PROTEIN-CONTAINING LIPOSOSOMES AND THEIR DISTRIBUTION AMONG THE ORGANS

E. F. Davidenkova,* O. A. Rozenberg, E. I. Shvarts, and S. A. Ketlinskii

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A comparative study was made of the distribution of creatine phosphokinase (CPK) among the organs of rats after its intravenous injection of the enzyme in liposomes and in the unencapsulated state. Uptake of CPK-^{125}I was found to be 5.5 times higher in the liver and spleen after lyosomal injection than after injection of the free enzyme. In the kidneys and lungs on the first day the enzyme level was higher after free injection, on account of its rapid elimination and high blood level. In all organs the enzyme remained detectable for much longer (from 4 to 6 days) in the tissues, whereas after free injection of the label none could be found after 24 h. The intracellular localization of the label above vacuoles of hepatocytes when the enzyme was injected in liposomes, and the absence of both label and vacuoles after free injection of the enzyme, were demonstrated autoradiographically. The enzyme activity was found to be preserved after liposomal injection in liver homogenates. Prospects for the use of liposomes as a transport medium for supplying proteins for the treatment of hereditary diseases are discussed.

KEY WORDS: liposomes; creatine phosphokinase; intravenous injection.

A promising trend in experimental research in the field of treatment of hereditary diseases is replacement therapy with homologous enzymes [5]. Previous attempts at parenteral injection of homologous and heterologous enzymes in lyosomal injection in man have been unsuccessful [4]. This is on account of biological degradation of the injected proteins, their poor penetration through the cell membrane, their rapid elimination from the body, and the impossibility of repeated injection because of the development of immunologic reactions.

The possibility of overcoming these obstacles in the way of enzyme therapy by the use of liposomes to protect and transport proteins into damaged organs and tissues has recently been discussed.

The writers have shown that liposomes with a high protein content and with 98-100% latent activity of the enzyme used can be prepared [2].

The object of this investigation was to compare the fate of creatine phosphokinase (CPK) when injected intravenously in the free state and encapsulated in liposomes.

EXPERIMENTAL METHOD

Egg lecithin was prepared by purification on alumina and silica gel columns [9, 10]. The purity of the lecithin was verified by thin-layer chromatography in systems described previously [1]. The cholesterol used was chemically pure and stearylamine was obtained from Eastman. A solution of phosphatidylcholine, stearylamine, and cholesterol in chloroform in the molar ratio of 7:2:1 was evaporated to dryness on a rotary evaporator. A solution of CPK containing 0.5 mg in 1 ml 300 mM glucose (pH 7.4) with the addition of labeled CPK-^{125}I was introduced into a flask with a thin film of lipids and emulsified at 37°C for 15 min. Iodination of CPK was carried out as in [6]. The quantity of emulsifying solution was calculated at the rate of 16.6 μmoles lipids to 1 ml [12] and labeled protein was added in a dose of (30-32) $\times 10^6$ cpm. To increase the uptake of protein into the liposomes, the emulsifying solution was used twice with a fresh batch of lipids. After swelling

*Corresponding Member, Academy of Medical Sciences of the USSR.

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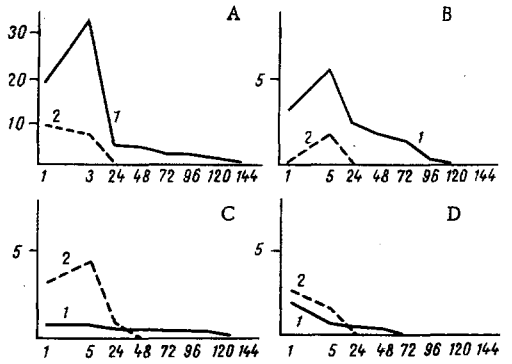

Fig. 1. Uptake of creatine phosphokinase by organs of rats after liposomal and free injection. Abscissa, time (in h); ordinate, uptake of CPK-^{125}I, per cent of injected dose; 1) CPK in liposomes; 2) after free injection; A) liver, B) spleen, C) kidney, D) lungs.

of the liposomes (2 h, 20°C) they were treated with ultrasound for 10 sec on the UZDN-1 apparatus at 22 kHz and at 4°C. The preparation was purified from unbound protein by centrifugation twice from physiological saline at 140,000g on the VAC-601 centrifuge (East Germany, 8 × 11 rotor) for 1 h. The liposomes were resuspended in 0.9% NaCl and kept in an atmosphere of argon at 4°C until use.

Under ether anesthesia, 1 ml of protein-containing liposomes (3.75 mg lipids, 75 μg CPK protein, 9×10^5 cpm) was injected into the femoral vein of noninbred albino rats weighing 200-220 g and the animals were decapitated at different times after the injection. The liver was perfused through the portal vein with physiological saline, and the spleen, heart, lungs, and brain were washed with the same solution and dried with filter paper. Small samples weighing 100-300 mg were placed in a γ-vial (Nuclear Chicago) and the radioactivity of the samples was counted on a Unilux (USA) scintillation counter. Each animal of the control group received an injection of 75 μg unlabeled enzyme with the addition of CPK-^{125}I (9×10^5 cpm).

To detect the degree of preservation of CPK enzyme activity (this enzyme is present in the liver in trace quantities) liposomes with unlabeled CPK (75 mg lipids, 2 mg CPK) was injected intravenously and activity of the enzyme was determined in liver homogenates by means of test systems (Boehringer, West Germany).

Autoradiography was carried out after the ordinary histological treatment of fragments of liver, and sections were cut and then coated with type M (Photographic Chemical Research Institute project) liquid emulsion. The sections were exposed for 1-3 weeks at 4°C, developed, and counterstained with hematoxylin-eosin.

EXPERIMENTAL RESULTS

The internal protein content in positively charged liposomes when the emulsifying mixture is used once and sonication carried out for 1 min, as the writers have shown [2], does not exceed 6-7%. Using the protein solution twice and reducing the time of sonication to 10 sec increased the uptake of protein into the liposomes to 25%. After intravenous injection of lysosome preparations into rats (3.75-75 mg lipids per animal) no sign of a disturbance of the general condition or behavior of the animals was observed at least for 1 month.

The distribution of labeled protein among the organs of the rats at different times after injection is shown in Fig. 1. The quantity of enzyme in the organs was considerably higher when it was injected in lysosomes than after free injection. The maximal level when unencapsulated protein was used was observed in the liver 1 h after injection, and after 24 h the label could no longer be detected in the organ. Injection of protein in liposomes led to a much higher uptake of enzyme by the organs. For instance, the uptake in the liver was 33% (of the injected dose) compared with 6.9% in the control, and a maximum was reached 6 h after injection. About 2.5% of protein (of the injected dose) was found in the liver 6 days after injection. Similar proportions, but at a lower level, also were found in the spleen. In the kidney and lungs label was found during the first day in large quantities after free injection, evidently because of the high blood level of the protein and its rapid excretion through the kidneys. Later, the period of detection of protein in these organs was definitely prolonged as a result of liposomal injection (Fig. 1).

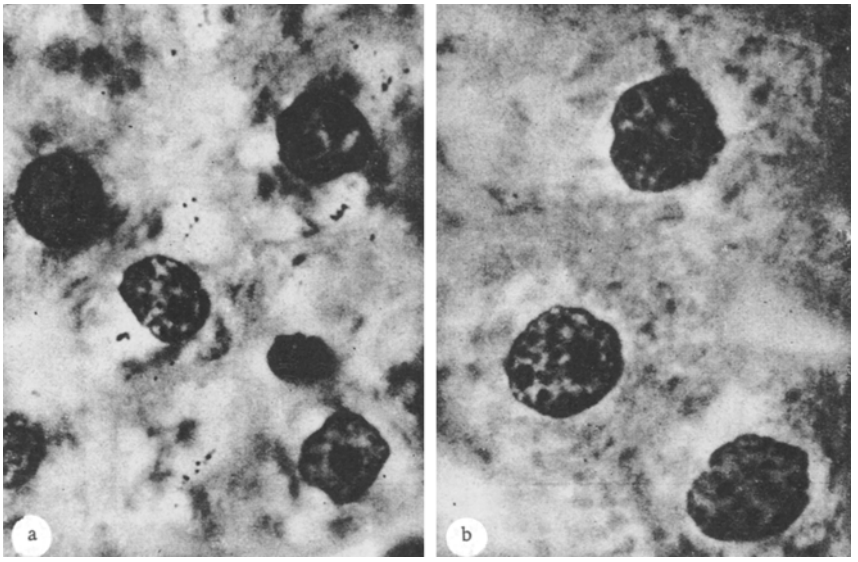

Fig. 2. Histoautoradiographs of liver after injection of CPK-^{125}I in liposomes (A) and in the free form (B), 1230×.

Autoradiography of the liver tissue 24 h after injection of enzyme-containing liposomes showed that the label was present mainly above the cytoplasmic vacuoles of the hepatocytes, probably containing material of liposomes. The intracellular localization of the labeled enzyme when injected in liposomes was confirmed by the absence of vacuoles and label in the control sections at all times of exposure of the histoautoradiographs (Fig. 2).

In a separate series of experiments CPK enzyme activity was tested in liver homogenates 6 h after injection of the preparation (see: Experimental Method). The same level of CPK – 450 and 432 μg respectively (per wet weight of liver) – was found in the control animals (receiving physiological saline) and in the animals receiving the free enzyme; after injection of protein in liposomes the value was 1382 μg (each point is the mean result of tests on 3 animals). These findings, on the one hand, confirm the level of protein uptake by the liver, determined as labeled protein (Fig. 1), and on the other hand, they show that enzyme injected in liposomes preserve their activity.

The experiments thus showed that when the enzyme was injected in liposomes there was a sharp increase in the retention of protein in the organs and in the time during which it could be detected in them. The intracellular localization of the protein was demonstrated autoradiographically and, in addition, the preservation of its enzyme activity was confirmed.

Most workers consider that after injection of positively charged liposomes they are taken up by the cells by endocytosis and are found in the lysosomes [7, 10]. Desnick et al. [5] found β-glucuronidase activity in the liver on the 8th day after its injection in liposomes into mice deficient in this enzyme. These observations confirm the good prospects for the use of enzyme-containing liposomes in order to correct genetic defects at the product translation level. With the recent introduction of techniques whereby liposomes can be "addressed" to particular organs, the attention of various specialists is being drawn increasingly closely to this problem. Orientation of administration is achieved by introducing molecules of antiserum against particular cells or tissues into the lipsomal membrane [3, 8].

Our own observations and data in the literature thus indicate the good prospects for the use of liposomal injection of substances for the treatment of a wide range of metabolic disturbances, and the urgency of research in this field will be evident.

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PATTERN OF HYPERLIPIDEMIA IN RATS WITH CHRONIC RENAL FAILURE

N. V. Nikiforova, I. K. Tananaeva, A. L. Pozdnyakov, and I. V. Sokolovskaya

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The character of the hyperlipidemia was studied in rats with chronic uremia caused by removal of five-sixths of the total weight of kidney tissue. The blood cholesterol and phospholipid concentrations were almost twice the normal level 13-30 days after subtotal nephrectomy in the rats with uremia. The hyperlipidemia was more marked when the blood nonprotein nitrogen level was high. The serum triglyceride concentration was not increased. The total concentration of serum β- and pre-β-lipoproteins, determined nephelometrically, was significantly increased only if the nonprotein nitrogen exceeded 80 mg%. Disk electrophoresis of the serum lipoproteins of the rats with uremia revealed a definite increase in the α-lipoprotein concentration and a very small increase in the β-lipoprotein concentration. Postheparin plasma lipolytic activity in the rats with uremia was normal. Massive proteinuria was observed in the experimental rats, but the hypoproteinemia was not significant.

KEY WORDS: hyperlipidemia; lipoproteins; atherosclerosis; proteinuria; postheparin plasma lipolytic activity.

There is convincing evidence at the present time in support of a disturbance of lipid metabolism and the more rapid development of atherosclerosis in patients with chronic renal failure (CRF), whose life can be supported by programed hemodialysis [5, 9].

Unlike in man, atherosclerotic changes in the vessels (aorta) do not develop in rats with experimental CRF, although in the late stages sclerosis of the media caused by deposition of calcium salts may take place [15]. In view of these differences it was decided to investigate the serum lipid concentration and lipoprotein spectrum and the postheparin lipolytic activity (PHLA) of the plasma in rats with long-lasting CRF and to compare any posssible changes with the lipid disturbances in patients described previously [2, 5]. There are no data in the literature on relations between the lipoproteins and PHLA of the plasma in rats with CRF, and only scattered reports of their blood lipid concentration [4].

EXPERIMENTAL METHOD

Experiments were carried out on 44 noninbred male rats weighing initially 140-160 g, 27 of which had experimental CRF whereas 17 acted as the control. Experimental CRF was induced by removal of five-sixths of the total weight of the kidney tissue in two stages [11]. Observations were kept for 13-30 weeks after the

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