Granule Enzymes of Polymorphonuclear Neutrophils: A Phylogenetic Comparison

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The functional significance of granule enzymes in polymorphonuclear leukocytes (PMN) is not fully understood because of the multiplicity of the enzymes and the rare occurrence of deficiencies in man. In order to select appropriate laboratory animals for functional studies, a phylogenetic comparison of enzyme levels in animal and human PMN was undertaken. Neutrophils were obtained from a variety of laboratory animals and man; the activities of alkaline phosphatase, lysozyme, myeloperoxidase, and β-glucuronidase were determined by histochemical and analytical techniques. Marked interspecies differences in enzyme activity were found; many species were deficient in alkaline phosphatase or lysozyme. Differences in the pH optima and metal requirements of alkaline phosphatase were not of sufficient magnitude to explain the variations of this enzyme.

WHEN POLYMORPHONUCLEAR leukocytes (PMN) phagocytize microorganisms, rapid degranulation occurs as cytoplasmic granules fuse with the phagocytic vacuole and discharge their hydrolases and bactericidal proteins.[1] Investigations of bactericidal and digestive functions of PMN have thus centered on the morphology of PMN granules and the biochemistry of their enzymatic constituents.[2] Studies in rabbits and man have identified two major types of granules, azurophilic and specific.[3] The former are larger and are synthesized in the promyelocyte; they contain myeloperoxidase (MPO), lysozyme (LZM), cationic antibacterial proteins, and a number of acid hydrolases including β-glucuronidase (βGU).[4] Specific granules are smaller and are formed only in the myelocyte; they contain lysozyme (LZM), lactoferrin,[5] and, in rabbits, alkaline phosphatase (AKP).[4,6] In human PMN, AKP is not contained in specific granules, but in a third particle.[7]

That PMN enzymes contribute to the overall bactericidal ability of the cell has long been assumed, but only in the case of MPO and LZM have specific functions been identified.[2] The functional importance of other PMN enyzmes has been difficult to determine because of their multiplicity and the inability to study them individually.

Deficiencies of one or more enzymes in the PMN of man[8-11] have been reported periodically; investigations of such patients afford one approach to functional analyses. However, the occurrence of such deficiencies is infrequent, and the variety of studies that can be performed is limited.

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Laboratory animals in which enzyme deficiencies naturally occur offer another opportunity to evaluate enzymatic function. Unfortunately, identification of suitable models for investigation has been difficult, since few interspecies comparisons have been published. Although semiquantitative histochemical comparisons of AKP and MPO are available,[12,13] biochemical activity or multiple enzyme comparisons have not been reported. The present study was undertaken to identify suitable animal models for assessment of the physiologic importance and function of PMN granule enzymes.

MATERIALS AND METHODS

Blood was collected in EDTA from humans and laboratory animals. Animals were healthy and free from infection at the time of phlebotomy. Erythrocytes (RBC) were lysed by exposure to 2 volumes of 0.87% NH_4Cl at 4°C for 10 min, and leukocytes were removed by centrifugation and washed twice in 0.15 *M* NaCl (NS). The leukocytes were suspended in 10 ml of Hepes-buffered Hank's balanced salt solution, pH 7.4 (HBSS-HEPES) containing 1% bovine serum albumin; they were layered on top of 5 ml Isopaque-Ficol solution (LSM, Litton Bionetics) and centrifuged at 400 *g* for 40 min.[14] The granulocyte pellet was removed, washed twice in NS, and diluted to 5×10^6 PMN per ml. Any preparation containing less than 90% PMN was discarded. Since the nucleated RBC in avian blood were resistant to lysis by NH_4Cl, they were first removed by Dextran sedimentation[15] and the leukocytes then processed as described above.

For cellular enzyme determinations, isolated leukocyte suspensions were sonicated in an ice bath at 80 W (Bronson Sonifier) with three 30-sec periods of exposure. Triton X-100 was then added to a final concentration of 0.1%. AKP (EC 3.13.1) was determined in 0.62 *M* 2-amino-2-methyl-1-propanol buffer (pH 10.2) with 15.2 m*M* *p*-nitrophenylphosphate and 0.1 m*M* $MgCl_2$ at 37°C for 30 min. The reaction was stopped with 1.25 *M* NaOH and absorbance read in a Beckman DU recording spectrophotometer at 410 nm.[16] Nanomoles of substrate hydrolyzed per minute were calculated using the molar extinction coefficient (1.62×10^4). The reaction was linear throughout the range obtained and achieved a sensitivity of 1.0 nmole/min. Results are expressed to the nearest whole integer. MPO (EC 1.11.1.7) was assayed with 3.3 m*M* *o*-anisidine in 0.1 *M* phosphate buffer (pH 6.8) with 10 m*M* hydrogen peroxide. Change in absorbance was monitored continuously at 460 nm and results expressed as change in optical density per minute.[17] β-glucuronidase (βGU) (EC 3.2.1.31) activity was determined at 56°C for 2 hr with 1.0 m*M* phenolphthalein glucuronide in 0.1 *M* acetate buffer (pH 5.0).[18] Nanomoles of substrate hydrolyzed per minute were calculated using the molar extinction coefficient of 2.52×10^4. Maximum sensitivity of the assay under the conditions employed was 0.25 nmole/min. LZM (EC 3.2.1.17) was determined by the lysoplate method of Osserman and Lawlor[19] using egg white LZM as standard.

Hydrogen ion optima for AKP were determined with a series of amino methyl propanol buffers prepared in increments of 0.2 pH units from pH 8.0–10.6. Metal requirements were determined by the inclusion of 0.1 m*M* Mg^{2+}, Zn^{2+}, or Fe^{2+} in the incubation medium. The per cent change was calculated from the activity determined without addition of metal ions.

Blood smears for the histochemical demonstration of MPO and AKP were processed and scored according to the methods of Kaplow.[20,21]

RESULTS

Marked interspecies variations in enzyme activity were detected (Table 1). βGU was present in variable amounts in PMN from all species. MPO was present in all species except chickens and geese. Monkeys, cattle, goats, sheep, cats, and hamsters were deficient in LZM, while AKP was undetectable in rhesus monkeys, cats, two strains of inbred mice, chickens, and geese. Cat and rhesus monkey PMN were deficient in both AKP and LZM.

Histochemical results correlated closely with biochemical activity for all species examined. In most species the MPO reaction product was visualized in

Table 1. Enzyme Activity in PMN From Various Species

Species		No. Tested	Units* per 5 × 10^6 PMN ± SEM			
			βGU	MPO	LZM	AKP
Primates	Human	7	7.7 ± 2.4	109 ± 44	86 ± 11	11 ± 4
	Monkey—Rhesus	7	3.0 ± 1.3	38 ± 7	<1	<1
	Cynomolgus	5	0.3 ± 0.1	68 ± 5	<1	114 ± 30
	Squirrel	3†	0.4 ± 0.1	127 ± 9	<1	87 ± 4
Ungulata	Cow	4	0.4 ± 0.2	27 ± 2	<1	38 ± 8
	Goat	9	0.6 ± 0.1	6 ± 1	<1	27 ± 11
	Sheep	9	1.1 ± 0.4	19 ± 3	<1	30 ± 4
	Horse	3	1.0 ± 0.3	39 ± 10	16 ± 1	471 ± 76
	Burro	11	1.7 ± 0.4	63 ± 9	8 ± 1	536 ± 201
Carnivora	Cat	3	2.9 ± 0.7	9 ± 1	<1	<1
	Dog	6	0.6 ± 0.2	95 ± 26	9 ± 2	8 ± 4
Lagomorpha	Rabbit	5	0.7 ± 0.2	13 ± 6	15 ± 3	99 ± 46
Rodentia	Rat	10†	5.1 ± 0.6	46 ± 4	10 ± 2	95 ± 34
	Guinea pig	6	2.1 ± 0.2	11 ± 3	15 ± 3	471 ± 91
	Mouse—AKR	2†	0.7	18	10	<1
	C57	2†	1.0	20	5	<1
	Hamster	3†	0.7 ± 0.1	16 ± 3	<1	273 ± 23
Aves	Chicken	3	1.1 ± 0.5	0	84 ± 14	<1
	Goose	4	0.9 ± 0.1	0	24 ± 4	<1

*βGU and AKP, nmole/min; MPO ΔOD/min (× 10^{-3}); LZM, μg.

†Number of pools tested.

neutrophils, eosinophils, and to a lesser extent in monocytes. In chickens and geese, however, MPO was present only in eosinophils. AKP, if present, was seen only in neutrophils; in burros, however, the enzyme was present in eosinophils as well.

With the exception of horses and sheep, AKP from all sources exhibited maximal biochemical activity at pH 9.8–10.0. Human, sheep, and rabbit AKP required Mg^{2+} for maximum activity, but enzymes from other species were activated by Zn^{2+} (Table 2).

There was no correlation (r = 0.170) between PMN enzyme content and the absolute number of PMN normally present in the peripheral blood of each species.

DISCUSSION

MPO in conjunction with H_2O_2 and oxidizable cofactors has been shown to exert potent bactericidal effects,[2,22] and clinical reports are now appearing of infections occurring in association with MPO deficiencies in man.[8,9] Avian PMN have previously been reported to be deficient in MPO[23] but kill bacteria without difficulty.[24] Since other bactericidal mechanisms must be operative in these cells, it is interesting to note that both chickens and geese have higher levels of LZM than most other species studied.

The functional importance of βGU in PMN granules has not been determined, but the enzyme may degrade β-glucuronic acid moieties present in the capsular polysaccharides of types II, III, and V of *Streptococcus pneumoniae* and some types of *Escherichia coli, Klebsiella,* and *Salmonella.*[25] Lysis of the bacterial capsule might then permit other bactericidal substances to take effect.

Table 2. Interspecies Comparison of pH Optima and Heavy Metal Requirements* for PMN AKP Activity (Values refer to per cent change from the biochemical activity determined at optimal pH without cation addition.†)

Species	pH Optimum	Per Cent Change			
		Mg^{2+}	Zn^{2+}	$Mg^{2+} + Zn^{2+}$	Fe^{2+}
Primates					
Human	9.8–10.0	+5	+4	+9	0
Cynomolgus monkey	—	0	+340	+270	−20
Ungulata					
Cow	9.8–10.0	0	+200	+100	+30
Goat	—	−50	+120	−40	0
Sheep	9.0–9.2	+5	−35	−10	0
Horse	9.0–9.2	−30	+260	+210	−30
Burro	9.8–10.0	−5	+160	+100	−10
Lagomorpha					
Rabbit	9.8–10.0	+5	−15	0	−15
Rodentia					
Rat	9.8–10.0	+2	+15	+15	−10
Guinea pig	9.8–10.0	+80	+680	+515	+65

*0.1 mM of metal ion.

†AKP undetectable in rhesus monkeys, cats, mice, chickens, and geese regardless of assay conditions.

Sly et al.[26] reported a case of a mucopolysaccharidosis secondary to βGU deficiency. While no PMN functional studies were reported, increased PMN granulations were noted, and the patient had three episodes of pneumonia in the first 30 mo of life. Both PMN and cultured fibroblasts were found to lack βGU.

To our knowledge, there have been no previous comparisons of βGU activity in the PMN of laboratory animals. Our finding that it is present in all species and the findings reported by Sly et al.[26] suggest that, regardless of mechanism, it may be essential to PMN function.

LZM is found in both azurophilic and specific granules[7] and is a constituent of both neutrophils and monocytes.[19] We have previously reported that LZM was deficient in rhesus monkey PMN but present in rhesus monocytes and macrophages.[27] Others have noted LZM deficiencies in cattle[28] and a strain of rabbits.[29] The present report confirms these findings and documents additional deficiencies occurring in the PMN of monkeys, goats, sheep, cats, and hamsters.

LZM hydrolyzes peptidoglycan components of cell walls of Gram-positive bacteria and occasionally Gram-negative organisms.[2] While the enzyme may be bactericidal, it is probably most important in aiding digestion of bacteria already killed by other mechanisms. Thus it is not unexpected that both rhesus[30] and bovine[28] PMN phagocytize and kill bacteria without difficulty.

Spitznagel et al.[10] demonstrated a bactericidal defect in PMN from a patient with combined deficiencies of LZM and lactoferrin. However, since the specific granules themselves were absent, the case is not comparable to our data. In future studies, the LZM-deficient species identified here may be useful for identification of the functional importance of LZM.

It should be noted that, although our cell preparations contained at least 90% granulocytes, contaminating monocytes could contribute some activity, and therefore the values in Table 1 cannot be considered absolute. However, since

the actual number of monocytes was low, we doubt that any error is of practical importance.

AKP cannot be demonstrated histochemically in feline, canine,[12] simian, or murine PMN.[31] We obtained similar results and also demonstrated AKP deficiency in avian PMN. The activity in burro eosinophils that we noted has been previously reported,[32] but the significance of this finding is unknown. Our biochemical analyses closely approximate the histochemical data in all species except the dog, in which low levels of enzyme activity were detected.

In human PMN, AKP content is variable, and deficiencies have been reported in a variety of clinical disorders. For a full discussion of these changes, the reader is referred to Kaplow's excellent review.[32] Neither the function nor physiologic substrate of AKP is known, but the marked increases in activity that occur during infection have long been of interest.[33] If induction is a physiologic response, it is valid to ask what purpose it serves and how AKP-deficient species compensate for its absence. Accordingly, it is interesting to note that PMN from experimentally infected rhesus monkeys contain high levels of AKP, and that induction is noted as early as 12 hr following inoculation.[27] Similarly, AKP is reported to increase during infection in patients with chronic myelogenous leukemia[34] and adult hypophosphatasia.[35] Strauss et al.[11] reported deficient bactericidal capacity and recurrent infections in a patient whose PMN contained abnormal specific granules. Although the authors did not test for specific granule markers, AKP was deficient and did not increase during infection.

Our data do not permit any conclusions regarding AKP function; nevertheless, they allow the choice of appropriate experimental animals for future investigations of activation mechanisms and functional significance of AKP.

Interspecies enzyme variations can be explained either by differences in content or by biochemical differences between enzymes which require methodologic differences in assay conditions. The AKP data suggest that biochemical differences are not sufficient to explain all the variations observed; but more significantly, for those species deficient in alkaline phosphatase, the deficiency was not artifactual, since the enzyme remained undetectable regardless of pH or metal ion changes. Because of limited sample volumes, similar studies were not performed for other enzymes, but presumably the same conditions apply.

Another possible explanation for these variations is that PMN enzyme activity in each species is related to the relative or absolute number of PMN normally present in peripheral blood. However, regression analysis of activity versus absolute PMN count revealed no correlation. Finally, some granule enzymes might actually be present but in an inactive form in certain species. To test this possibility, specific antibody studies will be required.

Investigations of the functional significance of individual PMN enzymes have been limited because of the infrequent occurrence of genetic enzyme deficiencies in human PMN. Identification of a large number of suitable animal models in which specific deficiencies naturally occur should facilitate the choice of appropriate animals for more detailed studies of PMN enzymatic function and bactericidal mechanisms. Further, these data emphasize the need for careful selection of animal models in experiments concerned with PMN function or enzymatic changes.

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