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LYMPHOCYTE PLASMA MEMBRANES II. CYTOCHEMICAL LOCALIZATION OF 5'-NUCLEOTIDASE IN RAT LYMPHOCYTES

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Summary

Cytochemical studies of thymic and splenic lymphocytes from rats showed that 5'-nucleotidase was restricted to the plasma membranes. Isolated plasma membranes contained the highest specific activity of 5'-nucleotidase of any cellular fractions. The results indicate that this enzyme can be used as a plasma membrane marker for lymphocytes.

Since various immunological functions of lymphoid cells involve their outer membrane, the characterization of isolated plasma membrane is under study in several laboratories [1—11]. The distribution of enzymes in various subcellular fractions has been a major criterion in assessing the purity of the isolated membranes, and 5'-nucleotidase (EC 3.1.3.5), which has been found in high concentration in membranes from several other tissues both analytically [12—15] and cytochemically [16—18], has been considered a specific marker enzyme. In lymphocytes, however, the subcellular localization of this enzyme has not yet been established. The present work reports cytochemical studies on the subcellular distribution of 5'-nucleotidase in lymphocytes from the spleen and thymus of inbred ACI and F344 rats.

Spleens and thymuses were taken from 8—10 weeks old rats, minced in 10mM Tris—0.15 M NaCl (pH 7.4) and passed through a screen [10]. The isolated lymphocytes were washed with the same buffer and fixed in 3% glutaraldehyde buffered to pH 7.5 with 100 mM Tris—acetate for 1—2 h at 4 °C. They were then washed with 6 changes of the cold buffer over a period of 3 h and incubated in the Tris—acetate buffer containing, in addition, 2 mM adenosine 5'-phosphate (AMP) (P-L Biochemicals), 2 mM $Mg(NO_3)_2$ and 2 mM $Pb(NO_3)_2$

at 37 °C for 0.5—1 h. Control samples consisted of cells incubated with AMP and $Mg(NO_3)_2$, with AMP and $Pb(NO_3)_2$ or with $Pb(NO_3)_2$ and $Mg(NO_3)_2$ under identical conditions. The cells were then washed with cold buffer, post-fixed with 1% OsO_4 for 2 h at 4 °C, dehydrated in graded cold ethanol and embedded in an Epon—Araldite mixture. Electron micrographs of the thin sections were taken with a Phillips E.M. 300 at 80 kV with and without staining by uranyl acetate and lead citrate. For analytical work, plasma membranes were isolated and the 5'-nucleotidase was assayed in the different subcellular fractions as described previously [9,10].

The cells incubated in the presence of lead, magnesium and AMP showed three kinds of plasma membrane staining: (a) most showed heavy staining with lead phosphate precipitate (Figs 1A and 1B); (b) some showed discrete patches of lead precipitate (Fig.2A); (c) a small number did not show any staining. These characteristics were observed in both stained and unstained sections. Cells incubated in the presence of lead and AMP, but without magnesium, did not have any precipitate in the cellular membrane, cytoplasm or nucleus (Fig.1C). Similarly, no reaction products could be detected in cells incubated with lead and magnesium or with AMP and magnesium. A large number of micrographs of cells incubated with lead, AMP and magnesium was scanned, and none showed any lead granules in the intercellular regions or in the cytoplasm. The nuclear membrane never showed any stain, but fine granules of reaction products were dispersed in the heterochromatin of many cells (Fig.2A) Fig.2B shows a late prometaphase cell where the mitochondria and other cytoplasmic organelles are unstained but the condensed chromatin is. Fig.2C illustrates another cell where lead grains are absent or barely visible in the nucleus. Heavy precipitates similar to those on the intact cell plasma membranes were found on isolated membrane vesicles (Fig.2D).

Lead ion itself was found to hydrolyze ATP and some other nucleosides but had no effect on 5'-AMP [19]. We also did not find any lead precipitate in the control samples. We have not yet studied the effect of glutaraldehyde on 5'-nucleotidase activity, but this fixative was used successfully for cytochemical localization of 5'-AMPase [20], glucose-6-phosphatase [21] and ATPase [22]. The fine granules observed in the heterochromatin in many cells were absent in all control samples. Similar nuclear staining was observed in cytochemical studies by the lead phosphate method during the localization of ATPase in liver tissue [23] and during the localization of AMPase in leucocytes, Kupffer cells and endothelial cells [17], liver cells [24] and Ehrlich ascites carcinoma cells [25]. Such nuclear staining was explained by Gomori [26] as an artifact due to diffusion of the reaction products and their selective affinity for certain reactive groups within the nucleus. Diffusion of the reaction products into the nucleus was reported to occur when the concentration of Pb^{2+} in the incubation medium was low or when the incubation time was prolonged [24,27]. In our studies, the lead ion concentration was the same as that of AMP. When the incubation time was varied from 0.5—1 h, we found the same pattern of grains in the nucleus.

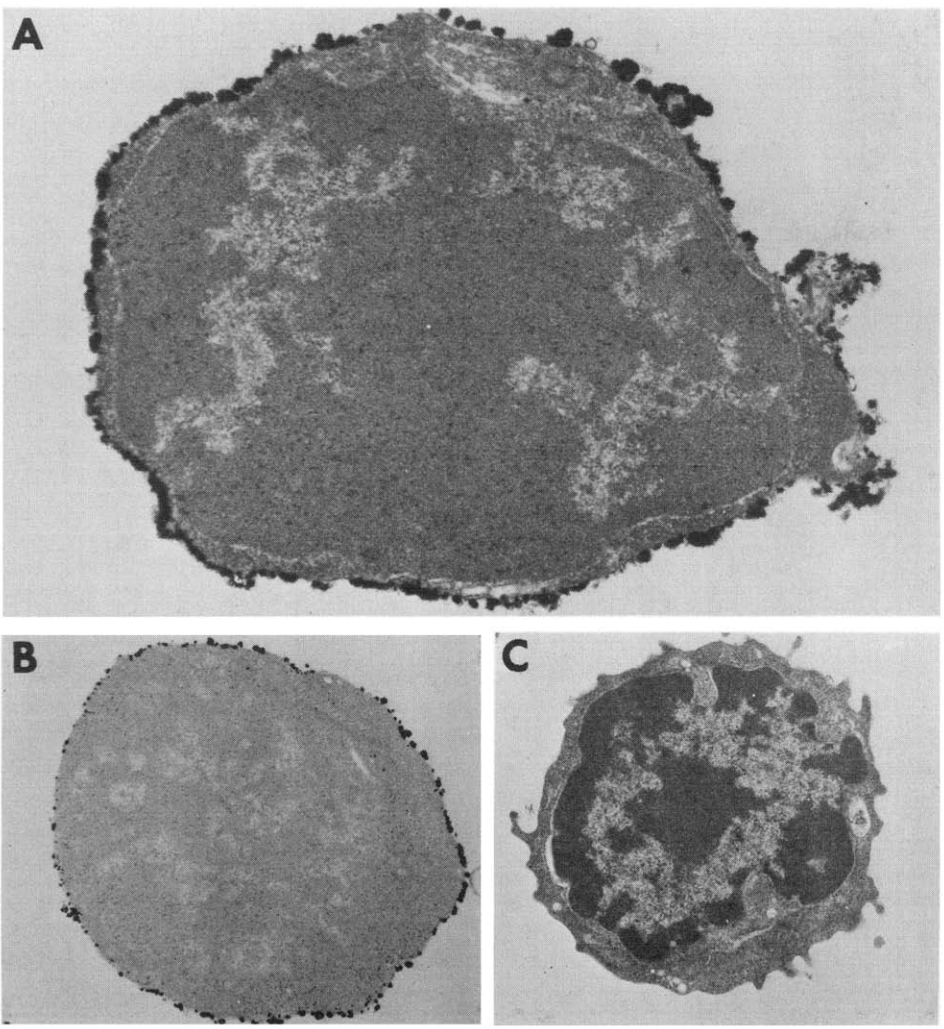

Fig. 1. A. Lymphocyte incubated in the presence of AMP, lead and magnesium showing a heavy precipitate of lead phosphate in the cell membrane and fine granules of reaction products in the nucleus. The section was stained with uranyl acetate and lead citrate. (X 22 500). B. Same as in A but unstained, showing the same characteristics as the stained section (X 10 400). C. Lymphocyte incubated in the presence of lead and AMP without magnesium. The sample does not show any reaction products. (X 10 200).

The 5'-nucleotidase activity of isolated lymphocyte membranes is summarized in Table I. The specific activity of the splenic lymphocyte membrane in ACI rats was higher than that of the thymic membrane. Using an identical isolation procedure, we consistently observed higher specific activity in the F344 thymocyte membrane than in the ACI thymocyte membrane. The specific activity of 5'-nucleotidase in membrane preparations increased consider-

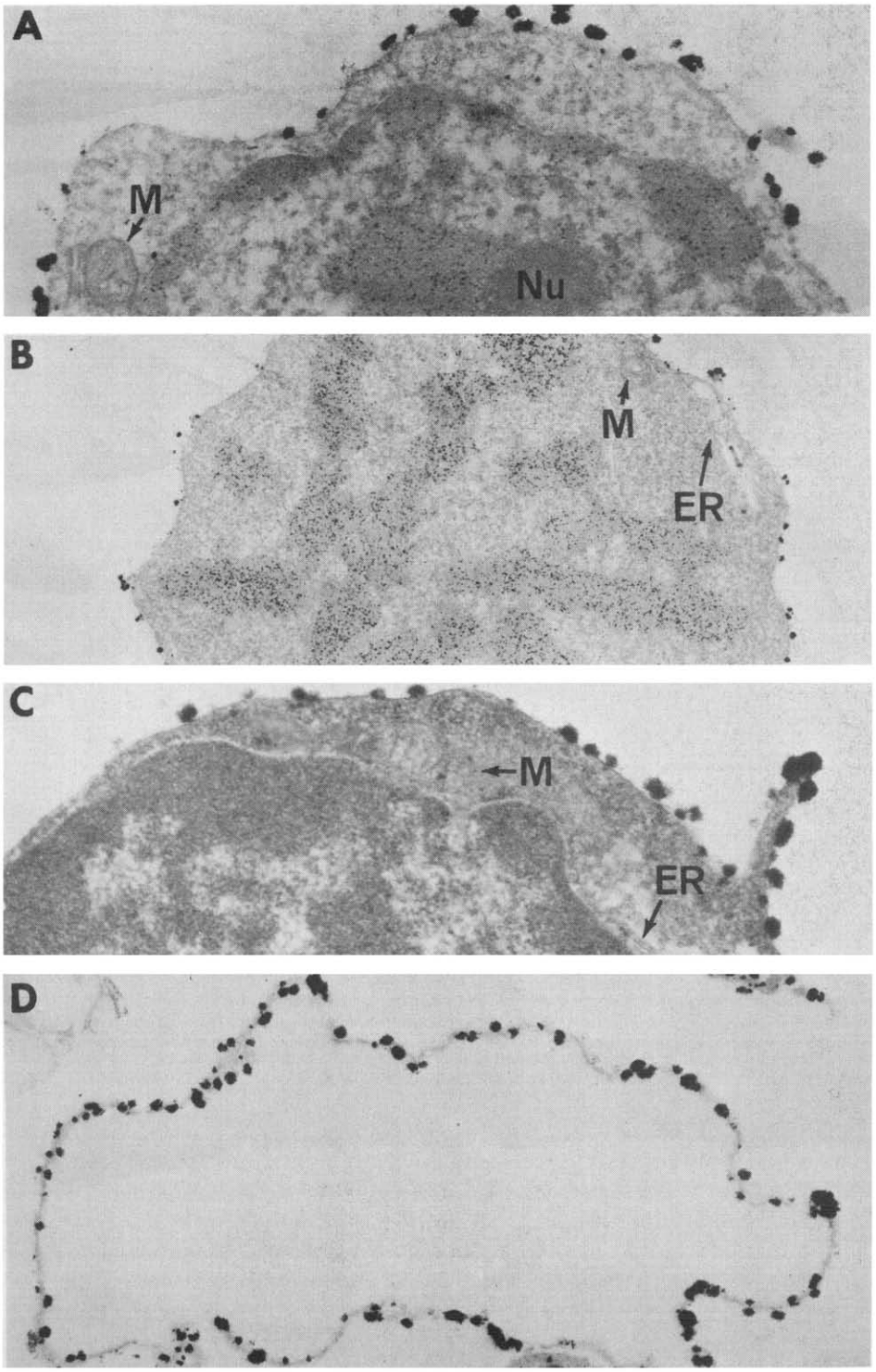
A
M
Nu
B
M
ER
C
M
ER
D

TABLE I

THE 5'-NUCLEOTIDASE CONTENT OF ISOLATED PLASMA MEMBRANES OF LYMPHOCYTES FROM DIFFERENT SOURCES.

The specific activity is expressed as μmoles of product liberated per h per mg of protein.

Source of lymphocytes	Specific activity	Enrichment in specific activity with respect to homogenate	Percent recovery of the total homogenate activity	Reference
Human thymus	3.9	16	10—15	[2]
Human tonsil	10.0	12.5	7.6	[4]
Human tonsil	14.0	13—14	12.3	[5]
Human leukaemic tissue	28.4	43	6.5	[6]
Calf thymus	2.6	8	5.4	[7]
Pig mesenteric lymph node	10.1	11.6	15.4	[1]
Pig mesenteric lymph node	7.5	25	44.7	[3]
Mouse mesenteric lymph node	3.4—11.6	2—23	0.3—1.6	[8]
Mouse leukaemic tissue	5.9, 11.6	1.8, 2.0	0.4, 2.0	[8]
Rat (ACI female) spleen	8.9	10	14.3	[9,10]
Rat (ACI female) thymus	5.2	14	17—18	[9,10]
Rat (ACI male) spleen	10.2	10—15	14	(Present work)
Rat (ACI male) thymus	5.8	14	18	(Present work)
Rat (F344 male) spleen	8.1	13	20	(Present work)
Rat (F344 male) thymus	11.4	15	15	(Present work)

ably compared to that in the homogenate, and the membrane fraction was much richer in this enzyme than the other fractions.

The recovery of 5'-nucleotidase in the membrane fractions from different lymphocytes (Table I) varies over a wide range. In the case of pig mesenteric lymph node lymphocyte and human thymocyte membranes, Allan and Crumpton [1,2] found a 10—15% recovery. Following a similar isolation procedure, we also found 14—20% of the total homogenate 5'-nucleotidase in the rat lymphocyte plasma membrane. Using the nitrogen cavitation method for cell disruption, however, Ferber et al. [3] found about 45% of the enzyme in isolated pig lymph node lymphocyte membrane. Van Blitterswijk et al. [7] used the same nitrogen cavitation method and found only 5.4% of the enzyme in calf thymocyte plasma membranes. Marique and Hildebrand [6] found a 43-

Fig.2. All samples were prepared as described for Fig.1A. A. Lymphocyte showing that grains are distributed in the heterochromatinic regions and no reaction products are in the cytoplasm. (X 27 400). B. A late prometaphasic lymphocyte showing grains distributed on the chromosomes. (X 12 100). C. This lymphocyte does not have any grains over the nucleus. (X 34 200). D. Plasma membrane vesicles showing heavy deposits of reaction products. (X 24 500). M, mitochondrion; Nu, nucleolus; ER, endoplasmic reticulum.

fold enrichment of 5'-nucleotidase in the plasma membrane fraction from human leukaemic lymphocyte, but the recovery of the enzyme was only 6.5%. Similarly, Warley and Cook [8] found a 23-fold enrichment in the specific activity of their preparation of membranes from mouse mesenteric lymph node lymphocytes, but the recovery in the membrane fraction was less than 2%. They also tried the method of Ferber et al. [3], but the recovery did not increase. They concluded that both in normal and malignant murine lymphocytes, 5'-nucleotidase is not exclusively located within the plasma membrane.

These results indicate either that 5'-nucleotidase is distributed in the subcellular fractions or that the subcellular fractions are contaminated by plasma membrane during isolation. Comparison of the results of Allan and Crumpton [1] and Ferber et al. [3] on the pig mesenteric lymph node plasma membranes, however, indicates that the recovery of the enzyme in the plasma membrane fraction can be increased. Recently, the latter group [28] found that 80% of the microsomal 5'-nucleotidase was recoverable in the plasma membrane fractions of calf and rabbit thymocytes. Table II shows that in many lymphocytes the microsomal pellet plus the microsomal supernatant contain 40—80% of the total 5'-nucleotidase. In many other tissues, 40—60% activity was recovered in the microsomal fractions [18]. In different lymphocytes, 13—47% activity was in the microsomal supernatant, and we found about 14% of the activity in this fraction, which could not be sedimented at 88 000 × g_{av}. Since 5'-nucleotidase is released by osmotic shock in *Escherichia coli* [29], some enzyme may be released from lymphocytes during cell fractionation, probably from the plasma membrane fraction. The nuclear pellets are contaminated with membrane vesicles so most of the enzyme activity (4—20%) probably comes from these vesicles.

TABLE II

DISTRIBUTION OF 5'-NUCLEOTIDASE AS A PERCENTAGE OF THE ACTIVITY IN THE TOTAL HOMOGENATE

Source of lymphocytes	Nuclear fraction	Microsomal fraction	Microsomal supernatant	Reference
Human tonsil	17.0	20.9	21.5	[5]
Human leukaemic tissue	20.0	44.6	33.6	[6]
Calf thymus	—	13.5	47.2	[7]
Pig mesenteric lymph node	4.4	33.8	25.6	[1]
Pig mesenteric lymph node	9.3	56.3	14.2	[3]
Rat thymus and spleen	6—9	37—38	13—15	(Present work)

The studies cited above indicate that the plasma membrane has the highest content of this enzyme, even though the exact distribution of this enzyme in the different subcellular fractions cannot be defined due to the different methods of membrane isolation used. Our studies showed that only very small amounts of this enzyme are in the nuclear pellet and that none is localized

cytochemically in the cytoplasm of the splenic and thymic lymphocytes. We conclude that 5'-nucleotidase is exclusively contained in the plasma membrane of rat lymphocytes.

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