Biochimica et Biophysica Acta, 972 (1988) 137–143
Elsevier

BBA 12360

Biochemical studies of neutrophils from male and female rats: a differential response to basement membrane treated with nephrotoxic antiserum

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(Received 27 May 1988)

Key words: Basement membrane; Nephrotoxic antiserum; Neutrophil; (Rat)

Rat neutrophils added to ^{3}H-labelled glomerular basement membrane (GBM) treated with rabbit anti-rat GBM antiserum degraded the GBM as judged by the release of ^{3}H-labelled peptides. Cells from female animals promoted a more marked degradation than cells from males. This correlated with measurements of higher levels of elastase in granule fractions from the cells. The subcellular distributions of granule marker enzymes was found not to differ between the sexes. Levels of myeloperoxidase, lysozyme, cathepsin G, alkaline phosphatase, γ-glutamyltranspeptidase and *N*-acetyl-β-glucosaminidase showed no sex-based differences. No α-mannosidase could be detected in the cells.

Introduction

Neutrophils react with immobilised antigen-bound antibodies via Fc-receptors on the cell surfaces [1]. Superoxide and its derivatives are generated together with arachidonic acid metabolites [2], and the cells also exocytose the content of the specific and then the azurophil granules [3]. When this chain of events is initiated in vivo tissue damage ensues. Neutrophils are known to be involved in the pathogenesis of some forms of glomerulonephritis in humans [4] and in animal models [5] of the disease; the cells can play a role in the initiation [6] as well as in the progression of the disease [4].

In modelling pathogenic events in vitro it has been shown that rabbit neutrophils respond to rat glomerular basement membrane (GBM), exposed to rabbit anti-rat GBM antiserum, by producing oxygen-derived radicals and by liberating proteinases as judged by the release of peptides from GBM [7]. Proteinase attack was prevented by elastase inhibitors in vitro [8]. In vivo proteinase action may be prevented or reduced by the presence of plasma proteinase inhibitors though myeloperoxidase, concurrently released, might inactivate proteinase inhibitors locally allowing damage to the GBM to occur [9,10].

Since there are notable species differences between neutrophils, those from rabbits do not contain cathepsin G for example, it was of interest to examine the responses of rat neutrophils to rat GBM after exposing the membranes to rabbit anti-rat antiserum. The antiserum was known to cause Masugi nephritis in rats and this combination of cells, membrane and serum offered a more exact analogy of the processes that may be occurring in vivo. It was discovered from these studies that neutrophils from female rats caused a more marked degradation of the basement membrane than was observed with cells from male rats. This observation prompted measurement of the enzyme contents and enzyme distributions in neutrophils from rats of either sex.

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Materials and Methods

Wistar rats, 150–200 g, were used throughout. Chemicals were obtained commercially. Rabbit anti-rat nephrotoxic antiserum was a gift from Dr. T. Twose, I.C.I. plc.

Preparation of rat peritoneal neutrophils. Rat peritoneal neutrophils were obtained 12–15 h after i.p. injection of Brewer's thioglycollate medium (3% w/v in distilled water). Peritoneal exudates were harvested by lavage of the peritoneal cavity with heparinised (1.7 U/ml) saline (0.15 M sodium chloride), the cells were centrifuged ($400 \times g$, 5 min) and resuspended in 2 ml of saline. The suspension was diluted with 5 ml of 0.2% (w/v) sodium chloride for 2 min to lyse any contaminating red blood cells; tonicity was then restored with an equal volume of 1.61% (w/v) sodium chloride [11]. The cells were washed twice more with phosphate-buffered saline: 8.1 mM disodium hydrogen orthophosphate, 1.5 mM potassium dihydrogen orthophosphate, 0.14 M sodium chloride, 2.7 mM potassium orthophosphate, pH 7.4 (PBS) and neutrophils were isolated by step gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Sweden) [12]. The cells ($5 \cdot 10^7$ cells/ml) were layered over 10 ml of Ficoll-Paque solution and centrifuged (4° C, $400 \times g$, 40 min). Neutrophils sedimented to the bottom of the tube while other cells remained at the interface. Following removal of the supernatant the cells, 95% neutrophils on the basis of differential counts after staining with Giemsa, were resuspended and washed twice in 15 ml of either 0.35 M sucrose solution or with phosphate-buffered saline if the cells were to be used in incubations. Using this procedure it was found that a single rat typically yielded $(1–2) \cdot 10^8$ neutrophils. Cell viability was greater than 95% as measured by trypan blue exclusion. In additional studies of viability the luminescent responses of lysed and unlysed cells, stimulated by phorbol myristate acetate, were tested and found to be comparable indicating that lysis had not markedly altered cell responsiveness.

Degradation of antibody-coated basement membrane by neutrophils. The method of studying the response of neutrophils to antibody bound to GBM employed ^{3}H-labelled rat basement membrane fixed to the bottom of microtitre plate wells and pretreated with rabbit anti-rat GBM antiserum according to Bray et al. [7]. Neutrophils suspended in Dulbecco's modified Eagle's medium (Flow Laboratories Ltd., U.K.) were added to the wells at the concentration of $5 \cdot 10^6$ cells per well. The extent of basement membrane degradation was assessed by measuring release of isotope to the supernatant and the degree of degranulation measured by the release of lysozyme. The release of lactate dehydrogenase was used as a measure of cell death during incubation.

Preparation of the neutrophil granule fraction. Attempts to disrupt the neutrophils using a Potter-Elvejhem homogeniser gave low yields of granules, approx. 50% as judged by lysozyme and myeloperoxidase measurements in whole cells and in subcellular fractions. Accordingly a method using higher shearing forces was employed. Neutrophil suspension, ($5 \cdot 10^7$ cells/ml) in 0.35 M sucrose was extruded through a small orifice against the pressure of a spring-loaded ball bearing [13]. With this spring-loaded pressure device, the shear force could be altered by varying the strength and compression of the spring and optimal conditions for cell disruption were established empirically. The homogenate was immediately centrifuged at $2000 \times g$ for 10 min and three fractions were collected: a pellicle containing nucleoprotein, a pellet containing cell debris, and a supernatant which consisted of both the cell cytosol and granules. This supernatant was used for isopycnic fractionation of the granules. A 'total' granule fraction was recovered from this supernatant by centrifugation ($160\,000 \times g$ for 30 min).

For enzyme assays all particulate fractions were washed twice in phosphate-buffered saline and then resuspended in detergent/salt solution (0.05 M Tris-HCl buffer pH 7.4, containing 1 M NaCl and 0.1% v/v Triton X-100) to give a concentration equivalent to a cell suspension of $5 \cdot 10^7$ cells/ml. An appropriate volume of 10× concentrated detergent and an appropriate weight of salt were added to the cytosol, to give final concentrations equivalent to those used to disrupt the granules. All the fractions were homogenised to ensure granule disruption, and aliquotted into appropriate volumes for enzyme assays before being stored at −70° C until required.

Fractionation of neutrophil granules. Fractionation of neutrophil granules was carried out by isopycnic density gradient centrifugation; the composition of all sucrose solutions are expressed as % (w/w), and densities were confirmed by weighing. A 30–60% linear sucrose gradient (30 ml) was loaded over 65% sucrose (2 ml) in 35 ml nitrocellulose tubes. Granule suspension (3.5 ml in 0.35 M sucrose, equivalent to $2 \cdot 10^8$ cells) was then layered on to the surface and the tubes centrifuged at 4° C in a Beckman SW 27rotor for 16–18 h at $80000 \times g$. Preliminary experiments showed that isopycnic distribution was achieved under these conditions. Fractions (2.5 ml each) were collected by displacement with 70% sucrose and parallel fractions from tubes were pooled after the dilution of the fractions to 10% sucrose concentration by addition of distilled water. Particulate material was then recovered by centrifugation for 2 h at $20000 \times g$ at 4° C. The pellets were resuspended in 2 ml of 0.15 M sodium chloride containing 0.1% (v/v) Triton X-100 in 0.01 M Tris-HCl buffer pH 7.4 and homogenized. These homogenates were assayed for enzymes.

Biochemical assays. Alkaline phosphatase [14], lactate dehydrogenase [15], lysozyme [16], myeloperoxidase (adapted from Ref. 17) *N*-acetyl-β-glucosaminidase [18], α-mannosidase [19], cathepsin G [20] and γ-glutamyltranspeptidase [21] levels were assayed using the methods described in the appropriate reference. Elastase activity was measured by following the hydrolysis of *p*-nitrophenyl *N-tert*-butoxycarbonyl-L-alanine (NBA) according to the method of Visser and Blout [22]. Enzyme activity was proportional to enzyme concentration, with the exception of myeloperoxidase, where only the initial slope of the absorbance curve was proportional. The degradative action of proteolytic enzymes on basement membrane was assayed by a modification of the method of Brown and Robinson [23].

The myeloperoxidase assay was based on the oxidation of 3,3′,5,5′-tetramethylbenzidine [17]. It was found necessary to establish optimum substrate and chloride ion concentrations in order to achieve maximum reaction velocities. The concentrations employed in routine analyses were: tetramethylbenzidine, 1.6 mM; hydrogen peroxide, 0.3 mM; sodium chloride, 70 mM. Reaction velocities declined with increasing incubation time but initial reaction rate measurements showed a linear dependence on the amount of enzyme added.

It was noted that amounts of enzyme measured were critically dependent upon the detergent used to lyse cells or cell granule fractions. These detergents were examined: 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 0.5% (w/v) cetyltrimethylammonium bromide, all in 0.05 M Tris-HCl buffer, pH 7.2. Low activities were recorded using deoxycholate; activities were 5-fold greater using Triton X-100 but 80% of the activity recorded remained associated with the particulate fraction. With cetyltrimethylammonium bromide total activities were 10–12-fold higher than those recorded with deoxycholate and 90% of the activity was released from the granules into the supernatants. The reasons for these differences were not investigated further, but indicate that myeloperoxidase is stored in an inactive form in azurophil granules. However in measuring myeloperoxidase activity it is important to note the conditions used, since measured values will vary depending upon the detergents employed.

The BCA protein assay system (Pierce Chemical Company, U.S.A.) was used to measure the protein content of the cell fractions, and bovine serum albumin served as a standard [24].

Units of enzyme activity are expressed as change in absorbance per min except in the cases of alkaline phosphatase, *N*-acetyl-β-glucosaminidase, cathepsin G and elastase, where 1 unit is the hydrolysis of 1 μmol of substrate per min.

Results

The degradative response of male and female neutrophils was measured by adding suspensions of neutrophils in Dulbecco's medium to microtitre wells containing isotopically labelled rat GBM pre-treated with nephrotoxic rabbit anti-rat GBM antibody. The amount of isotope released to the supernatant in a period of 2 h was measured and expressed as a percentage of total available GBM.

In each study the female cells showed about 25% higher degradative activity ($P < 0.01$) than the male cells (Table I). There was little variation in levels of lactate dehydrogenase and lysozyme

TABLE I

THE DEGRADATIVE ACTIVITY OF NEUTROPHILS FROM MALE AND FEMALE RATS ON ANTIBODY-COATED BASEMENT MEMBRANE

Tritiated rat GBM 100 μg/well; 10000 cpm/well) pretreated with nephrotoxic rabbit anti-rat GBM IgG (20 μg/well) was incubated with neutrophils (10^7 cells/well) at 37 °C for 2 h. Supernatants were assayed for the release of label and expressed as a percentage of total recovery per well. The cells were then removed by centrifugation (2000× *g*, 5 min) and the release of lysozyme and lactate dehydrogenase measured in the supernatants. The release of activity to the medium was expressed as a percentage of the total recovery by resuspending the cells in 0.01% (v/v) Triton X-100 in 0.01 M Tris-HCl buffer pH 7.4 and assaying for enzyme activity. The results represent the average of values obtained from four experiments (three animals of each sex) performed in triplicates.

Well contents	Degradation of GBM (% release of label)	Lysozyme (% release)	Lactate dehydrogenase (% release)
Antiserum + ♂ cells	16.6±1.2	20.2±0.4	7.6±0.8
♂ cells only	2.2±0.1	6.8±1.4	8.7±1.5
Antiserum + ♀ cells	22.4±1.5	18.2±1.3	8.1±1.2
♀ cells only	2.7±0.1	11.9±2.3	9.2±0.3

activity in the supernatants. In studying the specificity of the neutrophil response, the effects of non-specific rat and rabbit serum were compared with the effects of nephrotoxic serum; rabbit and rat non-specific sera induced a very low release of labelled GBM (about 3%), close to the levels of release in control wells containing neutrophils with no serum (2.5%).

Differences in the levels of activity exhibited by neutrophils from animals of either sex in degrading antibody-coated GBM prompted a separate analysis of the granule fractions from cells from male and female animals. To provide sufficient material for assay, neutrophils from five rats were pooled for each fractionation; hence each result reported represented a mean for 20 animals (four experiments). Table II shows protein and enzyme recoveries in the subcellular fractions obtained during granule isolation. Recoveries were good but latency was exhibited in the case of cathepsin G, where total activities for the fractions were higher than levels measured for lysed whole cells. Recoveries of enzymes in the granule fraction were still low despite the use of improved homogenisation procedures.

When the specific activities of lysozyme, myeloperoxidase, elastase, cathepsin G and alkaline phosphatase were compared in the subcellular fractions from both sexes (see Table II) it was found that granules from female neutrophils possessed about 20% more elastase activity ($P = 0.1$) than those from male cells, a difference which was also noted for the debris fractions. Cathepsin G and myeloperoxidase showed no similar consistent difference. The higher elastase activity seen in the female granules correlates with previous observations (Table I) that female neutrophils exhibited a greater capacity to degrade basement membrane than did male cells.

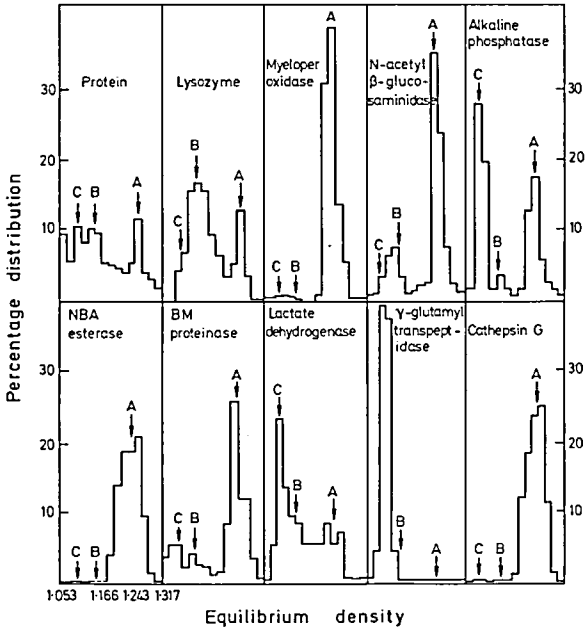

Fig. 1. Isopycnic equilibrium of rat neutrophil granules in sucrose gradient. Percentage distribution of enzymes was expressed as a percentage recovery of the total enzyme activity in all gradient fractions. Graphs are percentage distributions as a function of specific gravity. Percentage recovery of the total enzyme activity in all gradient fractions against enzyme activity in granules was as follows: protein 99.5%; lysozyme 76%; myeloperoxidase 65%; *N*-acetyl-β-glucosaminidase 74%; alkaline phosphatase 114%; elastase 56%; lactate dehydrogenase 42%; cathepsin G 68%; γ-glutamyltranspeptidase 96%. GBM-proteinase was measured by the release of radioactive peptides from ^{3}H-labelled basement membrane; elastase was measured by the hydrolysis of *N-tert*-butoxycarbonyl-L-alanine (NBA esterase).

TABLE II

FRACTIONATION OF MALE AND FEMALE RAT NEUTROPHILS: PROTEIN AND ENZYME DISTRIBUTION (SPECIFIC ACTIVITY, RECOVERY)

The data are based on four fractionation experiments. Neutrophils from five rats were pooled for each fractionation, and results therefore represent an average from populations of 20 animals. All enzymes were measured as described in the text. An equivalent concentration of $5 \cdot 10^7$ neutrophils per ml was maintained throughout the enzyme assays, for all cell fractions. wc, whole cells; gran, granules; debr, debris; pell, pellicule; cyt, cytosol.

	Protein (mg/ml)		Lysozyme (A/min per mg)		Myeloperoxidase (A/min per mg)		Elastase (μmol/min per mg)		Cathepsin G (μmol/min per mg)		Alkaline phosphatase (μmol/min per mg)	
	M	F	M	F	M	F	M	F	M	F	M	F
Specific activity (U/mg)												
wc	2.04	1.96	3.14	2.98	566	481	22.3	23.8	32	39	4.6	5.5
	±0.11	±0.11	±0.31	±0.37	±155	±148	±6.9	±2.1	±9	±4	±1.0	±1.4
gran	0.31	0.28	12.24	13.35	2254	2287	80.2	99.8	250	261	15.3	18.0
	±0.06	±0.05	±1.89	±1.97	±164	±203	±9.5	±10.1	±16	±53	±2.2	±2.3
debr	0.42	0.37	2.09	3.01	809	1009	34.2	52.1	127	147	4.5	3.2
	±0.06	±0.05	±0.44	±0.82	±290	±406	±8.1	±7.0	±29	±13	±0.2	±0.1
pell	0.22	0.13	3.58	3.29	873	743	37.3	43.4	129	125	6.9	4.5
	±0.03	±0.01	±0.54	±1.86	±163	±54	±4.5	±7.0	±24	±24	±1.7	±0.3
cyt	1.04	0.83	0.56	0.45	55	43	1.8	1.8	0	0	4.2	3.9
	±0.13	±0.11	±0.32	±0.06	±38	±17	±0.5	±0.7			±0.2	±0.3
Recovery (%)												
wc	100	100	100	100	100	100	100	100	100	100	100	100
gran	15	14	58	60	54	64	54	56	106	90	50	44
debr	21	19	14	19	29	41	31	42	82	73	20	11
pell	11	7	13	7	15	11	18	13	40	22	16	6
cyt	51	42	9	7	5	4	4	3	0	0	47	30
Total recovery (%)												
	98	82	94	93	103	120	107	114	228	185	133	91

The differences in enzyme distributions between the 'debris' and 'pellicle' fractions for male and female cells (Table II) suggested that there may be differences between the classes of granules found in the cells from the different sexes. Accordingly fractionation of granule fractions of cells from male and female rats was carried out separately by isopycnic equilibrium in sucrose gradients. The results of fractionation were similar for both sexes and the representative distribution pattern of male granules is shown in Fig. 1. A broad density range ($d = 1.05–1.32$) was chosen in order to resolve the specific and azurophil granules, though this meant that low density fractions were not separated well. Two enzymes were found associated with a low density fraction C ($d = 1.08–1.11$): these were alkaline phosphatase and γ-glutamyltranspeptidase which overlapped the cytosolic marker lactate dehydrogenase. Lysozyme showed a bimodal distribution with the bulk of activity in the intermediate density zone B ($d = 1.14–1.20$). The only other enzyme of note in this zone was a small amount of *N*-acetyl-β-glucosaminidase. Other enzymes were located in the heavy density lower zone A ($d = 1.23–1.27$) and significant alkaline phosphatase was also found in this region together with some lysozyme; the proteinases elastase, cathepsin G and GBM-proteinase were in this zone. Thus rat neutrophils show a similar spectrum of granules to those noted for other species [25]. It is worthy of note that α-mannosidase could not be detected in either lysed whole cells or in subcellular fractions.

Since myeloperoxidase within the granules from neutrophils is not readily extractable we decided to compare elastase activities extracted from neu-

TABLE III

VARIATION IN ENZYME ACTIVITY OBSERVED UNDER DIFFERENT EXTRACTION CONDITIONS

The results are based on two fractionation experiments, so each represents a pool of neutrophil granules from 10 animals, at an equivalent concentration of $5 \cdot 10^7$ cells per ml. The extracting medium consisted of 0.05 M Tris-HCl buffer pH 7.4, 0.1% (v/v) Triton X-100 and one of the following: 0.15 M NaCl, 1 M NaCl and 1 M NaCl with extra 0.1% (w/v) cetyltrimethylammonium bromide (CTAB).

Rat neutrophil granules	Elastase specific activity (U/mg)		
	0.15 M NaCl	1 M NaCl	1 M NaCl + 0.1% CTAB
Male	48.9 ± 8.8	79.7 ± 9.4	101.2 ± 28.7
Female	84.5 ± 8.7	92.5 ± 7.0	128.5 ± 16.9

trophil granules under three different conditions. Table III demonstrates the effect on the measurement of elastase by using a homogenisation buffer containing 0.1% (v/v) Triton X-100 with a salt concentration of 0.15 M, or 1.0 M NaCl or 1.0 M NaCl plus 0.1% (w/v) cetyltrimethylammonium bromide. All three methods of extraction released more activity from the female granule fractions than from male. Furthermore it can also be seen that increased activities are manifest when there is an increase in salt concentration and when a strongly positive detergent is used. It is of interest that the most marked difference between the sexes was under mild extraction conditions. This suggests that the enzyme may be differently bound within the granules in the two populations. This potential difference in solubility may be responsible for the sex difference observed when neutrophils react to antibody-treated GBM.

Discussion

The distribution of enzymes (Fig. 1) indicates the presence of two classes of granules in rat neutrophils, in common with all other species studied, and these findings extend the results of Calamai and Spitznagel [26]. Peak A, of high density and rich in degradative enzymes, by analogy with results from other species, represents the azurophil granule fraction [25]. Peak B contains lysozyme, slight glucosaminidase activity and also slight enzymatic activity against GBM proteins. Again by analogy with rabbits the fraction appears to represent the specific granules [25] and agrees with histochemical characterisations of rat neutrophil granules [27]. It is of some interest that these granules show activity against GBM though they appear to contain no elastase activity, at least as judged by photometric assay. Presumably several enzymes may be involved in degrading connective tissue, some of which are located in the specific granules; these latter enzymes might be released early in the neutrophil response to immune complexes [28]. None of the granule fractions contained detectable levels of α-mannosidase though this enzyme is present in the azurophil granules of human and rabbit neutrophils [29].

Alkaline phosphatase and γ-glutamyltranspeptidase were found located in a light granule fraction which presumably represents plasma membrane. Some alkaline phosphatase was found associated with the azurophil granules; however some lactate dehydrogenase was also found in this fraction, possibly entrapped in vesicles such as plasma membrane vesicles, formed during homogenisation. Smith et al. [30] have described the cofractionation of the γ-glutamyltranspeptidase and alkaline phosphatase in a light fraction from human neutrophils and have termed these organelles phosphasomes.

The finding that the elastase levels were higher in cells from females as compared with males (Tables II and III) and that this difference was also manifest when the cells were activated to degrade GBM was a surprising result. Whether this sex difference applies to other species remains to be established. However it has been noted that rheumatoid arthritis is more prevalent among females than males in the human population and this might reflect a difference in the degradative capacity of the neutrophils from the two sexes [31]. In addition there is a sex-dependent concentration difference in human α_2-macroglobulin, one of the main plasma proteinase inhibitors [32]. Clearly it would be of interest to explore sex differences in neutrophils in humans.

Acknowledgements

M.U.-J. is indebted both to the Wellcome Trust and to the Mason Medical Research Foundation

for financial support during this study. We are grateful to Miss J. Byrne for skilled technical help.

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