Membrane Fractions of Rabbit Granulocytes. 1. The Presence of Ouabain-Sensitive ATPase and the Electrophoretic Patterns

Study of the characteristics of the granulocyte surface may provide a better understanding of its specific functional roles in marrow egress, tissue entry and microbial particle ingestion. For this purpose, it is essential to purify the plasma membrane using some reliable markers such as ouabain-sensitive ATPase. Its presence in granulocytes, however, is not well defined.

In the present paper, we describe the experiments in which weak but definite activity of ouabain-sensitive ATPase was concentrated in one of two membrane fractions isolated from rabbit granulocytes. The electrophoretic patterns and the distribution of other various enzymes in these fractions were also shown.

Materials and methods. Granulocytes were obtianed from the peritoneal exudate of rabbits (3–4 kg) at 10 to 12 h after injection of 1% sterile sodium caseinate solution[1]. This exudate contained 7 to 9×10^8 nucleated cells of which about 95% were granulocytes relatively free of erythrocytes. Subcellular fractions were obtained by a procedure essentially identical with that of WIENEKE and WOODIN[2]. Shortly, the exudate was washed once with cold saline and twice with cold 11.6% (W/V) sucrose by centrifuging at 250 *g* for 5 min. Homogenization was carried out by vigorous pipetting (40 strokes, pipet opening 3 mm in diameter) in 10 volumes of 11.6% sucrose. This procedure lead to the preferential disruption of the granulocytes. Each 2 ml of the opalescent supernatant, obtained by centrifuging the homogenate at 400 *g* for 5 min was placed on the top of a discontinuous gradient, consisting of 1.5 ml of 30% and 1.5 ml of 40% sucrose. The tubes were centrifuged at 200,000 *g* for 90 min with Hitachi R ST40 rotor. The fractions at the interface between 11.6% and 30% and between 30% and 40% were referred to B-1 and B-11, respectively. Each of them and the pellet were recovered and resuspended in 5 ml of 11.6% sucrose and homogenized carefully with a Potter-Elvehjem homogenizer and centrifuged at 200,000 *g* for 30 min. Each pellet was resuspended in 11.6% sucrose. All the samples were kept ice-old during the procedure.

Enzymic assays. ATPase activity was assayed in the presence and the absence of 0.5 m*M* ouabain. The reaction mixture contained 5 m*M* $MgCl_2$, 140 m*M* NaCl, 14 m*M* KCl, 3 m*M* ATP and 0.1 *M* *Tris*-HCl buffer, pH 7.4[3]. 5′-Nucleotidase activity was assayed with 10 m*M* $MgCl_2$, 3 m*M* 5′-AMP and 100 m*M* glycine-NaOH buffer, pH 8.5. The incubation was carried out at 37 °C for 10 min and the liberated Pi was determined by the method of FISKE and SUBBAROW[4] after deproteinization with perchloric acid at a final concentration of 0.5 *N*. Alkaline and acid phosphatase activities were assayed, using *p*-nitrophenylphosphate as a substrate. The former reaction mixture contained 0.1 *M* carbonate buffer, pH 10.0, 5 m*M* $MgCl_2$, 0.5 m*M* $ZnSO_4$ and 3 m*M* substrate[5]. The latter reaction mixture contained 50 m*M* acetate buffer, pH 4.5, 5 m*M* $MgCl_2$ and 5 m*M* substrate. The reactions were allowed to proceed at 10 to 20 min and the liberated *p*-nitrophenyl was determined spectrophotometrically. Acid phosphatase activity was assayed also with 50 m*M* β-glycerophosphate as a substrate. Myeloperoxidase activity was assayed with *o*-dianisidine as a substrate. 3 ml reaction mixture contained 0.016% *o*-dianisidine, 0.1 m*M* H_2O_2 and 30 m*M* phosphate buffer pH 7.0. After addition of the enzyme source, the reaction was followed for 3 min at 420 nm with a Hitachi recording spectrophotometer, type 124.

Glucose 6-phosphatase activity was assayed as described by HÜBSCHER et al.[6] and succinate-cytochrome-*c* reductase activity was assayed by the method of TISDALE[7]. Each fraction was determined in duplicate. Protein was determined by the method of LOWRY et al.[8] using bovine serum albumin as a standard.

[1] K. KAKINUMA, Jap. J. exp. Med. *38*, 165 (1968).
[2] A. M. WOODIN and A. A. WIENEKE, J. gen. Physiol. *56*, 16 (1970).
[3] M. FUJITA, H. MATSUI, K. NAGANO and M. NAKAO, Biochim. biophys. Acta *233*, 404 (1971).
[4] C. H. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 375 (1925).
[5] M. FUJITA, H. OHTA, K. KAWAI, H. MATSUI and M. NAKAO, Biochim. biophys. Acta *274*, 336 (1971).
[6] G. HÜBSCHER and G. R. WEST, Nature *205*, 799 (1965).
[7] H. D. TISDALE, Meth. Enzymol. *10*, 213 (1967).
[8] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDAL, J. biol. Chem. *193*, 265 (1951).

Specific activities of various marker enzymes in subcellular fractions

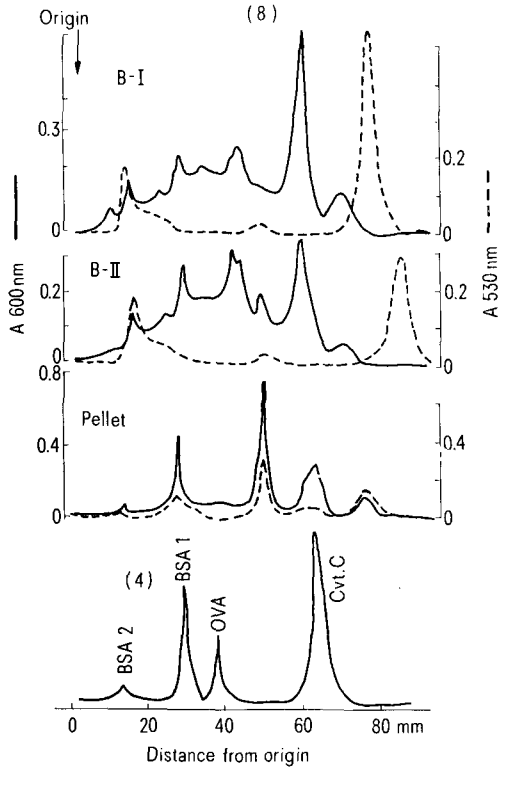
	ATPase		5′-nucleotidase	Acid PNPase	Alkaline phosphatase	Peroxidase	Protein
	Ouabain-sensitive	Ouabain-insensitive					
Homogenate	(+) (0.1)[a]	1.0 ± 0.2[a]	0.36 ± 0.06[a]	2.7 ± 1,4[b]	3.0 ± 0.9[b]	0.29 ± 0.06[c]	220 ± 3[d]
Soluble	n.d.	0.52 ± 0.03 (5.6)	0.22 ± 0.01 (6.9)	1.0 ± 0.5 (3.7)	trace	n.d.	22 ± 3 (11.2)
B-I	trace	2.20 ± 0.46 (0.7)	0.50 ± 0.05 (0.5)	9.5 ± 4.4 (1.1)	2.1 ± 0.7 (0.3)	0.13 ± 0.04 (0.2)	0.7 ± 0.2 (0.3)
B-II	1.0 ± 0.32	3.50 ± 0.88 (1.2)	0.52 ± 0.02 (0.5)	10.2 ± 6.7 (1.2)	4.2 ± 1.4 (0.5)	0.19 ± 0.06 (0.2)	0.7 ± 0.3 (0.3)
Pellet	n.d.	1.3 ± 0.46 (12.7)	0.81 ± 0.03 (10.1)	6.2 ± 3.0 (10.4)	9.9 ± 3.4 (18.6)	0.95 ± 0.28 (14.8)	9.7 ± 1.5 (4.5)

Values given are means ± standard deviation. n.d.: not detected. Those in parentheses are percent recoveries. [a] μmoles of Pi^{-1} mg $protein^{-1}$. [b] μmoles of PNP h^{-1} mg $protein^{-1}$. [c] Absorbance change min^{-1} mg $protein^{-1}$. [d] mg.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out as described previously[9]. Shortly, 10 to 20 µl of samples containing 50 or 100 µg protein was mixed and boiled at 80 °C for 1 min with 50 µl of the solubilizing mixture containing 2% SDS, 0.1% 2-mercaptoethanol and 20 m*M* phosphate buffer, pH 7.1. Gel system was made up of 6.7% polyacrylamide and 0.09% methylenebisacrylamide. Electrophoresis was carried out with a current of 3 mA per tube at room temperature for about 5 h. The developed gels were stained with Amidoblack 10B and with periodic acid-Schiff reagent (PAS).

Results and discussion. The distribution of ouabain-sensitive ATPase and the other enzymes in the subcellular fractions from more than 5 experiments are summarized in the Table. Ouabain-sensitive ATPase was obviously concentrated in B-II fraction with specific activity of about 1.0 mole Pi per mg protein per hour. In the homogenate its level was too low to be determined accurately. These findings support that the granulocyte should possess a small amount of this ATPase and that its plasma membrane should be concentrated in the B-II fraction. Some authors have failed to detect this activity, even in the subcellular fraction which was essentially identical with ours[10]. Although we cannot give any definite explanation for this discrepancy, our modification, such as induction of granulocytes with sterile casein or careful washing of the fraction, might account for the present findings. The specific activities of acid phosphatase and ouabain-insensitive ATPase were distinctly higher in both B-I and B-II fractions although a considerable portion of these activities was also found in the pellet and the soluble fraction. Most of myeloperoxidase, alkaline phosphatase and 5′-nucleotidase were concentrated in the pellet. The acid phosphatase of B-I and B-II fractions had little activity on β-glycerophosphate. Activities of succinate-cytochrome-*c* reductase and glucose 6-phosphatase were not detectable even in the subcellular fractions. Electron micrographic studies revealed that both B-I and B-II fractions were mainly composed of vesicles. These of B-I fraction seemed to be more homogeneous in shape and smaller in size, while those of B-II fraction were more diverse in both shape and size. The pellet was made up essentially of the primary and the secondary granules but often contaminated with membranous organelles as well as glycogen particles. These morphological findings were compatible with the enzymatic studies.

Representative patterns of SDS-polyacrylamide gel electrophoresis are shown in the Figure. Both of B-I and B-II fractions showed 10 to 11 bands for protein and 3 to 4 bands for carbohydrate. The most prominent PAS positive band appeared to be a glycolipid and the others to be glycoproteins. There were no significant differences between these two membrane fractions. In sharp contrast, the pattern of the pellet was relatively simple with only 5 prominent bands of glycoprotein. The electrophoretic pattern of the homogenate revealed decreasing intensity at one polypeptide band (MW 5×10^4) after incubation at 37 °C for 3 h, but such effect was not observed at ice-water temperature, even after prolonged standing. We also found no evidence for any changes occurring in the protein composition in each fractions stored at ice-cold temperature. For further purification of the plasma membrane and for studying the differences between the two membrane fractions, the phagosomal membranes likely to be present in these fractions would have to be separated from the plasma membranes.



The solid lines show the protein stains and the dotted ones carbohydrate stains. The reference proteins are bovine serum albumin (BSA), ovalbumin (OVA) and cytochrome *c* (Cyt. C).

Summary. In one of two membrane fractions isolated from the homogenate of casein-induced rabbit granulocytes ouabain-sensitive ATPase activity was found definitely. Electrophoretic patterns of these membrane fractions showed no significant differences in staining for carbohydrate and protein.

S. Asano, M. Fujita[11], F. Takaku and M. Nakao[11]

Department of Biochemistry, School of Medicine, Tokyo Medical and Dental University, Yushima-1 Bunkyo, Tokyo (Japan), and Institute of Hematology, Jichi Medical School, Minamikawachi-Machi, Tochigi 329-04 (Japan),
21 April 1975.

[9] M. Fujita, K. Kawai, S. Asano and M. Nakao, Biochim. biophys. Acta *307*, 141 (1973).

[10] A. M. Woodin and A. A. Wieneke, Biochem. biophys. Res. Commun. *33*, 558 (1968).

[11] Department of Biochemistry, School of Medicine, Tokyo Medical and Dental University, Yushima-1 Bunkyo, Tokyo, Japan.