M Cells Prefer Archaeosomes: An *In Vitro/In Vivo* Snapshot Upon Oral Gavage in Rats

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Abstract: The archaeolipids (lipids extracted from archaebacterias) are non saponificable molecules that form self sealed mono or bilayers (archaeosomes-ARC). Different to liposomes with bilayers made of conventional glycerophospholipids, the bilayer of ARC posses a higher structural resistance to physico chemical and enzymatic degradation and surface hydrophobicity. In this work we have compared the binding capacity of ARC exclusively made of archaeols containing a minor fraction of sulphoglycophospholipids, with that of liposomes in gel phase on M-like cells *in vitro*. The biodistribution of the radiopharmaceutical ^{99m}Tc-DTPA loaded in ARC vs that of liposomes upon oral administration to Wistar rats was also determined. The fluorescence of M-like cells upon 1 and 2h incubation with ARC loaded with the hydrophobic dye Rhodamine-PE (Rh-PE) and the hydrophilic dye pyranine (HPTS) dissolved in the aqueous space, was 4 folds higher than upon incubation with equally labeled liposomes. Besides, 15% of Rh-PE and 13 % of HPTS from ARC and not from liposomes, were found in the bottom wells, a place that is equivalent to the basolateral pocket from M cells. This fact suggested the occurrence of transcytosis of ARC. Finally, 4 h upon oral administration, ARC were responsible for the 22.3 % (3.5 folds higher than liposomes) shuttling of ^{99m}Tc-DTPA to the blood circulation. This important amount of radioactive marker in blood could be a consequence of an extensive uptake of ARC by M cells *in vivo*, probably favored by their surface hydrophobicity. Taken together, these results suggested that ARC, proven their adjuvant capacity when administered by parenteral route and high biocompatibility, could be a suitable new type of nanoparticulate material that could be used as adjuvants by the oral route.

Keywords: Adjuvant, archaeosomes, oral.

INTRODUCTION

In the last decade, the intervention of Nanotechnolgy into the field of Immunology has opened new avenues to novel nano-particulate material capable of inducing antigen-specific adjuvancy, particularly in non-parenteral delivery [1-5]. Up to the moment however, if well oral is the most friendly route of administration, satisfactory protective and memory responses can only be achieved employing live attenuated microoganisms [6, 7]. The generation of antigen-specific sIgA at the site of the infection upon oral immunization with inert particulate material, remains as an ambitious challenge, specially for developing countries [8, 9].

The inductive sites at the gut-associated lymphoid tissue (GALT) in the mucosa of small intestine are represented by the Peyer´s patches (PP) [10, 11]. Within the follicle-associated epithelial (FAE) cells of each PP are the M (microfold) cells, which are specialized in taking up particulate antigens from the intestinal lumen to transport them to the subepithelial dome, populated by immature myeloid dendritic cells (antigen presenting cells, APC) [12]. Epithelial mature dendritic cells also contribute to the direct uptake of particulate material from the lumen [13]. Binding and subsequent transcytosis of particulate material (instead of soluble) across M cells to gain access to the APC located in the follicule, are acknowledged as key steps to elicit effective immune responses by the oral route [14-16]. Hence, structural features of particulate material leading to enhanced uptake by M cells - that are present at a very low density in the gut- could contribute to improve potential immune reactions upon oral administration [17]. If well specific markers enabling active targeting of particulate material to human M cells remain unknown, the over expression of b1 integrins at the apical pole of human M cells was demonstrated [1, 18]. Very recently ovoalbumin (OVA) loaded PLGA nanoparticles grafted with analogs of the Arg-Gly-Asp motif were shown to be effective at targeting the M cells as well as in the subsequent generation of immune responses upon duodenal administration to mice [19].

Unfortunately, recent clinical studies using particulate material targeted to M cells resulted poorly reproducible [20, 21]. Several reasons have contributed to reduce the current expectancies on the use of particulate material as oral adju-

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vants [22]. For instance, the uptake by endothelial enterocytes and the M cells in PP is size dependent (higher for lower sized particles, a fact that limits the amount of loaded antigen) and limited at lower level than previously though, nearly 5% of the administered dose for particles below 100 nm [15, 23]. Conventional liposomes are poorly absorbed, in the order of 2 % the total dose, whereas polymerized liposomes are absorbed up to 6 % [24]. In this context, counting on new materials with structural characteristics tailored to allow an effective passive targeting and uptake by M cells becomes relevant.

Archaeosomes (ARC) are vesicles composed mostly by total polar lipids (TPL) extracted from micro organisms that belong to the Archaea domain of life, which are usual inhabitants of extreme environment [25]. The core structures of archaeal polar lipids consist of archaeol or diether lipid (2,3-di-*O*-diphytanyl-*sn*-glycerol) which contain 20 carbons per isoprenoid chain, and/or caldarchaeol or tetraether lipid (2,2´-3,3´- tetra-*O*-dibiphytanyl-*sn*-diglycerol) containing 40 carbons per isoprenoid chain, and modifications of these structures [26-28]. The different habitats of archaeas (extreme termophiles, extreme halophiles, sulpho acidophiles, methanogens (anaerobics) and psychrophiles) define the main composition of their TPL [29].

The purpose of this work was to estimate the binding and transcytosis of ARC and plain liposomes in gel phase, using an *in vitro* model of M-like cells (Caco-2/Raji co-cultures). Additionally, the biodistribution of the hydrosoluble radiopharmaceutical ^{99m}Tc-DTPA loaded into ARC and liposomes was determined upon oral gavage to rats.

MATERIALS AND METHODS

Materials

Hydrogenated phosphatidylcholine from soybean (HSPC) was obtained from Northern Lipids (Vancouver, Canada). Cholesterol, 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(Lissamine™ rhodamine B sulfonyl) (Rh-PE), *N,N*-dimethylformamide, pepsin, bile extract porcine, pancreatin from porcine pancreas, formaldehyde, Triton X-100, naphtol AS-MX phosphate, Fast Red TR salt and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). The fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was from Molecular Probes (Eugene, OR, USA). Acrylamide, bis-acrylamide and glycine were from ICN Biomedicals (Irvine, CA, USA). RPMI 1640 and MEM with non-essential amino acids (MEM-NEAA) culture media were purchased from Invitrogen Corporation (California, USA). Endotoxin-free fetal bovine serum (FBS), L-Glutamine, Trypsin, EDTA and penicillin/streptomycin (PEST) were provided by PAA Laboratories GmbH (Pasching, Austria). Tris buffer and all the other analytical grade reagents were from Anedra (Buenos Aires, Argentina).

Growth of Archaebacteria and Total Polar Lipids Isolation

Halorubrum tebenquichense archaeas were isolated from soil samples collected in Salina Chica, Península de Valdés, Chubut, Argentina as described in Gonzalez *et al*. [30]. Biomass was generated in 8 l batch cultures in basal medium [31], supplemented with yeast extract, glucose and antifoam (20µl/l). Cultures were monitored by absorbance at 660 nm and harvested in late stationary phase for storage as frozen cell pastes.

Lipids were extracted from frozen and thawed biomass, with Cl_3CH:CH_3OH:H_2O (1:2:0.9; v:v), and the total polar lipid (TPL) fraction was collected by precipitation from cold acetone [27].

The lipid extract was analyzed by two-dimensional chromatography, by using Cl_3CH:CH_3OH:H_2O (65:25:4 v:v) in the first dimension and Cl_3CH:CH_3OH: HCH_2COOH:H_2O (80:12:15:4 v:v) in the second dimension. Lipids were detected with the following spray reagents: (a) molybdenum blue Sigma spray reagent for phospholipids; (b) 0.5% orcinol/sulfuric acid for glycolipids; (c) azure A/sulphuric acid for sulphoglycolipids, (d) 5% sulfuric acid in ethanol, followed by charring at 120 °C, for all spots. Non-polar pigments were analyzed by mono-dimensional TLC using hexane/diethyl ether/acetic acid (70: 30:1 v:v) and stained with 5% H_2SO_4.

ARC Preparation and Physicochemical Characterization

Archaeosomes containing a double fluorescent label HPTS and Rh-PE (ARC-HPTS-Rh-PE were prepared by hydration of the thin lipid film. Briefly, 20 mg of TPL from $CHCl_3$: CH_3OH (9:1, v/v) solution was mixed with 5.10^{-5} mol of Rh-PE in $CHCl_3$ and further rotary evaporated at 40 °C in round bottom flask until organic solvent elimination. The thin lipid film was flushed with N_2 and hydrated at 40°C with 1 ml of 35 mM HPTS in 10 mM Tris-HCl buffer plus 0.9% w/v NaCl, pH 7.4 (Tris buffer). The resultant suspension was sonicated (20 min in a bath type sonicator 80 W, 40 KHz) and submitted to 5 cycles of freeze/thaw between - 80 and 37°C. Non-incorporated dyes were removed by centrifugation and washing with Tris buffer (3 times at 10000 x g for 20 min). Liposomes made of HSPC:cholesterol (1:1 mol:mol) were prepared in the same way (L-HPTS-Rh-PE).

Phospholipids were quantified by a colorimetric phosphate micro assay [32]. Fluorescence was measured in a spectrofluorometer PTI, Timemaster C-70, using λexc 550-λem 590 nm for rhodamine, and λexc 413- λem 510 nm for HPTS. Mean ARC size was determined by dynamic light scattering with a 90 Plus Particle size analyzer (Brookhaven Instruments) and Zeta potential was determined with a Zetasizer 4 (Malvern).

ARC and L were frozen at -80ºC for 24h and lyophilized using a Labconco Freeze Dry System / Freezone 4.5 (Kansas City, MO -USA), drying was performed at a pressure form 37.10^{-3} to 62.10^{-3} mbar for 12 h. Dried liposomal residua were re-hydrated to initial volume with distillated water and size was measured by dynamic light scattering.

Cytotoxicity

Cell viability, upon incubation with ARC and L was measured as mitochondrial dehydrogenase activity employing a tetrazolium salt (MTT) on Caco-2 cells. Caco-2 cells were seeded at a density of 4×10^4 cells/well in a 96 well plate and growth for 24h. Culture medium of nearly confluent cell layers was replaced by 100 µl of medium containing 10, 100, 500 or 1000 µg/ml of lipids. Upon 24 h at 37°C,

media were removed and replaced by fresh RPMI medium containing at 0.5 mg/ml of MTT. Upon 3 h incubation, MTT solution was removed, the insoluble formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm in a microplate reader.

Preparation of Caco-2/Raji Co-Cultures

Caco-2 cells (human colon carcinoma cell line was kindly provided by Dr. Osvaldo Zabal, INTA Castelar) were cultured in MEM-NEAA supplemented with 1mM L-glutamine, 1% pyruvate, 1% PEST and 10% FBS at 37°C, 5% CO_2 and 95% humidity.

Raji-B cells (human Burkitt´s lymphoma from Asociación Banco Argentino de Células) were cultured in RPMI 1640 supplemented with 1mM L-glutamine, 1% PEST and 10% FBS at 37°C, 5% CO_2 and 95% humidity.

Caco-2/Raji cells were co-cultivated following a previously described protocol [18]. Briefly, Caco-2 cells were seeded at a density of 3×10^5 cells per 24-well polyester inserts (3 μm pores, ThinCert™, Greiner Bio-One, Frickenhausen, Germany), the medium of the upper and bottom wells were changed every 2–3 days, and cells were cultured to confluence over 14-16 days.

Then, 3×10^5 Raji cells, suspended in supplemented MEM-NEAA plus 1% PEST and 10% SFB were added in the bottom wells. The co-cultures were maintained for 4–5 days. The medium of the upper wells were changed every other day. Monocultures of Caco-2 cells, cultivated as above except for the presence of Raji cells, were used as controls.

Monolayer integrity was evaluated by measuring transepithelial electrical resistance (TEER) using a Millicell Electrical Resistance System (Millipore Corp., Bedford, MA) connected to a pair of chopstick electrodes.

Additionally, the cell monolayer integrity was evaluated by red phenol exclusion [33]. In brief, culture media of upper and bottom wells were removed; cells were washed 3 times with HBSS, then 600 μl of buffer HBSS with phenol red (42 μM) was added to upper well and 700 μl of phenol red free buffer HBSS was added to the bottom well. The diffusion of phenol red was allowed for 2h a 37 ºC and aliquots of upper and bottom wells were removed, pH was adjusted to 11 with NaOH 1M, absorbance at 588 nm was measured and percent of diffusion was calculated. As 100% of phenol red diffusion an insert without cells was used.

Activity of the enzyme alkaline phosphatase was evaluated to show differentiation of Caco-2 cells to M-like cells [34]. Briefly, culture media of upper and bottom wells were removed and cells were washed 3 times with PBS, cells were fixed with 1% formaldehyde and incubated with X-100 triton 0.2 % for 15 sec. Then, cells were washed and incubated with reactive solution for 5 min (10 ml buffer pH 8.9 solution containing 5 mg naphthol AS-MX phosphate, plus 250 μl *N,N*-dimethylformamide and 10 mg Fast Red). Inserts membranes were cut and mounted on a Nikon Alphaphot 2 YS2 fluorescence microscope. Four photographs, each one corresponding to ¼ of the total area of the insert were taken using an objective of 4x and fluorescence intensity were quantified using ImagePro Plus software.

In Vitro Binding and Transport Studies

Upper wells media of Caco-2 cells and Caco-2/Raji co-cultures were replaced by 150 μl of fresh medium containing 50 μg of lipids as ARC-HPTS-Rh-PE or L-HPTS-Rh-PE. Cultures were incubated for 1 and 2 h at 37°C, and then media of bottom and upper wells were removed and stored for fluorescence measurements. Cells were washed 3 times with PBS and fixed with 1% formaldehyde, inserts membranes were cut and observed with a confocal laser microscopy Olympus FV300 equipped with an Ar laser (488 nm for HPTS excitation) and a He-Ne laser (543 nm for Rh-PE excitation).

In Vitro Digestion

Proteins of a homogenate of *Trypanosoma cruzi* epimastigotes (kindly provided by Dra. Patricia Petray, Servicio de Parasitología y Enfermedad de Chagas, Hospital de Niños Ricardo Gutierrez) were loaded in ARC (ARC-*T. cruzi*) and L (L-*T.cruzi*). In brief, thin lipid films were hydrated with 3 mg/ml of proteins en in PBS buffer, after sonication and frezze/thaw cycling; non-incorporated proteins were removed by centrifugation (3 times at 10000 g for 30 min at 4ºC). Protein contents were measured by Bradford assay [35].

An *in vitro* digestion process adapted from Patel *et al.* [36] and Jovaní *et al.* [37] was applied to ARC-*T. cruzi* and L-*T. cruzi*. Briefly, 500 μl of samples (0.5 mg proteins) were incubated with 2 ml of pepsin (1.5 mg/ml buffer Tris pH 2) for 30 min at 37°C. Then, pH of the mixtures was increased to 6.5 with $NaHCO_3$ 1M and 100 μl of bile extract and pancreatin solution were added (60 and 9 mg/ml, respectively) and incubated 30 min at 37 °C. Finally, pH was increased to 7.4 with $NaHCO_3$ 1M and samples were centrifuged (10,000 g for 30 min at 4ºC). Proteins from pellets were recovered [38] and analyzed by 15% polyacrylamide gel electrophoresis, proteins were visualized by silver staining.

Radiolabeling of Vesicles

First, ^{99m}Tc- sodium pertechnetate ($Na^{99m}TcO_4$), was eluted from a molybdenum-technetium generator (Tecnonuclear, Buenos Aires, Argentina) with 0.9% saline, then it was complexed with diethylenetriamine pentaacetic acid (DTPA) using a "^{99m} Tc-DTPA kit"(containing 5 mg of DTPA and 0.25 mg of stannous chloride). Then, thin archaeolipid film was hydrated with 11-12 mCi of ^{99m}Tc-DTPA with mechanical mixture and further sonication. Non-incorporated ^{99m}Tc-DTPA was removed by gel permeation chromatography in a PD-10 Sephadex G-25 column (GE Healthcare). Activity of fractions was measured by solid scintillation counter (Packard). Liposomes made of HSPC:cholesterol (1:1 mol:mol) were label in the same way (L-^{99m}Tc-DTPA)

The radiochemical purity and the label efficiency were determined by thin-layer chromatography on 2 x 20 cm strips, three different analyses were performed:

1) ITLC-SG using NaCl 0.9 % as solvent, L/ARC-^{99m}Tc-DTPA and reduced/hydrolyzed ^{99m}Tc remains at the original location, free ^{99m}Tc-DTPA migrates with the solvent in front.

2) ITLC-SG using pyridine:HCH_2COOH:H_2O (3:5:1.5 v/v) as solvent, L/ARC- ^{99m}Tc-DTPA and free ^{99m}Tc-DTPA migrates with the solvent in front, while reduced/ hydrolyzed reduced/hydrolyzed ^{99m}Tc remains at the original location.

3) Whatman No 1 using MEK (Methylethylketone) as solvent, L/ARC-^{99m}Tc-DTPA and reduced/hydrolyzed ^{99m}Tc remains at the original location, while free ^{99m}Tc-DTPA had a Rf of 0.1.

Oral Administration and Biodistribution

Wistar rats (180–250 g body weight) were fasted for 24 h and then received 500 µl of single dose of 40 µCi as ARC-^{99m}Tc-DTPA, L-^{99m}Tc-DTPA or free ^{99m}Tc-DTPA, through a cannula by the oral route. Animals were kept in separate metabolic cages for urine collection. After 4h of administration, animals were sacrificed and blood sample, liver, kidney, muscle, bones and brain were collected, washed, weighed and activity was measured by solid scintillation counter.

Statistical Analysis

The significance of the differences between the mean values of studied parameters was determined using the Student's *t*-test.

RESULTS

Vesicles Characterization

Total polar lipid extract was analyzed by 2D TLC and electrospray ionization mass spectrometry (ESI-MS) (negative ions). There were shown the presence of polar lipids 2,3-di-*O*-phytanyl-*sn*-phosphatydilglycerol methyl ester (PGP-Me), 2,3-di-*O*-phytanyl-*sn*-phosphatydilglycerol (PG), 1-*O*-[α-D-mannose-(2′-SO_3H)-1′→2′)-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol (S-DGD), *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerol-3-phospho-*sn*-di-*O*-phytanylglycerol (BPG) and the glycocardiolipin (3′-SO_3H)-Galpα1,6Man*p*-α1,2Glc*p*α-1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (GlyC), as already reported in Gonzalez *et al.* [30] Fig. (**1A**). Non-polar pigments such as bacterioruberin, retinal and caratenoids were also found Fig. (**1B**).

Size and Z potential of the L and ARC was shown in Table **1**. After lyophilization and reconstitution, no significant changes in ARC size were observed, while L immediately aggregated upon water addition with remarkably increased size.

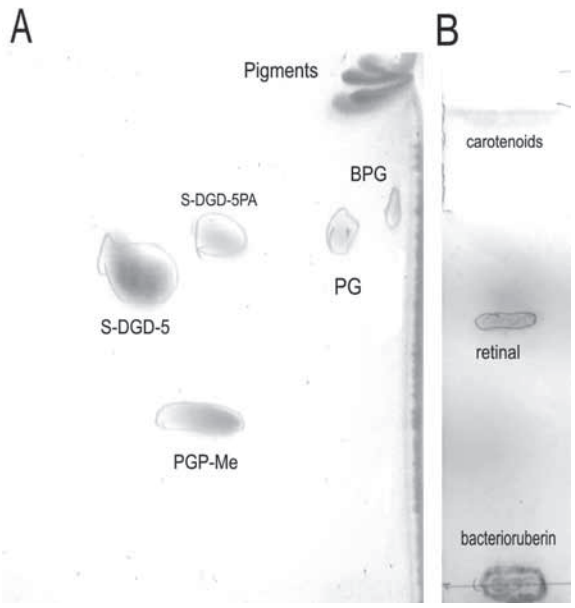

Fig. (1). (A) 2D-TPL pattern of TLC. The origin is in the bottom left corner. Lipids were identified by differential staining, comparison with reported Rf values (retention factor, calculated by dividing the distance traveled by the product by the total distance traveled by the solvent) and electrospray ionization mass spectrometry (ESI-MS). (**B**) Non polar pigments pattern by TLC, spots were identified by comparison with reported Rf values.

Cytotoxicity on Caco-2 Cells

None ARC neither HSPC:cholesterol liposomes significantly reduced the viability of Caco-2 cells upon 24h incubation at concentrations up to 1 mg/ml Fig. (**2**).

Binding and Transport Studies

TEER values were approximately 400 $\Omega.cm^2$ and 300 $\Omega.cm^2$ for mono and co-cultures, respectively (cells with lower TEER values were excluded from experiments), and 5.8% of phenol red diffused from the upper to the bottom wells after 2h incubation (it is acceptable up to a 6% diffusion on confluent Caco-2 cells [39]. On the other hand, the activity of alkaline phosphatase decreased 66% in Caco-2/Raji co-cultures with respect of Caco-2 cells mono-cultures Fig. (**3**). This drop is considered representative of a change from mature enterocytes to M-like cells phenotype [18].

ARC-HPTS-Rh-PE and L-HPTS-Rh-PE were incubated with mono- and co-cultures at the same lipid concentration. Since both had the same mean size distribution, we had assumed that the number of the two types of vesicles was the same.

Table 1.

	Z potential (mV)	Pre-Lyophilization		Post-Reconstitution	
		Mean Size (nm)	PI	Mean Size (nm)	PI
Liposomes	-11	530	0.36	2700	0.80
ARC	-49	570	0.40	740	0.60

PI: Polydispersity index, n:3

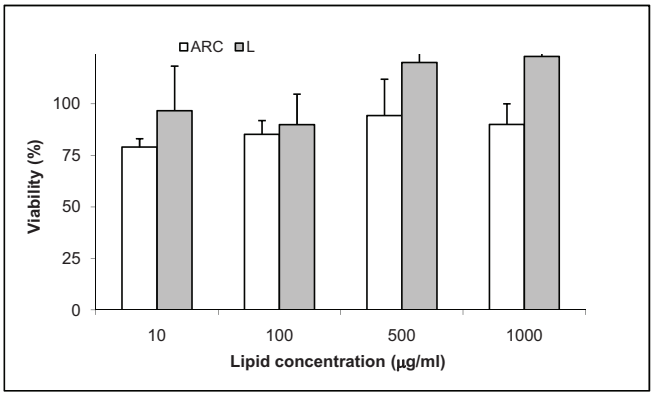

Fig. (2). Viability of Caco-2 cells upon 24h incubation with ARC or HSPC:cholesterol liposomes, as function of concentration. Values represent the average of triplicates ± S.D.

While no fluorescence was observed on Caco-2 mono-cultures upon incubation with ARC-HPTS-Rh-PE and L-HPTS-Rh-PE, a variable fluorescence was detected on Caco-2/Raji co-cultures.

The fluorescence intensity of the hydrophobic label Rh-PE and the hydrosoluble label HPTS on Caco-2/Raji co-cultures upon 1 h incubation with ARC-HPTS-Rh-PE were 4 folds higher than the corresponding fluorescence upon incubation with L-HPTS-Rh-PE Fig. (**4**). Upon 2h incubation, the fluorescence intensity of Rh-PE increased 2.5 folds both from ARC and L while the fluorescence intensity of HPTS remained unchanged. Also after 2 h incubation nearly 15% Rh-PE and 13% HPTS were found in the bottom wells of co-cultures incubated with ARC-HPTS-Rh-PE. On the other hand, no fluorescence was found in the bottom wells of co-cultures incubated with L-HPTS-Rh-PE.

In Vitro Digestion

After 1 h of simulated digestion, the free and loaded in liposomes total protein homogenate from *T cruzi* (L-*T. cruzi)* were completely digested On the contrary; several bands were observed in ARC-*T. cruzi*, that were coincident with the non digested homogenate controls (data not shown).

Biodistribution

The labeling efficiency was 50 and 40% for ARC and L, respectively. The highest percentage of the administered dose (93%) was found in the gastrointestinal tract (GIT) (stomach, small and large intestine) while a small proportion (2.9 ± 0.9 %) was found in bladder and urine, and no radio-activity was found in blood, 4 h after oral administration of free ^{99m}Tc-DTPA Fig. (**5A**). Similar biodistribution was obtained upon L-^{99m}Tc-DTPA administration; nearly 75% of the administered dose was found in the GIT, 2 ± 1.5% in bladder and urine, but 6 ± 2 % was found in blood additionally 2 ± 1.8% was found in lungs and 2.2 ± 1.3 % in muscles, Fig. (**5B**). In contrast, only 23% was found in the GIT, 21 ± 2% in bladder and urine and remarkably 22 ± 3 % was found in blood upon ARC-^{99m}Tc-DTPA administration (also 6.9 ± 2.7% and 1.6 ± 0.1% in muscles and lungs, respectively, Fig. (**5B**).

DISCUSSION

Conventional liposomes are weak adjuvants if used as depots for slow release of antigens or when the delivered antigen is processed by the MHC-II pathway upon phagocytosis of liposomes by APC. The poor adjuvancy of liposomes can be increased by including immunomodulatory agents, namely glycolipid monophosphoryl lipid A (MPL®, the first Toll-like receptor ligand and biological adjuvant approved for human use -i.e. the Hepatitis B vaccine Fendrix®-), muramyl dipeptide (the minimal unit of the mycobacterium cell wall complex that generates the adjuvant activity of complete Freund's adjuvant), or attaching ligands such as mannose [40, 41]. The inclusion of exogenous material to the lipid matrix however, is a main concern from the point of view of the industrial scaling up and regulatory issues in

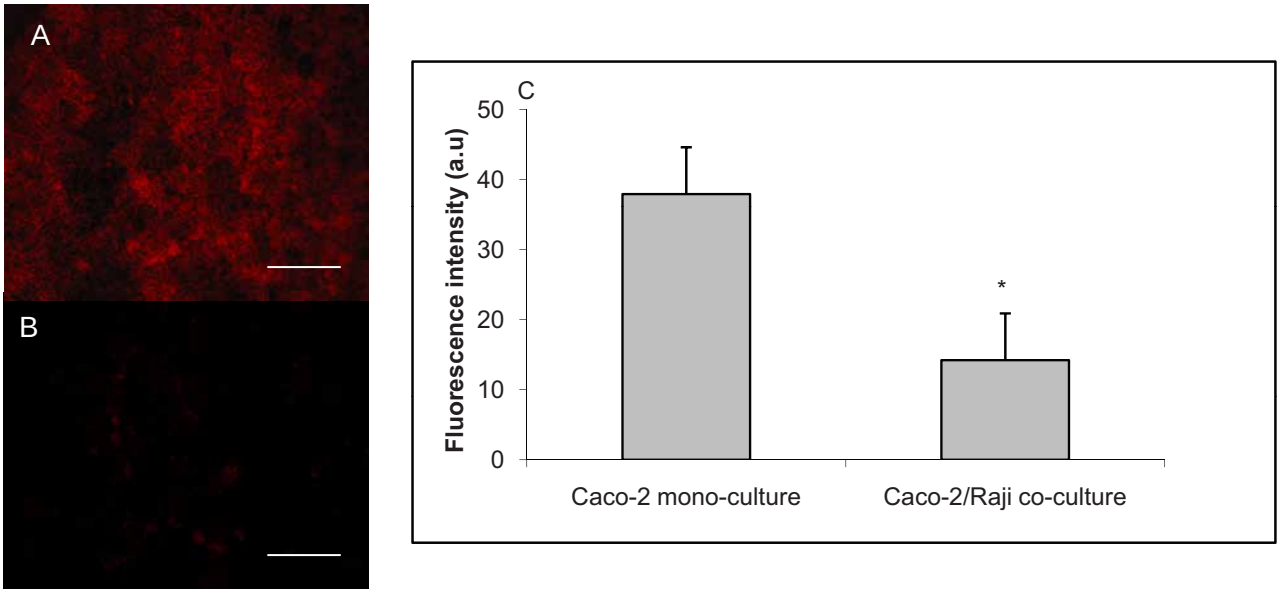

Fig. (3). Apical distribution o f alkaline phosphatase on (**A**) Caco-2 mono-cultures and (**B**) Caco-2/Raji co-cultures (**C**). The scale bar is 500 μm. Quantification of alkaline phosphate expression, intensity of cells was determinate using ImageJ imaging software. * $p < 0.01$

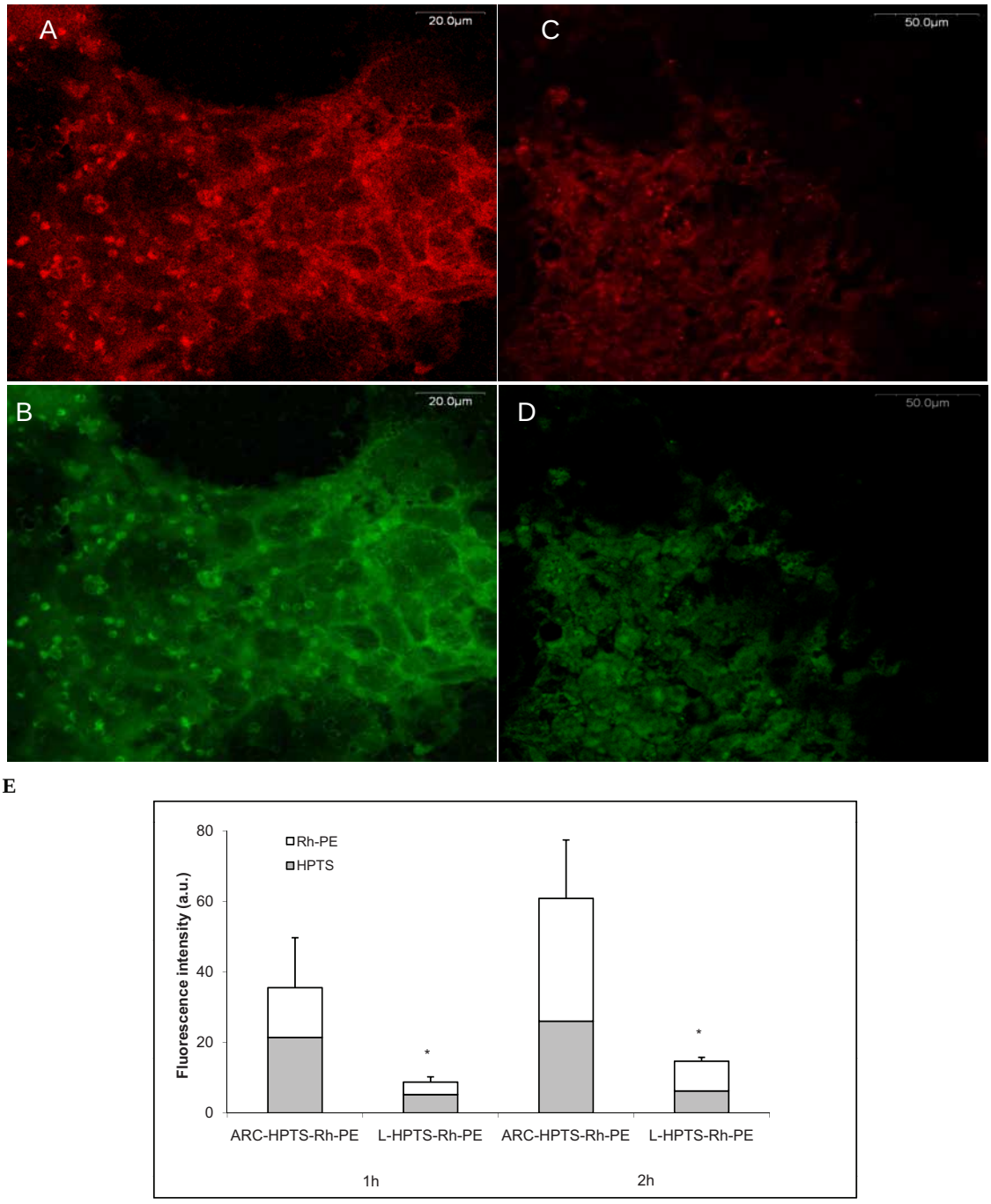

Fig. (4). CLSM images of Caco-2/Raji co-cultures after 1 h incubation with ARC-HPTS-Rh-PE (**A** and **B**) and L-HPTS-Rh-PE (**C** and **D**). Red (**A** and **C**) and green signals (**B** and **D**) from Rh-PE and HPTS, respectively. (**E**) Quantification of red and green fluorescence was determinate using ImageJ imaging software. * $p < 0.01$

vaccine development [42].

Archaea are non pathogenic microbes [43] that do not possess polysaccharides [44] nor murein [45] and that presumably would not have pathogen-associated molecular patterns to serve as danger signals that activate the innate immune system [46]. ARC in general also serve as depots and/or mediate the presentation of exogenous antigens by the MHC-II pathway. However, different to liposomes bearing immunomodulatory agents, ARC do lack of the capacity of activate Toll-like receptors [47]. Nonetheless, ARC constitute potent adjuvants for the induction of Th1, Th2 and CD8+ T cell responses to entrapped soluble antigen [48] in superior magnitude to other particulate vesicular systems such as conventional liposomes [49-51] and comparable, or superior to vaccination with acute bacterial live vectors such as *L. monocytogenes,* upon parenteral administration to mice [47]. Besides, pioneering research during the last 13 years carried out by the group of Sprott have shown that ARC prepared with TPL extracted from the metanogenous *Methanobrevibacter smithii* (containing 40 % caldarchaeols and 30 % wt archaetidylserine) [49] are potent inducers of adjuvancy due to their particular interaction with APC such as dendritic

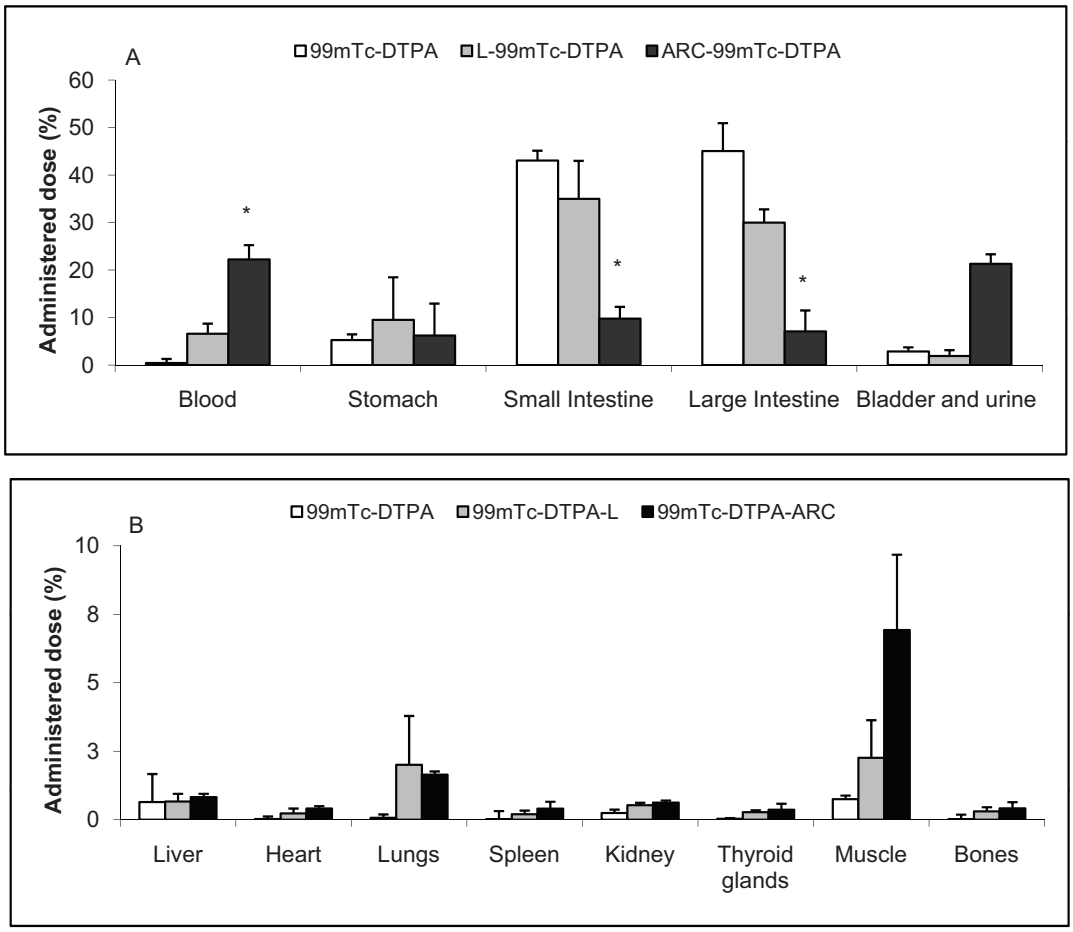

Fig. (5). Biodistribution of free ^{99m}Tc-DTPA, ARC-^{99m}Tc-DTPA and L-^{99m}Tc-DTPA upon oral gavage to rats. n: 4, * $p < 0.01$

cells or macrophages [52]. These ARC target phosphatidylserine-specific receptors on the cell surface of APC [53], resulting in the MHC-I cross presentation of soluble exogenous antigens. The expression of co-stimulatory molecules on APC, probably receptor mediated, which leads to up-regulation of cytokines and other immunological regulators, is induced [48, 54]. Several promising preclinical results upon parenteral administration of ARC indicate that these new type of vesicles could behave as ideal adjuvants [55].

In this work, a different type of ARC was prepared with TPL extracted from the extremely halophilic-non alkaliphilic *H. tebenquichense* strain isolated from the Argentinean Patagonia. The polar lipids of extremely halophilic Archaea exclusively contain archaeols and have several unique characteristics that vary little within specific genera [29, 56]. Between 50–80 mol% of extreme halophilic TPL is the methyl ester of phosphatidylglycerophosphate (PGP-Me), a diacidic phospholipid that maintains the bilayer structure at high NaCl concentration [57]. Phospholipids with choline, ethanolamine, inositol, and serine head groups are absent. In particular, the *Halorubrum* genus is rich in phosphatidylglycerol phospholipids and possesses mannosyl-glucosyl-diphytanylglycerol (DGD), a lipid derived from a basic diglycosyl archaeol by substitution of sugar or sulfate groups at different positions of the mannose residue, such as the SDGD-5 [58]. Besides of the characteristic lipids, this non-alkalyphikic *H. tebenquichense* strain contains PG, BPG and probably SDGD-5-PA (unpublished results). Recently, our research group has found that ARC prepared with TPL lacking of caldarchaeols and serine, extracted from this non-alkaliphilic strain and loaded with OVA, elicit a strong and sustained primary antibody response, as well as improved specific humoral immunity after boosting with the bare antigen. Both IgG1 and IgG2a enhanced antibody titers could be demonstrated in long-term (200 days) recall suggesting induction of a mixed Th1/Th2 response upon subcutaneous administration to mice [30].

The use of ARC as mucosal adjuvant has been recently described. ARC prepared from TPL extracted from *M. smithii, H. salinarum, T. acidophilum* or from 2,3-di-*O*-diphytanyl-*sn*-glycerolphosphate-*O*-methyl, complexed with Ca^{+2} and loaded with OVA, were intranasally administered to mice [59]. Anti-OVA IgA antibody responses in sera, feces, bile, vaginal and nasal wash samples were raised. Anti-OVA IgG, IgG1 and IgG2a antibody responses in sera, as well as cytotoxic T lymphocyte responses were also induced.

In the GIT, a chemical and enzymatically hostile medium for proteins and nucleic acids used as antigens, the balance between immunity and tolerance must be maintained [60]. An ideal particulate oral adjuvant must elicit proper immune reactions upon interaction with APC after uptake and transcytosis by M cells, but also to protect the structure of the carried antigen [61]. The higher structural stability of ARC over that of liposomes can be summarized as follows: a) ARC have the same water and ammonia permeability than

liposomes in liquid crystalline phase, but the presence of eter linkages reduces three folds its proton permeability [62]; b) eter linkages are resistant to acid/basic hydrolysis; c) stereochemical *sn-2,3* configuration instead of *sn-1,2* of glycerophospholipids from Bacteria and Eukarya domains [63] protect ARC from the stereospecific phospholipases attack [49] and d) the phytanyl group (3,7,11,15-tetramethyl hexadecyl) is a saturated polyisoprenoid chain stable in air that requires no special storage conditions. In this first approach, we observed that only when loaded in ARC the bands from *T cruzi* proteins remained comparable to the non digested band pattern. Also in the absence of lyoprotectant sugars, ARC could be successfully lyophilized and reconstituted without aggregation. Hence, the ARC could contribute to overcome the pharmaceutical design hurdles for antigen-loaded particles, such as antigen-stability issues and premature antigen release from particles in the intestine [22]. Two studies show the absence of toxicity of ARC upon oral administration up to 550 mg/kg day for 10 consecutive days in mice [36, 64]. However, data on ARC interaction with M cells or their fate upon oral administration remains unexplored.

In this work we have observed that only upon incubation with Caco-2/Raji co-cultures- but not with Caco-2 monocultures-, the Rh-PE and HPTS fluorescence of ARC remained associated to the cells, a fluorescence that was much weaker for liposomes. This should indicate a preferential binding of ARC on the M-like cells.

ARC are in a fluid state between -40 and 80 °C [65]. This means that the phytanil chains are in an unordered state over a broad range of T or a high entropy state at the air-water interfase [66]. Indeed, bilayers from ARC possess lower surface energy (32-37 mN/m) than that of liposomes composed by straight chain-lipids (54-56 mN/m). The low surface tension would arise from the unique packing mode of the highly branched hydrocarbons-chains as estimated from the abnormally large limiting occupied area of the archaeols with phytanyl chains (92-125 $Å^2$) which is nearly two folds that of conventional glycerophospholipids. Moreover, saturated polyisoprenoids such as phytanil chains are considered highly hydrophobic materials [67]. On the other hand, it is well known that M cells have more affinity by hydrophobic particles (polystyrene, polymethylmethacrylate, polyhydroxybutrate polycaprolactone) than for hydrophilic (composed of lactide and glycolide monomers, ethyl cellulose, cellulose triacetate and cellulose acetate hydrogen phthalate) [68-70]. It is uncertain if the presence of traces of non polar lipids in the TPL, or of sulphated sugars could influence the binding affinity/specificity. However, the unusual hydrophobicity of ARC could explain the higher uptake by M-like cells over that of liposomes. The finding of Rh-PE and HPTS only from ARC and not from liposomes in the bottom wells of Caco-2/Raji co-cultures, a compartment that is equivalent to the basolateral pocket of the M cells, could be owed to a transcellular passage of ARC. Hence, the ARC (whether in the intact form or devoid from hydrosoluble inner content) should have higher chances than liposomes in accessing the APC that *in vivo* locate under the PP dome.

Finally, the hydrosoluble radiopharmaceutical ^{99m}Tc-DTPA (a marker of gastric emptying time upon oral administration, which is neither adsorbed nor absorbed across the mucosa [71, 72]), was loaded in ARC and liposomes. The finding of 22 ± 3 % ^{99m}Tc-DTPA (nearly 3.5 folds more than from liposomes) in blood 4 h upon oral gavage of rats suggested that ARC were involved in a pronounced shuttling of the *impermeable* ^{99m}Tc-DTPA from the lumen to systemic circulation. *In vivo*, the particulate material taken up by the M cells is found at the extensive network of lymphatics underlying the microvilli [73]. If ARC were transcitosed by M cells, a potential leakage of ^{99m}Tc-DTPA during or at the end of intracellular traffic, at the level of basolateral pocket, could explain the presence of ^{99m}Tc-DTPA in blood circulation. The leakage should not occur at the luminal side; otherwise ^{99m}Tc-DTPA would not be absorbed. An evidence of the shuttling of free ^{99m}Tc-DTPA was the absence of radioactivity in liver, since if encapsulated in ARC, it should be cleared by the Kupffer cells.

These results obtained in rodents, where PP are composed of at least 10% of M cells, can not be straightly extrapolated to humans, that posses less than 5% of M cells, between other differences [17, 74]. However, taken together these first approaches suggested that ARC should have higher affinity for M-like cells than non targeted liposomes, and that are further responsible for the shuttling of a considerable amount of hydrosoluble material to blood circulation. Deeper studies are required to reveal the fate of the ARC lipid matrix and its potential as oral adjuvant, since the higher uptake of particulate material could overcome the normal tolerance elicited upon oral administration of repeated small doses of soluble antigen [75, 76].

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