PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 342-347 (1978)

Biochemical Properties of Bovine Granulocytes (40050)

R. GENNARO, C. SCHNEIDER, G. DE NICOLA, F. CIAN AND D. ROMEO

Department of Biochemistry and Department of General Pathology, University of Trieste, 34127 Trieste, Italy

A number of investigations carried out *in vitro* on isolated granulocytes have allowed an increase in knowledge on the molecular mechanisms of host defense against invading microorganisms (1, 2). In addition, these investigations have contributed to a better understanding of cell events of broader biological interest, such as those concerning signal transduction from the cell environment to the cytosol, activation of metabolic pathways, modulation of cell functions by cytoskeletal components, secretion of granule associated enzymes (3, 4).

For some of these studies, in particular those involving separation of either subcellular fractions or enzymes, fairly large amounts of cells are required. For this purpose, granulocytes isolated from exudates of laboratory animals are generally used. So far most of the biochemical studies on blood derived granulocytes have been performed on cells of human blood, either donated by volunteers or supplied by blood banks. This sometimes might cause limitations because of the amount of cells available. We, therefore, thought it might be useful to secure another source of blood granulocytes with a precise biochemical characterization. In this paper we describe the ultrastructure, enzyme content and features of the oxidative metabolism of granulocytes isolated from bovine blood, which can be obtained in unlimited amounts from slaughterhouses, and compare the biochemical properties of these cells with those of granulocytes of human blood.

Materials and methods. Granulocytes were isolated from bovine blood, essentially as described by Carlson and Kaneko (5), washed once and suspended in Ca^{2+}-free Krebs–Ringer phosphate (KRP), pH 7.4. The white cell fraction of human blood anticoagulated in ACD was isolated by a conventional technique, after dextran sedimentation of the erythrocytes. Differential counts were performed on May-Grünwald-Giemsa stained smears.

Protein was determined by the method of Lowry *et al.* (6), using bovine serum albumin (BSA) as standard. Myeloperoxidase was measured biochemically as described by Romeo *et al.* (7) and histochemically according to Graham and Karnovsky (8). β-Glucuronidase was determined fluorimetrically (9) in 0.5 ml of 0.05 *M* Na-acetate buffer, pH 3.8, containing 0.05% BSA with 0.32 m*M* 4-methylumbelliferyl-β-D-glucuronide (Sigma, USA) as substrate; the reaction was stopped with 1.5 ml of 0.2 *M* Na-glycine buffer, pH 10.45. β-Galactosidase was assayed by the same procedure except for the pH of the buffer, which was 4.5, the substrate being 0.54 m*M* 4-methylumbelliferyl-β-D-galactoside (Sigma, USA). Acid phosphatase was measured in 0.5 ml of 0.1 *M* Na-acetate buffer, pH 4.5, with 4.5 m*M* *p*-nitrophenyl phosphate (Sigma, USA) as substrate (10); the reaction was stopped with 0.5 ml of 1 *M* NaOH. The rate of splitting of 4.5 m*M* *p*-nitrophenyl phosphate by alkaline phosphatase was directly recorded at 410 nm in 1 ml of 0.1 *M* diethanolamine-HCl buffer, pH 9.7, with 1 m*M* $MgCl_2$. Lysozyme was determined nephelometrically by the rate of lysis of cell walls of *Micrococcus lysodeikticus* in Na-phosphate, pH 6.6 (ionic strenght = 0.5); crystalline hen egg-white lysozyme (S.P.A., Italy) was used as standard. The oxidation of NADH by oxalacetate was followed to measure the activity of malate dehydrogenase (11). Catalase was assayed by the rate of decrease of absorbance of H_2O_2 at 230 nm (ϵ_{230} = 0.067 cm^{-1} mM^{-1}) (12). The activity of glutathione peroxidase and glutathione reductase was determined at 37° in a final volume of 1 ml of 20 m*M* KH_2PO_4-Na_2HPO_4 buffer, pH 7.0, essentially as described by Reed (13). For assay of glutathione peroxidase, cuvettes contained, in addition to the above, 5 m*M* ethylenediaminotetraacetate (pH 7.0), 1 m*M* sodium azide, 0.01% BSA, 1 m*M* GSH, 0.1 m*M* NADPH, 30 mU of yeast glutathione reductase (Boehringer, Germany)

0037-9727/78/1573-0342$01.00/0

and 0.1 m*M* H_2O_2. For assays of glutathione reductase, cuvettes contained 0.01% BSA, 1 m*M* GSSG and 0.1 m*M* NADPH.

The determination of the activity of catalase, glutathione peroxidase and glutathione reductase was carried out with postgranular supernatants, obtained by centrifugation (10,000*g*, 10 min) of cells disrupted by homogenization in 0.34 *M* sucrose (7). All the other assays were performed with cells disrupted by sonication (Branson Sonifier, 3.5 amp, 10 sec). The reaction media of β-glucuronidase, β-galactosidase, acid and alkaline phosphatase, and malate dehydrogenase also contained 0.05% Triton X-100. All the enzyme assays were shown to be linear with respect to time of incubation and amount of cell extracts or sonicates added to the medium. The rates of reactions were corrected for nonenzymatic rate before the calculation of specific activity of enzymes.

Vitamin B_{12}-binding capacity of cell sonicates was measured by the method described by Kane *et al.* (14). The incubation mixture (1 ml) contained 0.55 ng of ^{57}Co-B_{12} (0.18 μCi/ng, Radiochemical Centre, England) and 3.5 ng cold vitamin B_{12}; excess free vitamin B_{12} was removed with 0.5 ml of albumin-coated charcoal and 1 ml of the centrifuged samples was counted.

Estimation of lactoferrin content of bovine granulocytes was carried out by Ouchterlony double immunodiffusion in agarose, and staining of the immune precipitates was performed with amido black according to Wieme (15). Cells were extracted either in KRP (pH 7.4) containing 0.3% cetyl trimethyl ammonium bromide (16) or in 1 *M* NaCl overnight at 4° (17) (an identical recovery of lactoferrin was obtained with the two extraction procedures). The extracts were reacted with antiserum to bovine milk lactoferrin (a gift of Prof. P. Masson), and purified bovine lactoferrin (a gift of Prof. B. Senft) was used as standard antigen. Twelve dilutions of the standard antigen (from 1 to 100 μg/ml) and at least six dilutions of granulocyte extracts (from 1 to 10 × 10^6 cells/ml) were tested against the antiserum.

Phagocytic stimulation of granulocyte metabolism was accomplished by exposing the cells at 37° to autoclaved *Bacillus mycoides*, opsonized with fresh bovine serum. Oxygen consumption, superoxide anion recovery, hydrogen peroxide accumulation and ^{14}C-glucose oxidation were measured as described by Dri *et al.* (18).

Escherichia coli J53 (K 12) and *Staphylococcus aureus* (strain Oxford) were grown overnight in nutrient broth and BHI respectively, washed once and suspended in KRP, pH 6.0 or pH 7.4, with 0.5 m*M* $CaCl_2$. Bactericidal activity was determined by adding 1 × 10^7 bacteria to 2 × 10^6 granulocytes disrupted by sonication, frozen at −70° and then thawed, in a final volume of 0.3 ml of KRP, pH 6.0 or pH 7.4, with 0.5 m*M* $CaCl_2$. After 30 min incubation at 37°, aliquots were diluted and plated to obtain viable cell counts. Controls were run in the absence of either bacteria or granulocyte sonicates.

For electron microscopic observations cells were fixed at 4° for 60 min in 2% glutaraldehyde (0.1 *M* cacodylate buffer, pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in ethanol, carried through propylene oxide, embedded in DER 332, and counterstained with uranyl acetate and lead citrate.

Results. We carried out thirty cell preparations with an average yield (± SEM) of 1.25 (± 0.03) × 10^6 white cells per ml of bovine blood. Of these cells 96.7 (± 0.1)% were granulocytes, the ratio between neutrophils and eosinophils varying from 200:1 to 5:1. By processing two or more blood samples at the same time, cell preparations with the highest content of eosinophils could be discarded. By following this procedure we were able to perform the biochemical assays on cell suspensions containing an average of 92% neutrophils (see Table I).

The electron microscope observation of bovine blood granulocytes (Fig. 1) shows that neutrophils are relatively small (diameter about 7 μm), rounded or slightly elongated cells with occasional short cytoplasmic projections. Several spherical, elongated, rod and dumb-bell shaped granules, are noticed in the dense cytoplasm. They are all highly and homogeneously electron-dense. In many cell sections a roughly circular, granule-free area is observed in the cytoplasm, close to the nuclear lobes, but sometimes in an eccentric position. This is the centrosphere region, circumscribed by stacks of four to nine smooth-surfaced, curved Golgi cisternae, oriented

TABLE I. ENZYME ACTIVITIES AND CONTENT OF BINDING PROTEINS OF GRANULOCYTES OF BOVINE AND HUMAN BLOOD.[a]

	Bovine granulocytes	Human granulocytes
1. β-Glucuronidase	12.8 ± 0.3 (8)	202.1 ± 18.0 (3)
2. β-Galactosidase	11.8 ± 0.6 (9)	62.4 ± 7.4 (3)
3. Myeloperoxidase	1.6 ± 0.1 (8)	4.7 ± 0.1 (3)
4. Acid phosphatase	36.7 ± 6.3 (5)	—
5. Alkaline phosphatase	230 ± 9 (8)	23.9 ± 7.0 (3)
6. Lysozyme	< 0.2 (3)	31.8 ± 3.6 (3)
7. Lactoferrin	74 ± 7 (7)	30–64 (Refs. 16, 33)
8. Vitamine B_{12}-binding capacity	26.7 ± 2.8 (3)	4–9 (Refs. 34–36)
9. Malate dehydrogenase	0.49 ± 0.04 (6)	—
10. Catalase	7.1 ± 0.5 (3)	108.2 ± 11.1 (4)
11. Glutathione peroxidase	14.1 ± 0.6 (4)	5.2 ± 0.4 (4)
12. Glutathione reductase	25.0 ± 0.8 (4)	13.2 ± 1.1 (4)
13. Protein	1.21 ± 0.03 (14)	—

[a] Sixteen preparations of bovine blood granulocytes and four preparations of human blood granulocytes were used for the assays, which were run as described in the section on Materials and Methods. The bovine cell preparations had the following average % composition (±SEM): neutrophils 92.1 ± 0.4; eosinophils 5.3 ± 0.3; monocytes 2.6 ± 0.1. Data are expressed as means ±SEM/10^7 cells; the number of experiments is shown in brackets 1 and 2: nmoles methylumbelliferone/30 min; 3: μmoles *t*-guaiacol/min; 4 and 5: nmoles *p*-nitro phenol/min; 6 and 7: μg; 8: ng vit B_{12}; 9: μmoles NADH/min; 10: μmoles H_2O_2/min; 11 and 12: nmoles NADPH/min; 13: mg.

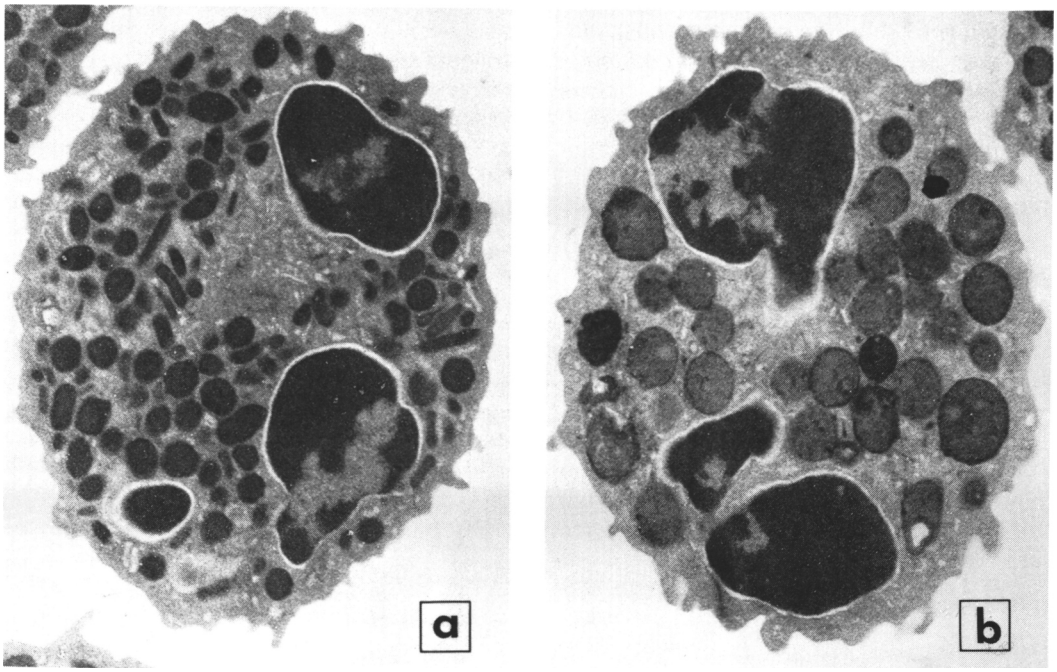

FIG. 1. Ultrastructure of a bovine blood neutrophil (a) and of a bovine blood eosinophil (b) (× 12,600).

around one or two centrioles. The cytoplasm also contains particulate glycogen and a few vesicular profiles of rough-surfaced endoplasmic reticulum but lacks appreciable quantities of most other organelles (e.g. mitochondria, ribosomes, microtubules).

Figure 1 also shows that bovine blood eosinophils have much larger granules than the neutrophils, as reported also by others (19). These granules lack the crystalloid structure characteristic for eosinophil granules in other species (20). In this regard, bovine eosinophils resemble eosinophils of horse (21), of mink (22) and of some domestic birds (23).

In Table I bovine granulocytes are compared with human leucocytes on the basis of

activities and/or content of a number of enzymes and binding proteins, selected on the basis of either their potential use as markers of subcellular fractions or their involvement in the oxidative metabolism.

The activities of β-glucuronidase, β-galactosidase and myeloperoxidase, which in other species are associated to azurophilic granules (10, 24, 25), are much lower in the bovine than in the human granulocytes. A histochemical survey on bovine granulocytes indicated that myeloperoxidase is particularly concentrated in eosinophils (data not shown), thus suggesting that the value of myeloperoxidase activity in the preparations of bovine neutrophils might in part depend on the contamination by eosinophils.

The concentration of lactoferrin and vitamin B_{12}-binding protein, markers of specific granules in other species (17, 25, 26), appears to be higher in the bovine than in the human granulocytes. As reported also by others (27), lysozyme activity is undetectable in bovine granulocytes.

With respect to the human leucocytes, the bovine granulocytes have a very low activity of catalase, the enzyme which provides an efficient system for H_2O_2 detoxification during phagocytosis (12). This deficiency, however, is counterbalanced by a higher specific activity of the two enzymes of the glutathione cycle (13), glutathione peroxidase and glutathione reductase.

The exposure of granulocytes to bacteria elicits a marked stimulation of oxygen consumption, O_2^- and H_2O_2 generation, and glucose oxidation through the hexose monophosphate pathway (HMP) (3, 13, 18, 28–30). An evaluation of the metabolic changes occurring in phagocytizing bovine leucocytes, and of the modulation exerted on them by the heme-enzyme inhibitor NaN_3 and by the O_2^- oxidizing agent ferricytochrome *c*, is reported in Table II. The significance of these data will be discussed below.

Finally, the oxygen-independent bactericidal activity of bovine granulocytes, as compared with the activity of human leucocytes, is shown in Table III. With *E. coli* the cidal efficiency of the two types of phagocytes is very similar, the activity of the lysates of bovine granulocytes being somewhat lower than that of human cell lysates at pH 7.4. On the contrary, at both pH's killing of *S. aureus* is carried out only by the bovine cell lysates.

Discussion. In this paper we have reported several biochemical features of preparations of bovine granulocytes containing an average of 92% neutrophils and less than 3% monocytes. Some enzyme properties of granules of bovine granulocytes have been already described by Hegner (19) and by Rausch and Moore (27). At least in the former case, however, cell preparations were heavily contaminated by mononuclear cells and had a high eosinophil/neutrophil ratio.

When compared to human granulocytes, the bovine cells appear to exhibit low activities of enzymes, which in other species are associated to azurophil granules (10, 24, 25), and to have a higher content of binding proteins, which belong to specific granules (17, 25, 26). Whether this is due to a different cell content of these two types of granules is difficult to assess on a morphological basis, since in bovine granulocytes azurophil and specific granules cannot be distinguished on the basis of electron density (31).

TABLE II. STIMULATION OF THE OXIDATIVE METABOLISM OF BOVINE GRANULOCYTES BY PHAGOCYTOSIS.[a]

	Resting granulocytes	Granulocytes + *B. mycoides*			
		—	+NaN_3	+cyt *c*	+cyt *c* +SOD
nmoles O_2/2 min	8.9 ± 1.4	77.9 ± 6.7	87.6 ± 8.3	70.1 ± 5.6	77.9 ± 8.2
nmoles O_2^-/2 min	—	—	—	26.1 ± 6.1	—
nmoles H_2O_2/2 min	—	8.6 ± 1.3	27.1 ± 3.3	—	—
$^{14}CO_2$ cpm/μCi 1-[^{14}C]Glc/10 min	6,665	100,525	—	—	—
$^{14}CO_2$ cpm/μCi 6-[^{14}C]Glc/10 min	110	190	—	—	—

[a] Data of O_2 consumption, O_2^- recovery and H_2O_2 accumulation are means of 4 experiments ±SEM (stimulated respirations are increments with respect to resting controls); a detailed description of the experimental conditions followed for these measurements is given in Ref. 18. Rates of glucose oxidation are results of a representative experiment. SOD: superoxide dismutase.

TABLE III. COMPARATIVE BACTERICIDAL ACTIVITY OF LYSATES OF BOVINE AND HUMAN BLOOD GRANULOCYTES.[a]

	E. coli		*S. aureus*	
	pH 6.0	pH 7.4	pH 6.0	pH 7.4
Bovine granulocyte lysates	66%	59%	13%	65%
Human granulocyte lysates	63%	85%	0	0

[a] 1×10^7 bacteria were incubated at 37° for 30 min with lysates of 2×10^6 granulocytes in 0.3 ml of KRP, pH 6.0 or 7.4, containing 0.5 m*M* $CaCl_2$. Data are average percentages of killing, calculated with respect to viable bacteria found at the end of incubations run in the absence of granulocyte lysates (two to three experiments).

The granules of bovine granulocytes appear to be endowed with bactericidal substances capable of killing *S. aureus* under conditions in which this microorganism is not killed by human leucocytes. The cidal activity of the bovine cell lysates on the gram-positive bacterium is more marked at pH 7.4 than at pH 6.0, thus suggesting that granule components with optimal activity near or above neutrality (32) are mainly responsible for the toxic effects on the staphylococci.

The exposure of bovine granulocytes to bacteria causes a stimulation of their oxidative metabolism, which is similar to that known to occur in human and guinea pig granulocytes (3, 13, 18, 28–30). The main product of the enhanced oxygen reduction in phagocytizing cells is likely to be O_2^-, which rapidly generates H_2O_2 by dismutation (18). The decrease of oxygen consumption by phagocytizing granulocytes observed in the presence of ferricytochrome *c* is due to the oxidation of the extracellularly released O_2^- to O_2. From the extent of the superoxide dismutase–inhibitable reduction of ferricytochrome *c* and on account of the stoichiometries of the O_2^- dismutation and of the catalatic degration of H_2O_2 (18), the amount of O_2^- released by phagocytizing bovine granulocytes under our experimental conditions can be calculated to be about 15%. This recovery is comparable to that obtained with human granulocytes under the same conditions (unpublished experiments).

The main mechanisms of H_2O_2 disposal in phagocytizing granulocytes involve catalase, myeloperoxidase and glutathione peroxidase (12, 13, 18, 28). By inhibiting the catalase-dependent back-conversion of H_2O_2 to O_2, NaN_3 enhances the overall O_2 consumption (18). In bovine granulocytes this enhancement is about fourfold lower than in human leucocytes (unpublished experiments) due to the deficient activity of catalase. The inhibition of catalase and myeloperoxidase by NaN_3 also causes an increased accumulation of H_2O_2, which in guinea pig granulocytes approaches 100% of the value expected from oxygen reduction (18). On the contrary, in phagocytizing bovine granulocytes, the average value of H_2O_2 measured in the presence of NaN_3 is only a small percentage of the amount calculated from the values of oxygen consumption. This suggests that in these phagocytes H_2O_2 is largely utilized by the NaN_3-insensitive glutathione cycle (13). This conclusion is consistent with (i) the low activity of catalase; (ii) the relatively high activity of glutathione peroxidase and glutathione reductase; and (iii) the remarkable activation of the HMP by phagocytosis, which is higher than that reported for granulocytes of man and guinea pig (28). The stimulation of the glutathione cycle leads, in fact, to increased generation of $NADP^+$ with activation of HMP (13).

In conclusion, bovine blood granulocytes, apart from the absence of lysozyme and from the different metabolic fate of H_2O_2, have biochemical properties very similar to those of human granulocytes. Since they can be obtained in unlimited amounts from slaughtered animals, bovine granulocytes can substitute for human cells in laboratory investigations, in particular when the isolation of large quantities of subcellular particles, enzymes and bactericidal factors is required.

Summary. Granulocyte fractions, containing an average of about 92% neutrophils, were isolated from bovine blood. The electron microscope observation of these fractions showed that neutrophil granules have different shapes, but are all highly and homogeneously electron-dense. With respect to the granulocytes of human blood, bovine cells appear to have a lower content of azurophil enzymes and virtually lack lysozyme. Lysates of bovine granulocytes efficiently kill both *E. coli* (at pH 6.0 and 7.4) and *S. aureus* (mainly at pH 7.4). When exposed to opsonized *B.*

mycoides, intact bovine granulocytes exhibit a marked enhancement in oxygen consumption, generation of O_2^- and H_2O_2, and glucose oxidation through the hexose monophosphate pathway. About 15% of the total oxygen reduced is recovered extracellularly as O_2^-. Hydrogen peroxide generated by phagocytizing cells is only partially utilized in reactions catalyzed by catalase and myeloperoxidase, and appears to mainly enter the glutathione cycle.

The authors thank Drs. P. Dri and P. Bellavite for their assistance in the measurement of oxidative metabolism of granulocytes.

Research supported by the National Research Council of Italy (CNR) (Grant No. 75.00607.04).

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Received July 8, 1977. P.S.E.B.M. 1978. Vol. 157.