LYSOSOMAL ELASTASE AND CATHEPSIN G IN BEIGE MICE

Neutrophils of Beige (Chediak-Higashi) Mice Selectively Lack Lysosomal Elastase and Cathepsin G

By KIYOSHI TAKEUCHI, HEATHER WOOD, AND RICHARD T. SWANK

From the Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

In humans, Chediak-Higashi Syndrome (CHS)[1] is a recessively inherited mutation with a wide variety of clinical symptoms, including lowered neutral proteinase activity in neutrophils (1), giant lysosomes in many tissues (2), diluted pigmentation (3), prolonged bleeding time (4), decreased chemotaxis in leukocytes (5), deficiency of functional natural killer cells (6), and increased susceptibility to infection (7). Homozygous patients are weakened by repeated infections and usually die before or during their teen years. Possible reasons for the increased susceptibility to infection include decreased natural killer cell (6) activity, neutropenia, and an inability of neutrophis to kill invading microorganisms (8).

The disease has been studied by many investigators, in part due to the availability of animal models including mutations in the mouse (9), cat (10), mink (11), killer whale (12), and cow (13). In the mouse, the recessive beige (*bg*) gene is on the proximal end of chromosome 13. Homozygous beige mice exhibit most of the phenotypic characteristics of human CHS patients (14). An important advantage in studying the mouse model is that in comparisons of beige mice to the normal inbred strain on which the spontaneous mutation arose, it is highly probable that genuine effects of the altered beige gene are being measured rather than unrelated effects of differing background genes. Also, the beige mutation has spontaneously arisen in several different mouse strains, so that it is possible to more definitively confirm any phenotypic effect of the gene.

One of the more striking quantitative observations of CHS is the very large decrease in neutral proteinase activity of neutrophils. When [^{125}I]-fibrin was used as substrate, Vassalli et al. (1) found a virtual absence of proteinase activity in leukocytes of three CHS patients, and only 4–7% of normal proteinase activity in C57BL/6J (bg^J/bg^J) leukocytes. These authors (1) suggested, based on inhibitor

This work was supported by National Institutes of Health (Bethesda, MD) grant HL 31698 and National Science Foundation grant PCM-8215808. Present address of H. Wood: Dept. of Genetics Trinity College, Dublin 2, Ireland. Address correspondence to R. Swank, Dept. of Molecular Biology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

[1] *Abbreviations used in this paper:* AMC, aminomethyl coumarin; CHS, Chediak-Higashi Syndrome; DIFP, diisopropylfluorophosphate; MCA, 4-methylcoumarinyl-7-amide; MeO-Suc, methoxysuccinyl; *p*NA, *p*-nitroanilide; *t*-boc, *t*-butyloxycarbonyl; Z, carbobenzoxy.

experiments, that the major proteinase responsible for fibrin hydrolysis in normal human leukocytes is elastase, and therefore that CHS patients are deficient in elastase. They (1) pointed out, however, that [^{125}I]-fibrin is not hydrolyzed in proportion to substrate concentration, so that enzyme levels were difficult to establish precisely. Also, there is some question whether such an apparently nonspecific assay would measure only elastase activity, leaving open the possibility that other neutrophil proteinases such as collagenase (15), plasminogen activator (16), or cathepsin G (17) might be decreased in CHS. Johnson et al. (18) confirmed that leukocytes of beige mice lack the ability to hydrolyze [^{125}I]-fibrin, but they were unable to detect elastase activity, even in normal leukocytes, using the more specific elastase substrate succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide. This raised the additional possibility that the missing proteinase in beige leukocytes may not be elastase.

In this paper, we present evidence from hydrolysis of specific substrates that both elastase and cathepsin G activities, which are well known neutrophil serine proteinases localized in azurophilic (lysosomal) granules (19), are profoundly decreased in peritoneal neutrophils of several independent beige mouse mutants. In contrast, other well-established marker enzymes of lysosomal granules (14, 19–22) are at normal or near normal levels in both normal and beige mice.

Materials and Methods

The following materials were obtained from the indicated sources: Ca^{2+}- and Mg^{2+}-free 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 aminomethyl coumarin (AMC) were purchased from Vega Biochemicals, Tucson, AZ; Z-glycyl-leucyl-phenylalanine chloromethyl ketone (Z-Gly-Leu-Phe-CH_2Cl), and methoxysuccinyl-alanyl-alanyl-prolyl-valine chloromethyl ketone (MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl) 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, and *o*-dianisidine-2HCl from Sigma Chemical Co., St. Louis, MO; 1,3-[^{3}H]diisopropylfluorophosphate (DIFP) (sp act, 5.2 mCi/mmol) from New England Nuclear, Boston, MA. All other materials were of the highest grade commercially available.

Animals. The C57BL/6J (+/+), C57BL/6J beige (bg^J/bg^J), C3H/HeJ (+/+), C3H/HeJ beige (bg^{2J}/bg^{2J}), DBA/2J (+/+), DBA/2J beige (bg^{8J}/bg^{8J}), SB/Le (+/+), and SB/Le beige (*bg/bg*) male and female mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME) and were later bred at the animal facilities of Roswell Park Memorial Institute. The beige mutations in the C57BL/6J, C3H/HeJ, and DBA/2J arose independently and spontaneously in the respective inbred lines in which they have been subsequently maintained. Thus, any abnormality in beige mutants compared to control strain mice likely is due to the beige gene. The independent mutation in the SB/Le inbred line was probably radiation induced (14). All mutations affect the same gene. Mice 3–10 mo old were used throughout the experiments.

Bone Marrow Transplantation. Before transplantation, hosts received 950 rad whole body ^{60}Co irradiation. Donor marrow was obtained from the long bones of 6–8 wk-old mice. 10^7 bone marrow cells from donor femoral marrow were transplanted via tail vein in a volume of 0.5 ml RPMI 1640 medium (Gibco Laboratories). Mice were routinely maintained on acid water to reduce infection. Assays were performed 100 d after bone marrow transplantation. Congenic mice that had electrophoretically altered forms of β-

glucuronidase were used (23) as controls. Assay of β-glucuronidase phenotypes in bone marrow cells of recipient mice at 100 d confirmed that the transplants were successful.

Induction and Purification of Peritoneal Neutrophils. Peritoneal neutrophils were induced by the copper wire implantation technique developed by McGarry (24). After 24 h of copper wire implantation, cells were recovered by peritoneal lavage after injecting Ca^{2+}- and Mg^{2+}-free HBSS containing 5 mM EDTA into the peritoneal cavity. The peritoneal exudate cells were harvested by centrifugation and resuspension of cells was according to the method of Czuprynski et al. (25). The cell suspensions were underlaid with Ficoll-Paque and centrifuged at 400 *g* for 30 min. The neutrophils were recovered in a pellet and washed three times with HBSS/5 mM EDTA at 20°C. We routinely obtained ~10^7 cells per mouse; 80–95% neutrophils. The cells were then resuspended with cold 0.2% Triton X-100, 0.2 M sucrose, 5 mM EDTA in 20 mM imidazole-HCl, pH 7.3 (homogenization buffer), and homogenized (on ice) with a sonicator (Ultrasonic Processor, W-220; Heat-Systems-Ultrasonic, Inc., Farmingdale, NY) at setting 2.5, twice for 5 s with a 60-s interval between each burst. The homogenate was centrifuged at 13,000 *g* for 10 min at 4°C, and the supernatant was used as a neutrophil elastase source or stored at −80°C until used.

Enzyme Assays. Elastase activity was measured with the fluorogenic substrate methoxysuccinyl-Ala-Ala-Pro-Val-4-methylcoumarinyl-7-amide (MeO-Suc-Ala-Ala-Pro-Val-MCA) by the method of Barrett (26), modified as follows. The regular reaction mixture contained, in a final volume of 100 μl, 40 mM Tris-HCl, pH 7.5, 8% (vol/vol) DMSO, 0.4 M NaCl, and 0.8 mM substrate. The assay was stopped by the addition of 1 ml of 5 M formic acid (pH 3.0). Fluorescence product, AMC, was measured by its fluorescence intensity at 460 nm with excitation at 370 nm on an Aminco Fluoro-colorimeter (American Instrument Co., Silver Spring, MD). 1 U of activity corresponds to release of 1 μmol of product per minute at 37°C. Cathepsin G was assayed using Suc-Ala-Ala-Pro-Phe-*p*NA as substrate according to the method developed by Barrett (17), and collagenase-like peptidase (CL-peptidase) activity was measured using Suc-Gly-Pro-Leu-Gly-Pro-MCA as substrate by the method developed by Kojima et al. (27). Plasminogen activator was assayed with *t*-Boc-Val-Gly-Arg-MCA by the modification of Nieuwenhuizen et al. (28).

β-glucuronidase and β-galactosidase were assayed according to the method of Brandt et al. (29). Myeloperoxidase was assayed according to the methods of Maelhly and Chance (30), with *O*-dianisidine as the dye. Cathepsin D was determined by the method of Ishikawa and Cimasoni (31).

Protein was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, VA), with ovalbumin as a standard protein.

Inhibition Studies. PMSF and *N*-ethylmaleimide were dissolved in DMSO. *p*-Chloromercuribenzoate was made up as a stock solution in ethanol. MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl and Z-Glu-Leu-Phe-CH_2Cl (32) were dissolved in methanol.

The protein extracts were preincubated with inhibitors for 30 min at 25°C before the addition of fluorogenic substrates. The preincubated mixtures were assayed for elastase or cathepsin G as described above.

Gel Electrophoresis. SDS-PAGE (12.5% polyacrylamide gel) was performed in 115 × 140 × 1.5 mm slabs using the method of Laemmli (33). The sample buffer (pH 6.8) contained 50 mM Tris-HCl, 2% SDS, 10% glycerol, 5 mM EDTA, and 0.1 M DTT. Bromophenol blue at a final concentration of ~0.002% was added to most samples just before electrophoresis, but was not present during the heat treatment. After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue.

[^{3}H]DIFP Labeling of Peritoneal Neutrophil Extract. Each protein extract was incubated with 2 μCi of 1,3-[^{3}H]-DIFP in a total volume of 35 μl at 25°C for 1 h, and processed for SDS-PAGE as described above. Partially purified intact neutrophils (1–2 × 10^7 neutrophils) from normal and beige mice in HBSS/EDTA were also incubated with 10 μCi [^{3}H]-DIFP in a total volume of 0.5 ml at 25°C for 2 h. The neutrophils were extracted as described above, and labeled proteins were subsequently analyzed by SDS-PAGE. After electrophoresis and staining for protein, fluorography was done according to the method developed by Bonner and Laskey (34). Enhanced gels were wrapped with

TABLE I

Activities of Elastase and Cathepsin G in Neutrophil Extracts of Normal Mice and Four Independent Beige Mutants, and Light Ear and Pallid Mutants

Strain	Activity (U/PMN × 10^6) (n)	
	Elastase	Cathepsin G
C57BL/6J (+/+)	4.26 ± 0.58 (9)	4.13 ± 0.39 (6)
C57BL/6J (bg^J/bg^J)	0.11 ± 0.05 (6)*	0.29 ± 0.04 (4)*
C3H/HeJ (+/+)	4.8 ± 0.7 (3)	5.35 ± 0.55 (2)
C3H/HeJ (bg^{2J}/bg^{2J})	0.04 ± 0.01 (4)*	0.20 ± 0.04 (2)‡
DBA/2J (+/+)	4.0 ± 0.7 (4)	4.95 ± 1.35 (2)
DBA/2J (bg^{8J}/bg^{8J})	0.1 ± 0.03 (3)*	0.56 ± 0.03 (3)§
SB/Le (+/+)	3.9 ± 0.8 (3)	3.92 ± 1.10 (2)
SB/Le (bg/bg)	0.09 ± 0.01 (4)*	0.16 ± 0.02 (4)*
C57BL/6J (le/le) (light ear)	4.30 ± 0.63 (4)	4.06 ± 0.42 (4)
C57BL/6J (pa/pa) (pallid)	4.53 ± 0.14 (4)	3.29 ± 0.09 (3)

Peritoneal neutrophils were isolated by Ficoll-Paque centrifugation as described in Materials and Methods. Elastase was assayed with the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA and cathepsin G with Suc-Ala-Ala-Pro-Phe-*p*NA. 1 U of elastase activity corresponds to release of 1 μmol of product per minute. Cathepsin G activity is expressed as ΔE/min at 410 nm (× 10^5). Specific activities are expressed as means ± SEM of assays of number of individual mice (n) in parentheses. No statistical difference for enzyme activity was found between male and female mice of the same strain.

* $p \leq 0.001$.

‡ $p \leq 0.02$.

§ $p \leq 0.05$.

Saran-Wrap with or without drying, and exposed to x-ray film (X-Omat AR, Eastman Kodak, Rochester, NY) at −80°C using Dupont Cronex Xtra Life intensifying screens (E. I. DuPont de Nemours Co., Wilmington, DE). The labeled protein components of corresponding bands in the gels were extracted, and radioactivity was measured by liquid scintillation counting (Beckman Model LS 7500) according to the method developed by Basch (35).

Results

Reduced Elastase and Cathepsin G Activities in Neutrophil Extracts of Beige Mice. Neutrophil elastase, assayed with the specific substrate MeO-Suc-Ala-Ala-Pro-Val-MCA, is drastically and uniformly reduced in four independent beige mutants in inbred strains C57BL/6J, C3H/HeJ, DBA/2J, and SB/Le (Table I). Elastase specific activity in normal neutrophils is 40–120 times that of neutrophils of the various beige mutants. Similarly, cathepsin G, assayed with Suc-Ala-Ala-Pro-Phe-*p*NA, is 9–26 times higher in normal neutrophils.

In contrast, normal neutrophil elastase levels are present in the genetically independent coat color mutant mice, pallid and light ear, which, like beige, exhibit lysosomal enzyme secretion deficiency (14) and storage pool deficiency (36), but which have not been reported to have the increased susceptibility to infection reported in the beige mutation.

The lysosomal serine proteinases, elastase and cathepsin G, were also examined in thioglycollate-induced macrophages (37), platelets from whole blood (4), and

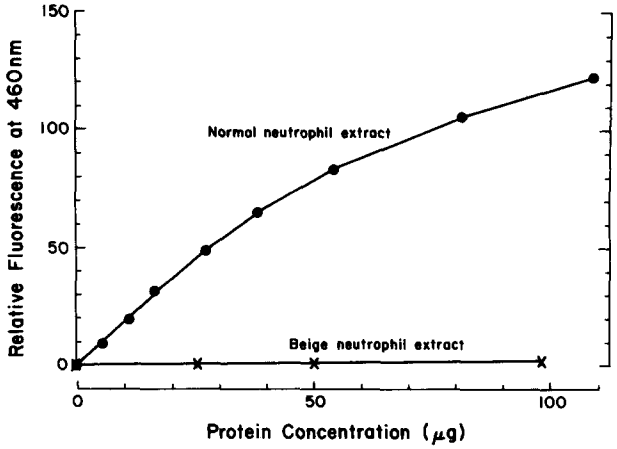

FIGURE 1. Protein dependency of elastase activity in normal and beige extracts. Normal neutrophil extracts (97% PMN in total cell preparation) and beige neutrophil extracts (94% PMN in total cell preparation) from strain C57BL/6J were assayed for elastase with the fluorogenic substrate MeO-Suc-Ala-Ala-Val-MCA.

lymphocytes from blood (38) in normal and beige mice. The elastase activities from these sources, assayed with the fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-MCA and expressed as a percentage of the specific activity in normal neutrophils, were very low, as follows: 0.03% in macrophages, 0.3% in lymphocytes, and 0.001% in platelets. Normal and beige extracts from these sources had the same level of activity. No significant cathepsin G activity was detected in any of the above preparations with Suc-Ala-Ala-Pro-Phe-*p*NA as substrate.

Several tests were performed to partially characterize the activity of the two proteinases in mouse neutrophils. The activities of both proteinases were proportional to time (up to 180 min, data not shown) and to 30–40 μg protein concentration in normal neutrophils (Fig. 1). When neutrophil extracts were pretreated with a series of typical proteinase inhibitors (Table II), elastase activity was completely inhibited by SDS. More importantly, the two serine proteinase inhibitors, PMSF and soybean trypsin inhibitor, were also effective inhibitors. ~48% of elastase was inactivated in the presence of 1 mM PMSF and 74% was inactivated at a concentration of 0.1 mg/ml soybean trypsin inhibitor. The inhibitory effect of DTT was also potent, with 24 and 52% of elastase activity being inactivated in the presence of 1 and 10 mM DTT, respectively. However, full activity was present with such reagents as *N*-ethylmaleimide, *p*-chloromercuribenzoate, and EDTA. These results suggest the absence of any thiol group(s) in the active site, and no metal ion requirement for activity.

Since elastase and cathepsin G were decreased to similar extents in beige neutrophil extracts, it was necessary to test that the two synthetic substrates were indeed measuring independent activities. For this purpose, we used the peptide chloromethyl ketones including MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, reported to be a specific inhibitor of elastase (32), and Z-Gly-Leu-Phe-CH_2Cl, a specific inhibitor of cathepsin G (32). Elastase activity (Table III) of normal and beige extracts was completely inhibited in the presence of 0.2 mM MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl but only slightly (~20%) in the presence of 0.2 mM Z-Gly-Leu-

TABLE II

Effects of Various Reagents on Mouse Neutrophil Elastase

Reagent	Concentration	Activity remaining (%)
PMSF	1 mM	52.0
N-Ethylmaleimide	1 mM	100
p-Chloromercuribenzoate	1 mM	98.5
Soybean trypsin inhibitor	0.1 mg/ml	26.0
DTT	1 mM	76.0
	10 mM	48.0
SDS	0.1%	0
EDTA	1 mM	100
	10 mM	100

The peritoneal neutrophils from C57BL/6J (+/+) mice were prepared as described in Materials and Methods. The extracts were preincubated in 20 mM imidazole-HCl, pH 7.2, containing 0.1 M NaCl, 0.2% Triton X-100, and 0.2 M sucrose at 37°C for 30 min with the reagents, and remaining activity was assayed by standard methods. Each value in the table represents the percentage of activity against a control experiment set at 100%.

TABLE III

Inhibition with Chloromethyl Ketone Inhibitors of Elastase and Cathepsin G in Peritoneal Neutrophil Extracts in Normal and Beige Mice

Enzyme	Inhibitor	Concentration (mM)	Activity remaining (%)	
			Normal	Beige
Elastase	None	0	100	100
	MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl	2×10^{-1}	0	0
	Z-Gly-Leu-Phe-CH_2Cl	2×10^{-1}	82	81
Cathepsin G	None	0	100	100
	Z-Gly-Leu-Phe-CH_2Cl	4×10^{-3}	1	0
	MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl	4×10^{-3}	98	98

Peritoneal neutrophils from C57BL/6J (+/+) and C57BL/6J (bg^J/bg^J) mice were prepared as described in Materials and Methods. 50 μg of each extract was preincubated with 0.1 mM Z-Gly-Leu-Phe-CH_2Cl or 0.1 mM MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, and this mixture was assayed for cathepsin G. 15 μg of each extract was preincubated with 0.5 mM Z-Gly-Leu-Phe-CH_2Cl or 0.5 mM MeO-Suc-Ala-Ala-Pro-Val-MCA, and this mixture was assayed for elastase. The mixture of extracts and inhibitors was incubated for 30 min at 25°C in 20 mM imidazole-HCl, pH 7.3, 0.2% Triton X-100, and 0.2 M sucrose. Elastase and cathepsin G were assayed with MeO-Suc-Ala-Ala-Pro-Val-MCA and Suc-Ala-Ala-Pro-Phe-*p*NA, respectively, as described in Materials and Methods. The final concentration of inhibitors is indicated in the table, and activity of enzyme is expressed as a percentage of activity against a control experiment set at 100%.

Phe-CH_2Cl. In contrast, 0.004 mM Z-Gly-Leu-Phe-CH_2Cl nearly completely inhibited cathepsin G in extracts of normal and beige mice, while 0.004 mM MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl caused little or no inhibition. Therefore, the elastase and cathepsin G assays exhibit nearly complete differential inhibition by the two inhibitors, suggesting that the two assays are specific for their respective enzymes.

Activities of Other Enzymes. Other typical lysosomal enzymes, including another proteinase, cathepsin D, the glycohydrolases, β-galactosidase and β-glucuroni-

TABLE IV

Enzymatic Activities of Peritoneal Neutrophils in Normal and Beige Mice

Enzyme	Substrate	Normal (+/+) (*n*)	Beige (bg^J/bg^J) (*n*)
Cathepsin D	Hemoglobin	3.32 ± 1.17 (3)	4.33 ± 1.13 (3)
Collagenase-like peptidase	Suc-Gly-Pro-Leu-Gly-Pro-MCA	0.438 ± 0.030 (4)	1.220 ± 0.220 (3)*
β-Galactosidase	4-methylumbelliferyl-β-D-galactoside	3.74 ± 0.40 (5)	4.32 ± 0.36 (3)
β-Glucuronidase	4-methylumbelliferyl-β-D-glucuronide	7.14 ± 0.46 (4)	10.98 ± 1.04 (3)
Myeloperoxidase	H_2O_2	2.36 ± 0.26 (3)	3.13 ± 0.60 (3)
Plasminogen activator(s)	*t*-Boc-Val-Gly-Arg-MCA	0.063 ± 0.005 (4)	0.103 ± 0.019 (3)

Peritoneal neutrophils from C57BL/6J (+/+) and C57BL/6J (bg^J/bg^J) mice were prepared as described in Materials and Methods. Activities are expressed as U/PMN ($\times 10^6$) ±SEM, with number of samples assayed (*n*) shown in parentheses. Cathepsin D activity is expressed as ΔE/min at 280 nm ($\times 10^3$); Units of activity: Collagenase-like peptidase and plasminogen activator(s) are expressed as 1 μmol of product per minute; myeloperoxidase as ΔE/s at 350 nm ($\times 10^4$); β-galactosidase and β-glucuronidase activities are expressed as 1 μmol of product per hour ($\times 10^3$). Tests for significance are between normal and beige mice.

* $p \leq 0.05$.

dase, and myeloperoxidase were not significantly different in normal and beige neutrophil extracts (Table IV). Two other proteinases, plasminogen activator(s) and collagenase-like peptidase, whose subcellular locations have not been established, were also assayed. There was no difference in plasminogen activator(s) activity in normal and beige extracts. There was, however, a relatively small (2.8-fold) but significant increase in collagenase-like peptidase activity of beige extracts.

Mechanism of Decreased Elastase and Cathepsin G Activities. Several indirect tests were performed to test for the presence of inactive proenzyme forms. Neither trypsin nor chymotrypsin treatment, however, activated elastase in beige extracts. Chaotropic reagents such as $NaClO_4$ and NaSCN (up to 1.5 M) or incubation of beige extracts at 37°C for 10–20 h likewise produced no activation of elastase activity.

We also tested, by reciprocal bone marrow transplantation, whether the mechanism of action of the elastase and cathepsin G deficiency involved the humoral environment of neutrophils, or was an intrinsic cellular defect in neutrophils of beige mice. We found that peritoneal neutrophils derived from beige mice transplanted with normal marrow retained high elastase and cathepsin G levels, while these activities were barely detectable in peritoneal neutrophils derived from normal mice transplanted with beige marrow (Table V). It is likely, therefore, that the lowered proteinase activity in beige neutrophils is a cellular defect intrinsic to bone marrow cells.

We wished to determine whether the beige mutation affected other serine proteinases. Incubation of extracts with labeled DIFP, which is known to covalently bind to the active site of serine proteinases, is one method of testing this question. Accordingly, extracts were labeled with [^{3}H]DIFP and individual labeled proteins were detected and compared in normal and beige extracts after SDS-PAGE and exposure to x-ray film. There was no difference in the fluoro-

TABLE V
Reciprocal Bone Marrow Transplantation Between Beige and Normal Mice

Donor	Recipient	Mouse	Activity (U/PMN × 10^6)	
			Cathepsin G	Elastase
+/+	bg^J/bg^J	1	2.89	2.87
		2	3.39	5.79
bg^J/bg^J	+/+	1	0.11	0
		2	0.14	0.14

Bone marrow was transplanted between C57BL/6J (+/+) and C57BL/6J (bg^J/bg^J) as described in Materials and Methods. Peritoneal neutrophils were prepared from C57BL/6J (+/+) and C57BL/6J (bg^J/bg^J), as described in Materials and Methods. Cathepsin G and elastase activities were assayed from each preparation by the standard procedure, with the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA for elastase and Suc-Ala-Ala-Pro-Phe-*p*NA for cathepsin G.

graphic patterns of labeled protein, whether [^{3}H]DIFP was added before or after homogenization of neutrophils of normal and beige mice. In agreement with the elastase and cathepsin G assay results, beige neutrophil extract is markedly deficient in one major and two minor [^{3}H]DIFP-labeled bands (2,500 vs. 30,300 cpm total in the three bands in normal extracts) at M_r 25,000, 27,000, and 30,000 (Fig. 2). This result is in agreement with the deficiency of elastase and cathepsin G activities, since similar M_r have been reported for elastase and cathepsin G of human neutrophils (17, 26, 39). In contrast, there were no significant differences between normal and beige mice in seven other relatively minor [^{3}H]DIFP-labeled proteins at M_r 90,000–95,000, 75,000–80,000, 50,000, and 22,000. There were likewise no major differences in total neutrophil proteins of normal and beige mice, as determined by Coomassie Blue staining (Fig. 2, *b* and *c*).

Evidence that the mechanism responsible for the deficiency in elastase and cathepsin G involves processing genes rather than alterations in the structural genes for elastase and cathepsin G was obtained from analysis of these activities in F_1 heterozygous mice from a cross between C57BL/6J (+/+) and C57BL/6J (bg^J/bg^J). The values of cathepsin G and elastase activities in neutrophils of F_1 mice, rather than being intermediate between normal and mutant values as expected for a structural gene mutation (40), are not significantly different from normal levels (Table VI).

Discussion

These findings show that both elastase and cathepsin G, as assayed with specific (17, 41, 42) and sensitive synthetic substrates, are nearly completely missing in inflammatory neutrophils of four independent beige mouse mutants. This finding confirms and extends the observation of Vassalli et al. (1), who pointed out the leukocyte neutral proteinase deficiency in CHS patients and beige mice, using fibrin degradation as an assay.

The elastase plus cathepsin G deficiency appears to be intrinsic to the cells of

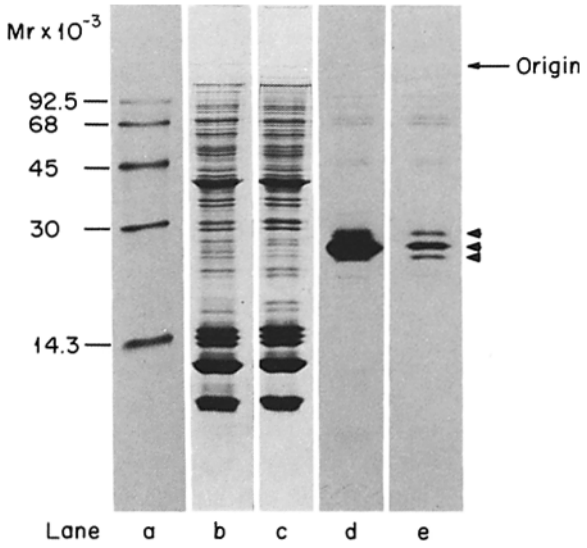

FIGURE 2. SDS-PAGE of [^{3}H]DIFP-labeled protein from peritoneal neutrophils of normal and beige mice. [^{3}H]DIFP-labeled proteins from peritoneal neutrophils of normal and beige mice were analyzed on 12.5% polyacrylamide gel containing 0.1% SDS. (*a*) [^{14}C]-labeled standard proteins: phosphorylase B (M_r, 92,500), BSA (M_r, 68,000), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 30,000), and lysozyme (M_r, 14,300), respectively. Lanes *b* and *d*, peritoneal neutrophil extract from normal mice (1.3×10^6 neutrophils); lanes *c* and *e*, peritoneal neutrophil extract from beige mice (1.3×10^6 neutrophils). Lanes *b* and *c* were stained for protein with Coomassie Brilliant Blue. After staining for protein and enhancing, the gels (lanes *b* and *c*) were processed for fluorography to obtain lanes *d* and *e*. Fluorograms were obtained by exposure of the gel to x-ray film for 6 d. Horizontal arrows indicate [^{3}H]DIFP-labeled bands at M_r 25,000, 27,000, and 30,000, which are decreased in neutrophils of beige mice.

TABLE VI

Peritoneal Neutrophil Cathepsin G and Elastase Activity of Heterozygous (+/bg^J) Mice

Genotype	Activity (U/PMN × 10^6) (*n*)	
	Cathepsin G	Elastase
C57BL/6J (+/+)	4.13 ± 0.39 (6)	4.26 ± 0.58 (9)
C57BL/6J (+/bg^J)	5.87 ± 0.41 (6)	5.31 ± 0.75 (9)
C57BL/6J (bg^J/bg^J)	0.29 ± 0.04 (4)*	0.11 ± 0.05 (6)*

Elastase was assayed with the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA and cathepsin G with Suc-Ala-Ala-Pro-Phe-*p*NA. Activities are expressed as mean ±SEM of assays of the number of individual mice (*n*) in parentheses.

* $p \leq 0.001$.

beige mice rather than humoral, since bone marrow transplantation from normal to beige corrected the proteinase deficiency. This general conclusion confirms studies of other properties of the beige mutation (reviewed in 14) that the primary CHS defect is a true cellular defect rather than an altered diffusible regulatory product. Also, recent studies have successfully corrected several characteristics of CHS by transplantation of normal marrow into an affected

patient (43) and into beige mice (23, 44). It remains possible that a diffusible factor produced by other bone marrow derived cells could explain these results.

The deficiency of elastase and cathepsin G in beige neutrophils is specific in that other lysosomal enzymes including β-glucuronidase, β-galactosidase, myeloperoxidase, and cathepsin D remain at normal levels. We have likewise shown in previous studies (29) that β-glucuronidase, β-galactosidase, and hexosaminadase are found at normal levels in six organs of beige mice. The normal levels of cathepsin D indicate that there is not a general proteinase deficiency. Also, there is not a general deficiency of serine proteinases, as determined by the fact that [^{3}H]DIFP bound at normal levels to a large number of serine proteinases at M_r different from that of elastase and cathepsin G. Likewise, major neutrophil protein components, separated by one-dimensional gel electrophoresis, are identical in normal and beige mice.

As Vassalli et al. (1) pointed out, a decrease in elastase activity could be responsible for the increased susceptibility to infection that is characteristic of this disease. The peptidoglycan structure of an organism such as *Staphylococcus aureus*, to which CHS patients are specifically susceptible (45) contains an elastase-sensitive bond. Our results show that the proteinase deficiency in this disease is more severe than simply a loss of elastase activity. It is possible that the additional deficiency of cathepsin G may exacerbate the inability to kill invading microorganisms associated with the elastase deficiency. In other systems, Boudiere et al. (46) found that the elastolytic activity was stimulated five to six times higher than that of elastase alone or cathepsin G alone when a mixture of both enzymes was used. A more modest 1.1–2.9-fold synergism of elastolysis was reported by Reilly et al. (47).

Our results also show that the deficiency of elastase and catepsin G is unlikely to be the primary genetic defect in CHS. First, two enzymes (rather than one as would be expected for a primary defect) are affected. Second, F_1 heterozygous mice do not show intermediate levels of either enzyme, as would be expected for a mutation in the primary structure of the enzyme (40).

Summary

A profound decrease in activities of the two lysosomal serine proteinases, elastase, and cathepsin G, was found in neutrophils of four independent beige mutants. Elastase and cathepsin G activities were assayed with the specific synthetic substrates MeO-Suc-Ala-Ala-Pro-Val-MCA and Suc-Ala-Ala-Pro-Phe-*p*NA, respectively. The defect is intrinsic to cells of beige mice, since transplantation of bone marrow from normal to mutant mice restored normal proteinase activity, and normal mice transplanted with beige marrow produced neutrophils with a deficiency of proteinase activity. The loss of elastase and cathepsin G activity was confirmed by separation of [^{3}H]diisopropylfluorophosphate-labeled proteins on denaturing gels, which also revealed that other serine proteinases are at normal levels in beige neutrophil extracts. The deficiency of lysosomal proteinase activity appears specific, in that four other common neutrophil lysosomal enzymes, plus the spectrum of major neutrophil proteins are not affected by the beige mutation. The deficiency of proteinase activity is likely not the primary genetic alteration of the beige mutation, since more than one proteinase

is affected, and heterozygous F_1 mice have normal rather than intermediate levels of both proteinases. The lowered proteinase activity may contribute to the high susceptibility of beige mice and Chediak-Higashi patients to infection.

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