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Rapid Report

Interaction of radiolabelled Na,K-ATPase-liposomes with human peripheral blood mononuclear cells

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Abstract

Artificial phospholipid vesicles (liposomes) containing in their membrane about eight Na,K-ATPase (sodium pump) molecules per vesicle were incubated in the presence of [^{110m}Ag]silver nitrate to label the membrane protein; silver binds specifically to the Na,K-ATPase protein. When such silver-labelled liposomes were incubated with freshly isolated human peripheral blood mononuclear cells, a large number of liposomes was found in cells as evidenced by their ^{110m}Ag content after washing them with powerful silver chelators. Thus, liposomes containing an integral membrane protein can be transferred to human peripheral blood mononuclear cells rapidly and without toxicity.

Key words: Proteoliposome; Silver-Na^+/K^+-ATPase-liposome; ATPase-liposome; Peripheral blood mononuclear cell; Cellular uptake; Subcellular distribution

Na,K-ATPase-liposomes were developed in our laboratory by incorporating purified active Na,K-ATPase (NKA) into artificially formed phospholipid vesicles [1]. A metal-chelating component in the NKA molecule has been described recently [2–4]. NKA strongly binds ^{230}Hg or ^{110m}Ag; the binding is irreversible in the absence of mono- or dithiols [2,5,6]. NKA-liposomes bind about two silver ions per NKA molecule [5]. In the present study the interaction of ^{110m}Ag-NKA-liposomes with cells is described. To our knowledge, interaction of liposomes containing transmembrane protein with cells has not yet been studied. Yet, such extracellular vesicles resemble morphologically and physicochemically certain viruses, i.e., the immune deficiency virus [7]. However, the mechanism of internalisation and the intracellular fate of internalised vesicles or viruses components are still poorly understood.

To see whether proteoliposomes are taken up by human peripheral mononuclear blood cells (PBMC) in vitro, radiolabelled silver, tightly bound to the NKA inserted in artificial membranes, was used in an original way. Such silver-labeled liposomes were incubated with freshly isolated human PBMC. A large fraction of the silver was found in the cells and remained associated with them after fractionation or washing with powerful silver chelators indicating that silver-NKA-liposomes can interact rapidly with human PBMC cells in vitro without toxicity.

ATP-filled liposomes reconstituted with functional Na,K-ATPase (NKA) were prepared as previously described [8]. Briefly, 300–800 μg purified Na,K-ATPase was suspended in 100 μl of a solution containing (in mM) 50 Na_2ATP, 5 $MgCl_2$, 30 histidine, 1 Tris-EDTA, pH 7.2 (solution A) and 23 mM Na-cholate and centrifuged for 15 min at 100 000 $\times g$ in an Airfuge Beckman. The resulting supernatant containing about 50% protein in soluble form was mixed with an equal volume of lipid solution containing 1.6 mg phosphatidylcholine and 0.4 mg phosphatidylserine per ml in 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA, 23 mM Na-cholate (pH 7.2). Ultrastructurally defined proteoliposomes were formed by dialysis in 8 ml solution B containing (in mM) 100 NaCl, 5 $MgCl_2$, 30 histidine, 1 Tris EDTA (pH 7.2) [10].

For silver labelling, 2 μl of a 20 μM solution of $^{110m}AgNO_3$ was added to 100 μl NKA-liposomes and incubated for 30 min at 25°C. The free isotope was removed by washing the liposomes four times at 95 000

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Table 1
Interaction of ^{110m}silver-NKA-liposomes with peripheral blood mononuclear cells in vitro

No. of expts.	Incubation time (h)	^{110m}Ag NKA-liposomes (No. added)	PBMC (No.)	^{110m}Ag NKA-liposomes (cpm)	^{110m}Ag in PBMC (cpm)	^{110m}Ag-NKA-liposomes	
						in PBMC (No.)	per cell (No.)
21	2	$3.1 \cdot 10^{12}$	$10 \cdot 10^6$	25504 ± 2300	1189 ± 213	$3.0 \cdot 10^{10}$	3114 ± 215
4	16	$3.3 \cdot 10^{12}$	$6 \cdot 10^6$	42792 ± 8090	2326 ± 985	$5.2 \cdot 10^{10}$	15500 ± 2810

Freshly isolated human lymphocytes in PBS containing about $(16–17) \cdot 10^6$ cells were incubated with ^{110m}Ag-NKA-liposomes for 2 or 16 h at 35°C. Data represent means ± S.E.

$\times g$ for 60 min in 200 μl solution B. The NKA-liposomes consistently incorporated about 60% of the total radioactivity [5]. The radiolabelled silver in the four supernatants was measured to control the washing procedure until a stable background value was obtained. The pellet containing the silver-labelled liposomes was suspended in phosphate-buffered saline (PBS) and stored at 0°C. No silver labelling of NKA-free liposomes was observed.

Freshly isolated human lymphocytes in PBS containing about $(16–17) \cdot 10^6$ cells were incubated with equal volume of ^{110m}Ag-NKA-liposomes for 2–18 h at 35°C on a rotating axis or by gentle shaking in a water bath. Aliquots were taken to count the total radioactivity of the sample. To remove the external liposomes the suspension was centrifuged at $400 \times g$ and the pellet suspended in PBS. The washing procedure was repeated at least three times followed by counting of radioactivity in the supernatants until background values were obtained. The final PBMC pellet was suspended in PBS. The incorporated radioactivity was expressed as% of total radioactivity added with ^{110m}Ag-NKA-liposomes. The viability of PBMC that have been incubated with silver-liposomes for 1 to 2 h was assessed by Trypan blue exclusion test.

To further analyse liposome internalisation, PBMC pellets obtained from above experiments were subjected to differential centrifugation. The cell suspension was homogenised in a Dounce tissue grinder with about 20–25 strokes at 20°C. The suspension was centrifuged for 5 min at $900 \times g$ and the resulting supernatant for 8 min at $1300 \times g$. The supernatant was again centrifuged for 30 min at $100000 \times g$. At each step the corresponding pellet as well as the last supernatant were collected, suspended in PBS and counted for radioactivity.

For electron microscopy, cells incubated with or without silver-NKA-liposomes were washed three times to remove unbound liposomes, prefixed in 2% glutaraldehyde in cacodylate buffer, postfixed in 2% OsO_4-collidine, dehydrated in ethanol and embedded in epon. Ultrathin sections stained in uranyl acetate and lead citrate were examined in a Philips 400 electron microscope.

PBMC were incubated with labelled NKA-liposomes to see whether they interacted with the cells. The data presented in Table 2 show that 10–20% radioactivity was transferred from the solution containing the ^{110m}Ag-NKA liposome to the cells. Washing of the cells with the strong silver-chelator DMPS did not remove the cell radioactivity (not shown) indicating that the labelled liposomes were not merely bound to the cell surface. From the total ^{86}Rb radioactivity entrapped by a defined volume of liposome suspension, their size and number can be estimated [4]. Together with the stoichiometric silver binding to NKA, the number of liposomes taken up by the cells can be calculated: within 2 h of incubation about 3000 liposomes were found per cell on the average and about 15500 within 16 h of incubation (Table 1).

To make sure that the silver-liposomes were taken up by the cells, the cells were homogenized and fractionated at $900 \times g$, $13000 \times g$ and $90000 \times g$. The radioactivity taken up within 2 h incubation was measured in pellets supernatants and expressed as cpm and % (Table 2). The majority of the radioactivity re-

Table 2
Distribution of ^{110m}Ag-NKA-liposomes in cell fractions

No. of expts.	^{110m}Ag added (cpm)	Total ^{110m}Ag								
		before fractionation (cpm)	in $900 \times g$ pellet		in $13000 \times g$ pellet		in $90000 \times g$			
			cpm	%	cpm	%	pellet		supernatent	
							cpm	%	cpm	%
5	133854 ±5670	16505 ±712	7754 ±298	72.6 ±3	238 ±14	3.4 ±0.5	203 ±12	2.0 ±0.15	1169 ±74	12 ±3

PBMC had been incubated with labelled liposome for 2 h as shown in Table 1 were washed to remove external liposomes and fractionated by differential centrifugation. Data represent means ± S.E.

mained associated with the $900 \times g$ pellet composed by nuclei, broken cells without plasma membrane and some intact cells as verified by electron microscopy (not shown). The $13\,000 \times g$ pellet (predominantly mitochondria) retained 3 to 4% radioactivity. The $90\,000 \times g$ pellet (microsomes) contained 2% radioactivity; 12% remained in the supernatant corresponding to the cytosol. Thus, the majority of the incorporated liposomes appeared to have reached the interior of the cells.

When cells were incubated with silver NKA-liposomes for 2 h at 37°C, washed by the procedure described above and then processed for ultra-thin sectioning and electron microscopy, interesting structures were seen at the cell surface constituted by grouped vesicles (Fig. 1). The vesicles had the same morphological aspect as the added liposomes (insert) and were not seen in control preparation incubated without liposomes (not shown). Thus, the clustered vesicle-filled structures are in relationship with the presence of liposomes but could be either silver-liposomes interacting with cells or natural vesicles excreted because of cellular liposome entry. More refined labeling procedures are required to define this process.

Silver NKA-liposome uptake is non-toxic since viability of PBMC incubated with silver-treated liposomes for 1 to 2 h was 97% of controls (Table 3). By contrast, when silver nitrate was entrapped in the absence of metal-binding NKA in the liposome membrane, about 90% of the cells died (Table 3) although the free silver

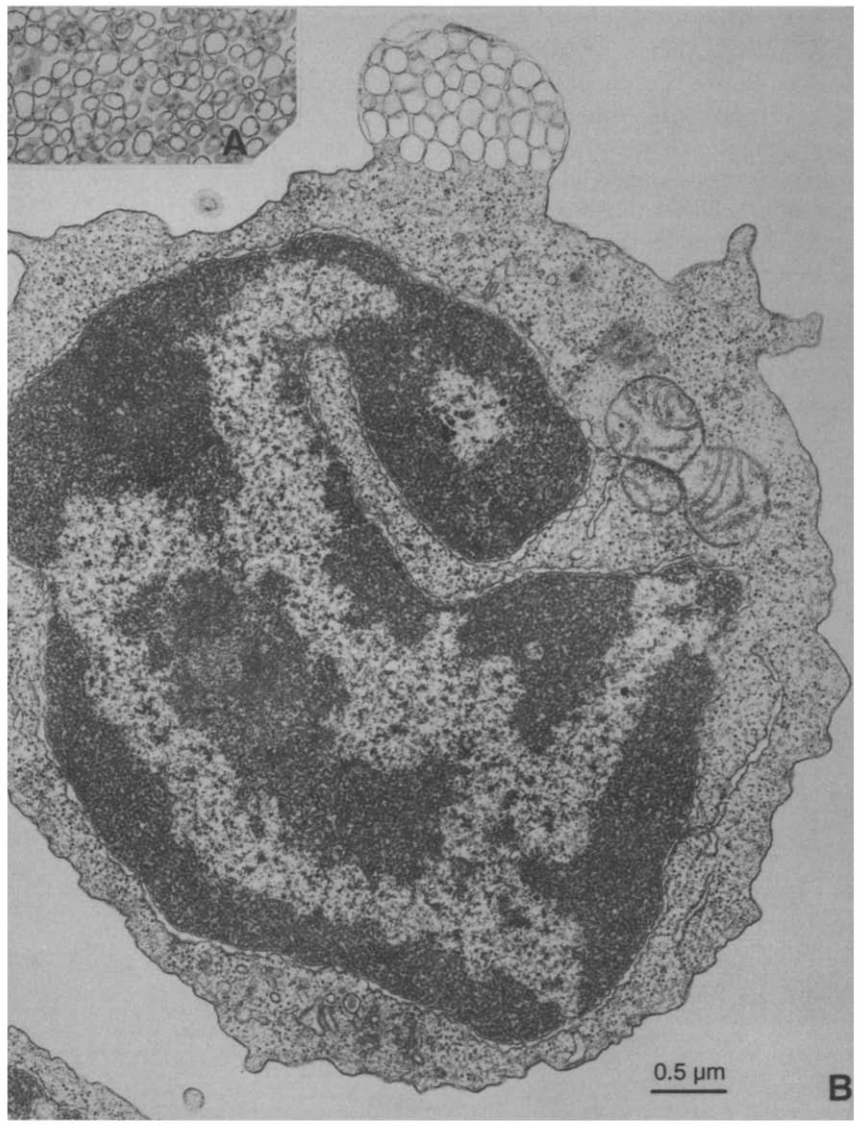

Fig. 1. Electron microscopic evidence for NKA–liposome interaction with PBMC. Peripheral blood mononuclear cells (PBMC) were incubated with silver-NKA-liposomes and washed as described for the labeling experiments shown in Table 1. They were then processed for electron microscopy as described above. A typical cell is shown at 36 000 × magnification.

Table 3
Non-toxicity of silver-NKA-liposomes

Liposomes	Silver	PBMC viability (%)
+NKA	+	96±2 (4)
+NKA	−	93±3 (4)
−NKA	+	12 (1)

Liposomes were prepared with (four preparations) or without (one preparation) NKA in their membrane; 5 mM $AgNO_3$ was trapped in the NKA-free liposomes. The external silver nitrate was removed by two centrifugations at 95000× *g* and added to cells for 2 h at 37°C. NKA-liposomes were incubated with 20 to 70 μM of silver nitrate and the external silver nitrate removed as above and they were added to cells for 1 to 2 h at 37°C. Data represent means ± S.E.

nitrate concentration, if released, would be at most 20 μM. Thus, the liposomal NKA protein protects cells from silver toxicity.

In summary, the present study describes uptake of ^{110m}Ag-NKA-liposomes by freshly isolated human PBMC in vitro. From the analysis of the interaction of PMNC with liposomes by fluorescent activated cell sorter [9] we know that the lymphocytes present about 90–95% of the cells and that the monocytes incorporate about twice the amount of liposomes; all lymphocytes take up liposomes whether the fluorescent label is contained in the aqueous [9] or in the lipid phase (unpublished results). In the present work we show that radioactive silver bound to a transmembrane protein can also be used as a marker of the liposome membrane. Dissociation of the silver from the NKA in the cell is unlikely since a high cysteine/NKA ratio is required to remove the silver from the enzyme [5]. Interestingly, the viablitiy of the target cells is not altered despite the uptake of apparently thousands of liposomes with NKA and silver in addition to the phospholipids. Thus, isolated human lymphocytes are able to incorporate metal-proteoliposomes without toxic effects.

Finally, the NKA-liposomes appear to enter the nuclei since the majority of the radioactivity remains associated with broken cells and isolated nuclei. Electron diffraction of ultrathin-sectioned intact cells revealed silver within the nucleus in support of this contention (Burrus, C., Lacotte, D., Moosmayer, M., Hussain, S., Anner, B.M., manuscript in preparation). The wide use of liposomes as efficient gene transfectors also speaks in favour of nucleus-liposome interaction [10]. In conclusion we show that artificial phospholipid vesicles containing an integral membrane protein can be used to demonstrate liposome-cell interaction, in particular liposome uptake by human PBMC ex vivo.

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