteinase inhibitors (Table IV), the elastase activity was almost completely inhibited by serine proteinase inhibitors such as PMSF, soybean trypsin inhibitor, and by  $\alpha_1$ -antitrypsin, and was weakly inhibited by aprotinin. More importantly, the synthetic peptide chloromethyl ketone MeO-Suc-Ala-Ala-Pro-Val-CH<sub>2</sub>Cl (24), a specific neutrophil elastase inhibitor (19, 20, 24), was found to be a potent inhibitor of bone marrow elastase. In fact, no elastase activity was detected from bone marrow extracts after treatment with this synthetic peptide. However, full activity was present with thiol reagents such as *N*-ethylmaleimide and *p*-chloro-mercuribenzoate, and with the chelating agent, EDTA. These results suggest the absence of any thiol group(s) in the active site and no metal ion requirement

TABLE IV  
Effect of Various Reagents on Mouse Bone Marrow Elastase

Reagent	Activity remaining	
	Normal	Beige
	%	
EDTA (5 mM)	100	100
<i>N</i> -Ethylmaleimide (1 mM)	100	100
Aprotinin (0.1 mM)	98	96
<i>p</i> -Chloromercuribenzoate (1 mM)	92	93
<i>Z</i> -Gly-Leu-Phe-CH <sub>2</sub> Cl (0.1 mM)	91	88
$\alpha_1$ -Antitrypsin (0.1 mM)	0	0
MeO-Suc-Ala-Ala-Pro-Val-CH <sub>2</sub> Cl (0.1 mM)	0	0
PMSF (1 mM)	7	6
Soybean Trypsin inhibitor (0.1 mM)	0	0

Bone marrow extracts from normal and beige mice were prepared as described in Materials and Methods. The extracts were preincubated in 20 mM imidazole-HCl, pH 7.2, containing 0.15 M NaCl, 0.2% Triton X-100, and 0.2 M sucrose at 25°C for 30 min with the reagents, and remaining elastase activity was assayed with MeO-Suc-Ala-Ala-Pro-Val-MCA as substrate. The final concentration of inhibitors is indicated in parentheses and activity of enzyme is expressed as a percentage of activity against a control experiment with no inhibitor added set at 100%.

TABLE V  
Bone Marrow Enzyme Histochemistry With Naphthol AS-D Chloroacetate Activity Scores

Bone marrow	Activity				
	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocytes	PMNs
Normal	1.00 ± 0.52 (10)	3.35 ± 0.25 (68)	3.24 ± 0.07 (127)	3.12 ± 0.04 (249)	3.11 ± 0.05 (300)
Beige	0.88 ± 0.30 (8)	3.28 ± 0.11 (74)	3.28 ± 0.07 (118)	2.89 ± 0.08 (299)*‡	0.73 ± 0.05 (401)‡

Bone marrow smears from four normal and four beige mice were prepared and naphthol AS-D chloroacetate hydrolytic activity was examined as described in Materials and Methods. The activity score in normal and beige marrow smears was determined microscopically by identifying neutrophil developmental stages and visually grading the intensity of enzyme stain of each cell from 0 to 4+ (lowest intensity to highest). Values represent the mean score ± SEM for the number of neutrophils indicated in parentheses.

\* Beige myelocytes vs. Beige metamyelocytes,  $p \leq 0.01$ .

‡ Beige metamyelocytes vs. normal metamyelocytes,  $p \leq 0.025$ .

‡ Beige PMNs vs. Normal PMNs,  $p \leq 0.001$ .

for activity. The negative inhibition with *Z*-Gly-Leu-Phe-CH<sub>2</sub>Cl shows that activity is not due to cathepsin G (24). Also, the effects of the inhibitors were equivalent in both normal and beige marrow suggesting that marrow elastase, as measured by MeO-Suc-Ala-Ala-Pro-Val-MCA, is predominantly in neutrophils in both types of mice.

*Neutral Proteinase Activity at Specific Developmental Stages of Marrow Neutrophils.* The lowered activities of elastase and cathepsin G in marrow neutrophils of beige mice prompted us to examine if this loss of activity occurred at a particular developmental stage(s). Immature neutrophils of beige marrow had readily visible neutral proteinase activity (Fig. 1*b*). The activity was visually estimated and scored accordingly (Table V). The activity score in normal marrow was quite consistent with the range between 3.11 and 3.35 at all stages of neutrophil development except for the expected much lower levels at the myeloblast stage (Table V). Similar activity scores were found in normal and



TABLE VI  
Percent Distribution of Neutral Proteinase Scores in  
Bone Marrow PMNs

Score	Percent with corresponding score	
	Normal	Beige
0	1	57
1+	4	29
2+	6	10
3+	9	3
4+	80	1

Bone marrow smears from normal and beige mice were histochemically stained for neutral proteinase activity (see Materials and Methods and Table V for details). Individual PMNs were scored for neutral proteinase activity as described in Table V. Numbers indicate the percent of normal or beige PMNs in each score category. 78 normal PMNs and 124 beige PMNs were scored. The values in this table are a representative result from three separate experiments.

TABLE VII  
Enzymatic Activities of Bone Marrow Extracts in Normal  
and Beige Mice

Enzyme	Normal (+/+)	Beige ( $bg^1/bg^1$ )
Cathepsin D	26.8 ± 0.97 (4)	24.4 ± 1.17 (4)
Collagenase-like peptidase	1.35 ± 0.11 (4)	1.23 ± 0.08 (4)
$\beta$ -Glucuronidase	153 ± 5.4 (3)	169 ± 6.2 (5)
Plasminogen activator(s)	1.27 ± 0.14 (4)	1.47 ± 0.05 (5)

Bone marrow extracts from C57BL/6J (+/+) and C57BL/6J ( $bg^1/bg^1$ ) mice were prepared as described in Materials and Methods. Cathepsin D was determined with hemoglobin as substrate. Collagenase-like peptidase activity was measured using Suc-Gly-Pro-Leu-Gly-Pro-MCA as substrate.  $\beta$ -Glucuronidase was assayed with 4-methylumbellifery- $\beta$ -D-glucuronide as substrate. Plasminogen activator(s) was assayed with *t*-Boc-Val-Gly-Arg-MCA as substrate. Activities are expressed as U/mg protein ± SEM with number of samples assayed shown in parentheses. Units of activity were expressed as described in (7).

beige neutrophils in early development stages including myeloblast, promyelocyte, and myelocyte, although there was a slight (7%) but significant lowering of activity in beige metamyelocytes. In beige marrow, however, a precipitous decrease in activity was detected between the metamyelocyte and PMN stages (Table V). PMNs of beige marrow demonstrated 23% of the activity of normal PMNs (Table V). When the activities of individual PMNs of beige marrow were more closely examined (Table VI), it was found that the large majority (86%) have either undetectable (0) or very low (1+) activity levels. Only a few percent had the normal activity scores (3+ or 4+) characteristically found in PMNs of normal marrow.

*Other Enzymes in Bone Marrow.* As was found in mature neutrophils (7), three proteinases, cathepsin D, collagenase-like peptidase, plasminogen activator(s) and  $\beta$ -glucuronidase, were not significantly different in normal and beige bone marrow extracts (Table VII).

TABLE VIII  
*Blood Neutrophil Elastase of Normal and Beige Mice*

Genotype	units per PMN	
	Elastase	Cathepsin G
	$\times 10^7$	
C57BL/6J (+/+)	3.241 $\pm$ 0.228 (4)	5.12 $\pm$ 0.45 (4)
C57BL/6J ( <i>bg</i> <sup>1</sup> / <i>bg</i> <sup>1</sup> )	0.100 $\pm$ 0.003 (3)*	0.17 $\pm$ 0.05 (3)*

Neutrophils ( $3-5 \times 10^6$ /mouse) were isolated from whole blood (see Materials and Methods). The purity of neutrophils was >99%. Elastase was assayed with the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA and specific activity is expressed as mean  $\pm$  SEM of assays of the number of individual mice in parentheses. Equivalent concentrations of neutrophils were found in whole blood of normal and beige mice.

\*  $p \leq 0.001$

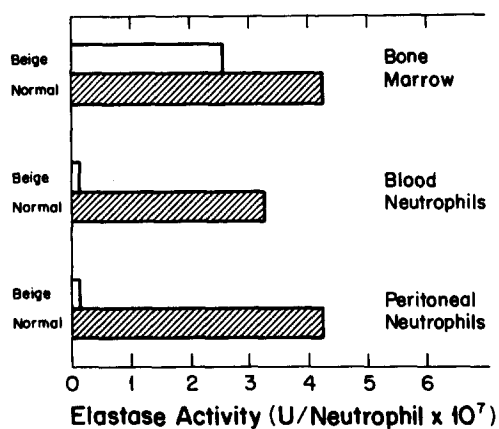


FIGURE 3. Summary of mouse neutrophil elastase activity for three neutrophil developmental stages. The results for bone marrow and blood neutrophils are from this report, while those for elicited peritoneal neutrophils were taken from Takeuchi et al. (7). Elastase activity was assigned with the standard enzyme assay procedure described in Materials and Methods.

*Elastase and Cathepsin G Activities of Peritoneal and Blood Neutrophils.* To determine if circulating neutrophils (i.e., mature, but recently derived from bone marrow) have elastase activity, blood neutrophils from normal and beige mice were purified and examined for elastase and cathepsin G activities (Table VIII). Interestingly, the blood neutrophils of beige mice contained very little elastase and cathepsin G activities (<4% of normal levels). Therefore, our data suggest that elastase and cathepsin G are present in immature and maturing bone marrow neutrophils but are nearly completely lost by the time these neutrophils release into the circulation (Fig. 3 and Table VIII). The loss in bone marrow is principally at the mature PMN stage.

### Discussion

Fig. 3 summarizes our findings (from this paper and reference 7) on elastase activity of peritoneal and blood neutrophils and of bone marrow of normal and beige mice. Our findings have also indicated that cathepsin G follows the same scheme in its activity. The striking observations are that these activities are virtually absent in beige blood and peritoneal neutrophils, but are only partially (40%) lower than that of the C57BL/6J normal mouse in bone marrow. Normal

neutrophils, however, have nearly equal activity in bone marrow, blood, and peritoneal neutrophils. The elastase and cathepsin G genes are expressed in bone marrow of beige mice, but there follows an inactivation or loss of each in bone marrow neutrophils mainly at the mature PMN developmental stage (though some loss is detectable at the metamyelocyte stage) just before their release into the blood.

The significant levels of elastase and cathepsin G activities in bone marrow neutrophils of beige mice are important because they indicate that the mechanism of absence of neutral proteinase activity in blood and peritoneal neutrophils (7) is not due to a lack of genetic expression (for example, deleted elastase and cathepsin G genes, or inability to produce elastase and cathepsin G mRNAs or active elastase and cathepsin G protein). Instead, the defect likely involves accelerated degradation or inactivation of the active enzymes. In fact, we have detected (Takeuchi, K. H., in preparation) increased levels of elastase inhibitory activity in extracts of beige neutrophils. Rausch et al. (12) have likewise presented evidence that elastase and cathepsin G are detectable by immunocytochemical methods in bone marrow neutrophils of CHS patients. Evidence based on beige and normal mice with reciprocally transplanted bone marrow (7) indicates that extrinsic factors in the circulation or other humoral environments of the beige mouse are not responsible for elastase and cathepsin G inactivation. Rather, the lowering of elastase and cathepsin G activities in mature beige neutrophils is a cellular defect.

Three types of experiments suggest that the activities measured in bone marrow are derived from neutrophils and their precursor cells. First, histochemical enzyme staining in bone marrow indicated that the enzyme is restricted to neutrophils and neutrophil precursors. Second, when mature neutrophils were increased in bone marrow by copper-rod implantation in normal mice, a corresponding increase in marrow elastase specific activity occurred. Third, elastase activity of bone marrow was highly sensitive to inhibitors of neutrophil elastase (19), but not to inhibitors of other major elastases such as those of macrophages and platelets (21-23). The lack of inhibition by EDTA rules out a contribution of the metalloelastase of macrophages (21, 22). In addition, the complete inhibition of bone marrow elastase by  $\alpha_1$ -antitrypsin (Table IV) strongly indicates that bone marrow elastase is not the same as platelet-type elastase (23). The weak inhibition by aprotinin is consistent with the activity being in neutrophils since others (25) have reported aprotinin has only weak inhibitory activity toward granulocyte elastase. Thus, the inhibitor evidence shows that bone marrow elastase is a serine proteinase with characteristics identical to neutrophil elastase and therefore likely derives from neutrophils and neutrophil progenitors in bone marrow. Other indirect observations are consistent with a neutrophil source. First, lymphocytes, another major class of cells present in bone marrow, have been suggested to have insignificant elastase levels (7) as measured with the fluorogenic substrate. In fact, lymphocytes, monocytes, and other cells in bone marrow failed to demonstrate enzyme activity with the histochemical substrate detection used here. Second, when elastase activity in marrow is calculated on a per neutrophil basis (Fig. 3), the activity in marrow is nearly identical to that in blood and peritoneal neutrophils in normal mice, a fact that is consistent with a

common cellular origin. Third, elastase activity in a putative minor unidentified cell type of marrow would have to be remarkably high compared with that calculated per neutrophil, since neutrophils in the case of mice implanted 6 d with copper-rod form 80% of total marrow cells.

The lowered activities of elastase and cathepsin G in bone marrow of beige mice could be due to: (a) a decreased activity in all marrow neutrophil cells at all stages of marrow development or (b) a preferential inactivation of activity of the more mature neutrophils. For the first possibility to hold, a precipitous inactivation of elastase and cathepsin G activity would be required upon release of mature neutrophils since blood neutrophils have only 4% the activity of beige bone marrow neutrophils. The results of the histochemical tests directly support the second possibility. It was found that all developmental stages of beige neutrophils including myeloblast, promyelocyte, myelocyte, and metamyelocyte have equal activities in normal and beige marrow with the exception of a slight decrease in beige metamyelocytes. In the myeloblast stage, both normal and beige indicated very low enzyme staining scores probably because these cells had just initiated synthesis of azurophilic granule enzymes (10). In contrast, mature PMNs of beige marrow had only 23% (on average) of the normal activity. Most PMNs of beige marrow, in fact, had no detectable activity. Thus the inactivation/loss of neutral proteinase activity in neutrophils of beige marrow occurs at a specific late point in neutrophil maturation, possibly commencing at the metamyelocyte stage and reaching completion early in the PMN stage. Mature PMNs released into the circulation, therefore, have almost no activity. The second possibility is also indicated by the results of the copper-rod implantation experiment. It was found that while the percentage of mature neutrophils in bone marrow increased in beige marrow from ~45 to 78% after 6 d implantation, the specific activity of neutrophil elastase per milligram total marrow protein did not significantly change at 3 d and decreased significantly (~45%) from day 3 to day 6 (Table III). Also, on a per neutrophil basis the specific activity in beige marrow decreased from day 0 to day 6 (Table III), consistent with a preferential loss in activity in mature neutrophils. The elastase activity in bone marrow of beige mice more closely parallels the change in percentage of immature neutrophils. The percentage of immature neutrophils is significantly higher at day 3 than either day 0 or day 6. While elastase activity is not significantly different between days 0 and 3 in beige marrow, it is significantly lower on both a per milligram protein and a per neutrophil basis on day 6. Taken together, these results suggest a preferential loss of activity in more mature neutrophils of beige bone marrow, a result in agreement with the direct histochemical tests.

It is interesting that the time of appearance (promyelocyte stage) (10) of giant granules in beige mice and CHS patients is dissociated by a large time interval from the time of loss (mature PMN) of elastase and cathepsin G activities. Therefore, the loss of these enzyme activities (which reside in giant granules) is not a direct result of the formation of giant granules. Several authors (12, 26, 27) have pointed out that formation of aggregates, curvilinear arrays, and ring-shaped structures occurs in late stages of neutrophil maturation in bone marrow of CHS patients and beige mice. It is likewise of interest that the time of loss of neutral proteinase activity in beige marrow (late metamyelocyte/early PMN

stages) is close to the time of termination of mitotic activities (10) in neutrophils. Another major metabolic change occurring during this time period is a large reduction in protein synthesis as evidenced by a reduction in morphologically identifiable endoplasmic reticulum and free ribosomes (28).

The finding of a virtual lack of elastase and cathepsin G in circulating neutrophils is important in regard to the etiology of CHS. Since elastase of CHS neutrophils is inactivated before their arrival at infection sites, this may explain their lowered ability to kill invading bacteria (9). If bone marrow neutrophils of CHS patients have, like beige mice, significant elastase and cathepsin G activities, then therapies that prevent inactivation of these enzymes would be clinically significant. Additionally, it would be of interest to determine elastase and cathepsin G activities in circulating neutrophils of CHS patients during periods of peripheral left shift in neutrophil granulocytes as, for example, during mobilization and premature release in response to infections or other leukemoid reactions (29). The immature neutrophils released from marrow under these conditions may contain significant neutral proteinase activity.

The absence of elastase and cathepsin G activities in circulating and tissue neutrophils of beige mice suggests this would provide an interesting model system to study other physiologically important processes. For example, emphysema has been associated with damage of lung tissue by neutrophil serine proteinases (19, 30) that are virtually absent (7) in beige mice. The beige mutant, therefore, potentially could be used to test the involvement of other factors such as macrophage elastase in the absence of neutrophil serine proteinases in emphysema.

### Summary

Elicited peritoneal neutrophils of beige (Chediak-Higashi) mice essentially lack activities of the neutral serine proteinases elastase and cathepsin G, which may explain the increased susceptibility to infection of beige mice and Chediak-Higashi patients. We have examined neutrophils of beige mice at earlier points in their development to determine if the proteinase genes are never expressed or whether they are expressed and then lost during neutrophil maturation. Surprisingly, bone marrow of beige mice had significant elastase and cathepsin G activity (~60% of normal). The results of several experiments indicate that neutrophils were the sole source of elastase and cathepsin G in bone marrow. Neutral proteinase activity was readily demonstrable by histochemical procedures in beige marrow neutrophil precursors up to and including the metamyelocyte stage. However, mature neutrophils of beige marrow had greatly decreased activity. Also mature neutrophils (PMNs) of the peripheral circulation, like peritoneal neutrophils, had very low elastase and cathepsin G activities. Thus we conclude that beige neutrophil precursors express neutral proteinase activity, which is largely and irreversibly depleted by the time they fully mature in marrow.

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