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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

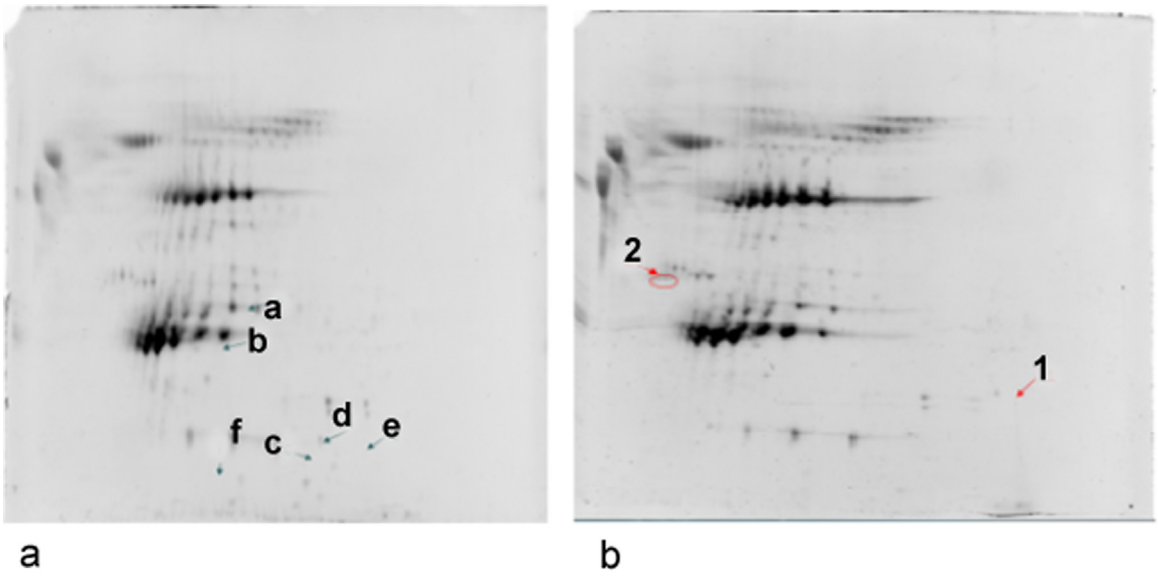

Fig. 1. Bidimensional electrophoresis of proteins of released virtosomes obtained from control (a) and stimulated with PHA (b) lymphocytes.

RAD apparatus.

using 50 μg protein per lane for each sample. Spots were analysed by SPECTROMETER ESI TRAP, LCQ Deca XP plus THERMO ELECTRON and a MASCOT SEARCH. Only the bands indicated in with the numbers 1–5 in Fig. 2 were so analysed.

3. Results

3.1. Lymphocyte cultures

3.1.1. Stimulated v non-stimulated lymphocytes

As expected, the number of lymphocytes increased more rapidly after PHA stimulation rising from 100,000 ml^{-1} to ~1000,000 ml^{-1} by 96 h whilst non-

stimulated cells augmented by only ~680,000 cells ml^{-1} (Fig. 3).

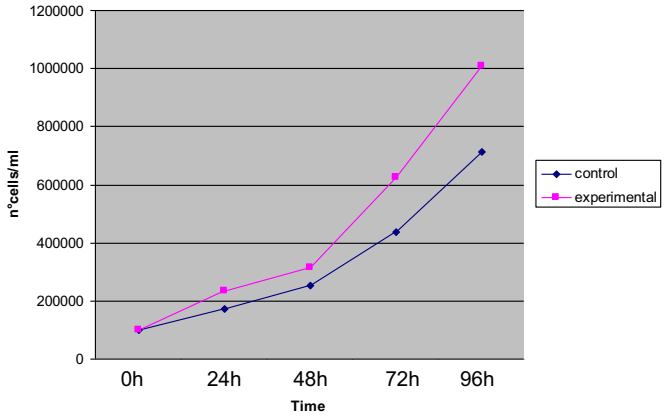

Fig. 3. Difference in the growth curve of non- stimulated (control) and PHA stimulated lymphocytes.

3.1.2. Effect of virtosomes released from stimulated lymphocytes on non-stimulated lymphocytes

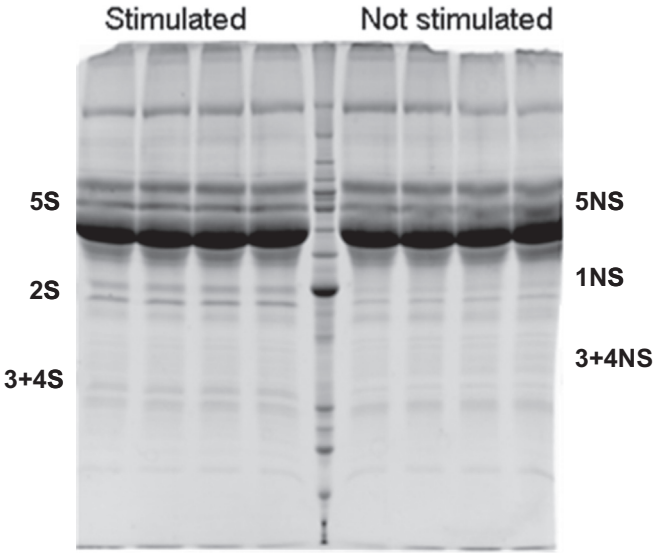

Fig. 2. Monodimensional electrophoresis of non- stimulated and PHA stimulated lymphocytes. The numbers indicate the bands analysed.

Whilst non-stimulated lymphocyte numbers increased from 100,000 ml^{-1} to 668,000 ml^{-1} cells, those cultured in the presence of the virtosome fraction from stimulated lymphocytes increased from 100,000 ml^{-1} to 821,000 ml^{-1} (Fig. 4).

3.1.3. Effect of virtosomes released from non-stimulated lymphocytes on stimulated lymphocytes

Little difference was observed between the cell number increase between non-stimulated lymphocytes and stimulated lymphocytes in the presence of virtosomes released from non-stimulated lymphocytes (Fig. 5).

3.2. Analysis of non-stimulated lymphocyte cytosolic and released virtosomal fractions

3.2.1. General analysis

The percentages of DNA, RNA, protein and phospholipids present in the two fractions are given in Tables 1 and 2 (Table 3 and 4).

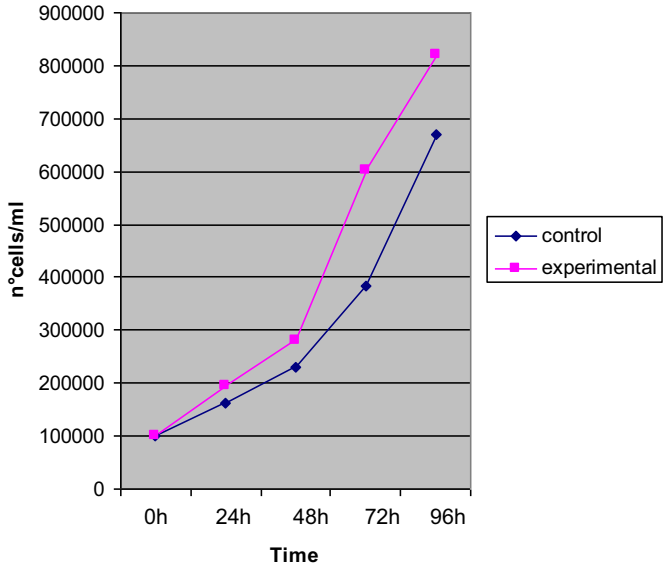

Fig. 4. Cell growth in the presence of virtosomes from PHA stimulated lymphocytes with respect to control.

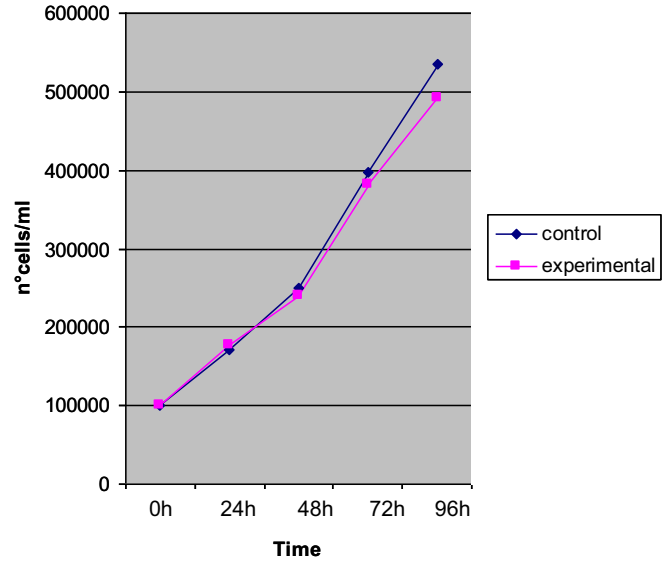

Fig. 5. Cell growth of PHA stimulated lymphocytes in the presence of virtosomes from non-stimulated lymphocytes (control).

Table 1
Composition of Virtosomes isolated from the supernatant.

Supernatant	%	γ/mg proteins
Proteins	27.44 ± 4.20	
DNA	3.92 ± 1.85	139 ± 33
RNA	36.41 ± 2.31	1345 ± 204
PLs	32.21 ± 3.69	1196 ± 223

The same analysis was made on the virtosomes isolated from the cytoplasm (Table 2).

3.3. Phospholipids and cholesterol

There is an increase in both the SM and PS contents of the released fraction compared with the cytosolic fraction. The PC and PE percentages remain relatively unchanged whereas PI increases.

Table 2
Composition of Virtosomes isolated from the cytoplasm.

Cytoplasm	%	γ/mg proteins
Proteins	41.01% ± 1.91	
DNA	3.45% ± 0.39	95 ± 10
RNA	35.09% ± 4.40	859 ± 142
PLs	19.90% ± 2.16	484 ± 42

Table 3
Comparison of the values calculated per mg of proteins.

	Cytoplasm	Supernatant
DNA	95 ± 10	139 ± 33
RNA	859 ± 142	1345 ± 204
PLs	484 ± 42	1196 ± 223

Comparison of the percentage values shows no significant differences in the DNA and RNA content from the two fractions (Tables 3, 4). An increase in PLs is observed in the released fraction.

Table 4
Comparison of the percentage of virtosome composition.

	Cytoplasm	Supernatant
Proteins	41.01%	27.91%
DNA	3.45%	3.92%
RNA	35.09%	36.41%
PLs	19.90%	32.21%

However, the protein concentration is diminished and the phospholipid percentage increased in the released fraction.

Table 5
PLs composition of virtosomes isolated from the supernatant.

	average%	μg PLs values of 2 experiments
PS	14.78%	PS: 0.15 μg → 14.56%PS: 0.15 μg → 15.0%
PI	16.25%	PI: 0.16 μg → 16.0% PI: 0.17 μg → 16.50%
SM	27.57%	SM: 0.27 μg → 27.0% SM: 0.29 μg → 28.15%
PC	20.69%	PC: 0.21 μg → 21.0% PC: 0.21 μg → 20.38%
PE	20.69%	PE: 0.21 μg → 21.0% PE: 0.21 μg → 20.38%

The same analysis, performed on virtosomes isolated from the cytoplasm, confirm the increase of SM and PI. The results of two experiments are very similar (Table 6).

Table 6
PLs composition of virtosomes from the cytoplasm.

	Average%	μg PLs values of 2 experiments
PS	12.5%	PS: 0.15 μg → 14.42% PS: 0.09 μg → 10.58%
PI	23.11%	PI: 0.20 μg → 19.23% PI: 0.23 μg → 27%
SM	19.23%	SM: 0.18 μg → 17.30% SM: 0.18 μg → 21.17%
PC	22.58%	PC: 0.25 μg → 24.0% PC: 0.18 μg → 21.17%
PE	22.5%	PE: 0.26 μg → 25.0% PE: 0.17 μg → 20.0%

CHO was present but at a level, which when referred to the PLs levels, is very low. In the released fraction, the ratio of CHO: PLs is 0.29 whilst that for the cytosolic fraction is 0.19This results is confirmed by the ratio CHO/SM which is very different with respect to membrane and is more similar to the values find in the chromatin (Table 7).

Of interest is the similarity of the PC levels that are less than would be expected to be present in a standard membrane (Tables 5 and 6) [30] Table 7.

Table 7
Values of CHO in relation to PLs and SM.

	Supernatant	Cytoplasm
CHO /PLs	0.29	0.19
CHO/SM	1.08	1.02

3.4. Protein levels and composition

The difference in percentage of protein composition between cytosolic fraction and supernatant is mainly due to an increase in PLs than a real decrease in protein (Table 4) which concentration/ml remains practically constant.

The bi-dimensional electrophoretic separation of the proteins shows a large number of proteins of >5000 Da to be present in both released and cytosolic fractions (Fig. 1(a), (b)). These include human RAI1-retinoic acid-induced protein present in the non-stimulated lymphocyte fraction (spot 1 of Fig. 1(a)) and human NFRKB-nuclear factor related to kappa-B-binding protein present in the stimulated lymphocyte fraction (spot f of Fig. 1(b)).

The mono-dimensional electrophoretic gels (Fig. 2) also show a large number of proteins present in both the non-stimulated and stimulated lymphocyte fractions. Six human proteins, present in the fraction from stimulated lymphocytes, but absent from the non-stimulated lymphocyte fraction, include CHM2A-charged multi-vesicular body protein 2a (line 3), CLMP-CXADR-like membrane protein (line5), ZFAN4-AN1-type zinc finger protein 4 (line 4), ZN160-zinc finger protein 160 (line5), DYHC1-cytoplasmic dynein 1 heavy chain 1(Line 4) and PNPH-purine nucleoside phosphorylase (Line4).

Human proteins present in both non-stimulated and stimulated lymphocyte fractions include ACTB-cytoplasmic 1 actin (Line1), ACTA-aortic smooth muscle actin (line2), POTEE-POTE Ankyrin domain family member E (line2), POTEF-POTE Ankyrin domain family member F(line2), FETUA-alpha-2-HS-glycoprotein and UBE4B-ubiquitin conjugation factor E4B(line5).

4. Discussion

The incubation of non-stimulated lymphocytes in the culture medium containing virtosomes from stimulated lymphocytes resulted in an increase in their level of multiplication (Fig. 4). No effect was seen when the stimulated lymphocytes were incubated in the culture medium from non-stimulated lymphocytes (Fig. 5). This response was in agreement with the data from earlier experiments [18] when DNA synthesis was increased in non-stimulated lymphocytes by virtosome preparations from tumour cells while DNA synthesis was inhibited by the virtosome preparations from non-stimulated lymphocytes and hepatocytes.

Hence, it was of interest to analyse the virtosome fractions from non-stimulated and stimulated lymphocyte populations to determine their composition and to note any differences. Similarly, a comparison of cytosolic and released virtosome fractions would indicate any differences between them. In addition, it would help to determine whether or not a standard limiting membrane was present since earlier studies had indicated that there was no such membrane [16,18].

Earlier workers had demonstrated that the virtosomes form in the cytosol and are released into the external medium after formation [13]. Hence, a biochemical analysis was performed on the cytosolic and released virtosome fractions from non-stimulated lymphocytes. The major differences concern the percentage increase in PLs contents with a lower percentage of proteins in the supernatant virtosomes (Table 4). Nevertheless, there is little difference between the percentage contents of the single PLs, the major change being an increase of 8% in the SM content and a decrease in PI with minor change of PC and PE. If compare the percentages of single PLs with that present in the nuclear membrane, chromatin and nuclear matrix [30] it is clear that they do not correspond to any of these structures especially for the large amount of SM and the low percentage of PC. This specific aspect excludes the possibility that the material analysed in the supernatant may be derived from membranes nuclear matrix or chromatin of dead cells.

This conclusion is also confirmed by the similarity with the PLs found in the cytoplasm, thus in agreement with the hypothesis that virtosomes are formed inside of the cells and thereafter excluded. The high percentage of SM is similar to that found in the chromatin and may have a possible role in transcription by protecting RNA from degradation. Previous experiments have demonstrated that chromatin SM protects RNA from RNase digestion [31].

The presence of CHO confirms the previous difference with values very low with respect to membranes. The only affinity is the value of CHO/SM which is similar to that found in the chromatin. The role of CHO is crucial for membrane biogenesis, but also for trafficking and signalling and is indispensable for cell migration and invasion. In this case it may have a role in favouring the invasion of cells by the virtosomes [32,33].

Earlier studies [16,18] have indicated that the virtosomes do not appear to have a standard limiting membrane. This is reinforced by the low amount of PC (Tables 5,6). Furthermore, the ratios of CHO: PLs are too low for the presence of a standard membrane. In the released fraction, the ratio is 0.29 whilst that for the cytosolic fraction is 0.19. This compares with a ratio of almost 1.0 for standard membranes. The comparison of the values expressed in percentages show a decrease of proteins in the released virtosomes with respect to that isolated from the cytoplasm with an increase in percentage of PLs. The protein decrease implies that they are formed inside the cells and released thereafter.

In order to confirm this supposition and to explain the difference of the effect of virtosomes from stimulated lymphocytes and that from nonstimulated on the lymphocytes, we have analysed the proteins of the supernatant.

Only the lanes which indicate possible differences were analysed. Bi- and uni-dimensional gel electrophoresis of the proteins showed a large number of proteins of >5000 Da to be present in the fractions measured (Figs. 1a, b, 2).

A small number of the proteins have been identified including five proteins present in the stimulated lymphocyte released virtosome fraction that are absent from the supernatant of the control.

Bidimensional electrophoresis showed the presence of NF-kB (nuclear factor related to kappa B–binding protein) which is involved in transcriptional regulation, DNA replication and probably DNA repair [34].

Monodimensional electrophoresis has permitted the identification of many proteins. Some of which are common to both stimulated and non-stimulated cells and some that are specific to one or other of the two groups of lymphocytes. Actin and Ankyrin are common and perhaps may function to anchor of structure.

Concerning the proteins present only on virtosomes released from stimulated lymphocytes, the most important are, the ZNF160 zinc finger protein 160 involved in transcriptional regulation [35], the DYHC1 dynein, cytoplasmic 1, heavy chain 1 potential transmembrane proteins that may play a role in either the transport between the endoplasmic reticulum and the Golgi complex or organization of the Golgi in cells. The dynein-dynactin complex is necessary for protein transport, positioning of cell compartments, mobility of structures within the cell and many other cell processes [36]. The PNPH purine nucleoside phosphorylase has a

central role in purine metabolism [37].

In conclusion, on the basis of these results, we can exclude the possibility that the isolated particles are due to dead cell fragments, since they have specific characters which correspond to those found in the cytoplasm and so confirm earlier findings that the complexes are not released by dead cells [15]. The composition change in relation to function such that the virtosomes are capable of influencing other cells.

Aknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.04.006

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