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Components of the cytosolic and released virtosomes from stimulated and non-stimulated human lymphocytes

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ABSTRACT

Abstract aim: This work intends to analyse the structure and the composition of virtosomes and their role.
Background: Virtosomes are newly synthesized DNA-RNA-lipoprotein complexes released from living cells in a regulated and energy-dependent manner.
Methods: Virtosome fractions were isolated by ultracentrifugation from human lymphocytes cytoplasm and from culture medium before and after stimulation with phitoemoagglutinin (PHA). The composition in DNA, RNA, protein and lipids was determined. The virtosomes present in the culture medium were put in contact with lymphocytes.
Results: Virtosome fractions released from non-stimulated lymphocytes are shown to reduce replication of stimulated lymphocytes and those from stimulated lymphocytes to increase multiplication of non-stimulated lymphocytes. Biochemical analyses of the virtosomal complexes have shown that those from stimulated lymphocytes have five proteins that are absent from non-stimulated virtosome fractions. A comparison of the cytosolic versus released virtosome fractions from non-stimulated lymphocytes indicated that there is a greater percentage of phospholipids in the released virtosomes with a corresponding decrease in the percentage of protein.
Conclusion: Although there is a presence of cholesterol in the virtosomes, the low levels of phosphatidylcholine and cholesterol, together with the low ratios of cholesterol: phospholipids leads to a confirmation of the apparent lack of a limiting membrane around the virtosomes.
General significance: Virtosomes are structural particles formed in the cytoplasm, released from the cells and capable to be transferred in other cells influencing their behaviour.

1. Introduction

A number of early investigators demonstrated that both stimulated and non-stimulated lymphocytes released DNA [1–9]. Subsequently, Stroun and Anker showed the released DNA to be newly synthesized with ^{3}H-thymidine labeling studies [3]. Furthermore, the DNA was associated with RNA [10]. Since both nucleic acids were resistant to nuclease activity, it was considered that they were protected by lipoprotein. The presence of protein was identified when RNAse activity affected RNA only after a prior treatment with either pronase or proteinase k [2] while that of lipids was identified from the complex's low density during upward sucrose density gradient centrifugation, freezing and thawing and the incorporation of radioactive phospholipid precursors [2]. Subsequent studies using radioactive precursors permitted the demonstration that the RNA, protein and associated phospholipids were (a) newly synthesized and (b) synthesized at about the same time. Similar results were obtained with other cell types [11,12]. This DNA/RNA-lipoprotein complex has an estimated size of $\sim 5 \times 10^5$ Da [3] although the complex released from stimulated rat lymphocytes had a higher density than that released from non-stimulated rat lymphocytes [1]. The complex, termed a virtosome [13] is released in an apparently energy-dependent step [2], only from living cells [2,3] in a controlled manner [3]. Experiments employing radioactive precursors have shown that the DNA, RNA, phospholipid and proteins appear in the cytoplasm at about 3 h after commencing labeling and that the complex is released from cells 3–6 h later, depending on which cells were studied i.e. human, other mammalian, avian, amphibian and plant

Abbreviations: PHA, phytohaemaglutinin; PLs, phospholipids; CHO, cholesterol; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphayidylcholine; PE, phosphatidyiletanolamine; SM, sphyngomyelin

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cells [1,3,12,14–16]. The complex does not appear to have a limiting membrane as shown by studies on the uptake and release of virtosomes between chick embryo fibroblasts [17] and on release from J774 cells and their uptake by non-stimulated lymphocytes [18]. Importantly, virtosomes released from one cell type can enter a different cell type resulting in a biological modification of the recipient cells e.g. transformation of NIH 3T3 cells on uptake of released mutant k-ras from SW480 cells [19], an allogenic T–B lymphocyte co-operation involving lymphocyte subsets from human donors with different allotypes [20,21] and DNA synthesis initiation in non-stimulated lymphocytes on uptake of virtosomes released by J774 and P497 tumour cells [18]. Thus, the virtosome appears to be a novel cytoplasmic component that may act as an inter-cellular messenger.

However, the full structure of the complex has not been ascertained. In the present study, experiments were designed (a) to identify the lipids and proteins associated with both the cytosolic and released complexes, (b) the comparative amounts of proteins, lipids, DNA and RNA in cytosolic and released virtosomes and (c) the nature of the proteins present in the released virtosomes from stimulated lymphocytes as opposed to those absent in non-stimulated lymphocytes. However, as a first step to ensure that the virtosomes released from stimulated and non-stimulated lymphocytes were biologically active, the released virtosomes were fractionated and tested for their biological activity, using a modification of the previously described method [17,18].

In addition to obtaining the overall content of DNA, RNA and phospholipids, the analysis of the individual phospholipids gave further confirmation for the absence of a classical membrane limiting the virtosome.

2. Material and methods

2.1. Lymphocyte separation

The lymphocytes were obtained from buffy coats kindly donated by the Immunotransfusion Laboratory (Ospedale Santa Maria della Misericordia, Perugia).

Blood samples were stratified on Ficoll-Plaque and centrifuged at 1600 rpm for 30 min The lymphocyte layer was collected and the cells resuspended in saline and sedimented by centrifugation at 1600 rpm for 30 min This treatment was repeated twice and the cells were counted using a Burker chamber. Dead cells (1.0–1.5%) were identified by trypan blue staining.

2.2. Lymphocyte experiments

2.2.1. Growth of lymphocytes

This was evaluated by seeding the isolated lymphocytes in RPMI medium in 50 ml flasks, either with or without PHA, at a concentration of 100,000 ml^{-1}. In some experiments, the lymphocytes were incubated in RPMI medium either with or without serum +PHA. Cell proliferation was estimated by cell counts every 24 h for 96 h.

After incubation for 96 h, the lymphocytes were recuperated by centrifugation at 600 g for 10 min, washed twice with RPMI medium in order to eliminate any serum and PHA after which they were ready for use in subsequent experimental procedures.

2.2.2. Released and cytosolic virtosome isolation

~350,000,000 lymphocytes were place in RPMI for 3 h and incubated at 37 °C for 3 h prior to centrifugation at 600 g for 10 min to sediment the cells. Cell death (< 1.5%) was monitored by trypan blue.

The supernatant so obtained was further centrifuged at 10,000 g for 10 min to remove organelles and cell debris. The supernatant was further centrifuged at 120,000 g for 1 h to sediment any remaining debris. The virtosomes will have remained in the supernatant. The cells and the final supernatants were saved for the analysis of the virtosomes that remain in the supernatant.

The supernatant to be used for the analysis of DNA, RNA and protein (350–390 ml) was lyophilized. The powder obtained was resuspended in distilled water and dialysed overnight at 3–4 °C against diluted PBS to decrease the saline concentration.

Cytosolic virtosomes were isolated by gently homogenizing the cells in PBS (10 strokes with a plastic pestle) [18]. The suspension was treated as described (above) for the released virtosomes. The supernatant so obtained was lyophilized and dialysed as described above.

2.2.3. Analysis of the cytosolic and released virtosomes

Protein was determined with the colorimetric method Folin [22] using albumin as a standard. The DNA was determined by the method of diphenilamine (Burton [23], using DNA (the highly polymerized calf-thymus DNA-preparation-Sigma) at scalar quantities for quantification. The RNA was measured using the orcinolo method, Ribonucleic acid type IV from calf-liver-(from Sigma for quantitative evaluation) at scalar quantities for quantification [24].

Total lipids were extracted by the method of Folch et al. [25] and their concentration determined by measuring the amount of inorganic phosphorus using Fiske and Subbarow method [26].

2.2.4. Chromatographic separation and quantification of phospholipids

The supernatant was placed directly upon thin layer chromatography plates (Merk) and the phospholipids separated using chloroform: methanol: ammonia (65:25:4 v/v) and the spots identified with iodine.

The individual phospholipids were scraped from the plates, collected and the amount of inorganic phosphorus present was determined [26]. The single phospholipids were identified using a standard phospholipid solution as reference [27].

2.2.5. Cholesterol determination

After total lipid extraction, chromatographic plate separation of cholesterol was made using a solution of ethyl ether: petroleum ether: acetic acid (50:50:1 v/v) and cholesterol was identified using cholesterol as a standard reference. After removal from the chromatographic plate, the cholesterol amount was determined with 0.05% o-phtaldeyde in acetic and sulphuric acids [28].

2.2.6. Protein analysis

Proteins present in the culture medium supernatants derived from cultures of both stimulated and non-stimulated cells were concentrated by ultra-filtration with AMICON CENTRIKON, Millipore that excludes proteins of < 5000 Da. The protein concentration was determined by the method of Bradford [29]. One hundred μg of the protein concentrate for each sample were analysed by bi-dimensional electrophoresis using a GE HEALTHCARE apparatus.

In bi-dimensional electrophoresis the first run is based on IEF, the pH range is 3–10, the direction is vertical from anode (+) to cathode (-), the second run is an SDS-Page, from cathode to anode is the direction and discriminates on the basis of molecular weight (the higher on top and the lower on back).

Each spot was analysed with a SPECTROMETER ESI TRAP, LCQ Deca XP plus THERMO ELECTRON and a MASCOT SEARCH system (Fig. 1).

The bands indicated with the letters are present in both samples, whereas that indicated with numbers are present only in stimulated lymphocytes.

Mono-dimensional electrophoresis was performed with a BIO