BACTERIAL EFFECTORS AND HOST MOLECULAR TARGETS WHICH CONTROL THE ORIGIN, MATURATION, AND FINAL DESTINY OF PHAGOCYTIC VACUOLE CONTAINING THE INTRACELLULAR BACTERIAL PATHOGEN

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Intracellular bacteria and parasites have evolved many different strategies to elude host defense mechanisms. Although some of the pathogens including *Listeria*, *Shigella*, and *Rickettsia* escape into the cytoplasm to avoid lysosomal digestion, the major group is capable to adapt to intracellular environment and reside in membrane bound vesicles – phagosomes. The phagosomes containing intracellular parasites can arrest in the early (*Mycobacterium* spp., *Ehrlichia chaffeensis*) or late (*Leishmania*) endosomal stage of maturation and even mature to phagolysosome and adapt to this environment (*Coxiella burnetii*). In addition, several of the "parasitophorous vacuoles" remain completely separated from the conventional endocytic pathway and appear to interact with host non-endocytic organelles, such as Golgi apparatus (*Chlamydia* spp.), mitochondria (*Toxoplasma*, *Chlamydia psittaci*) or endoplasmic reticulum (*Legionella*, *Toxoplasma*, *Brucella*) [1].

Phagosomes containing inert particles such as latex beads or killed bacteria are rapidly trafficked through the series of transient interaction with endosomal network to phagolysosomes. After internalization, they carry the classic sign of rapid recycling pathway, transferrin receptor, which indicates their communication with the cell plasma membrane. Furthermore, early endosomal antigen (EEA1) and Rab5 are typical markers of early phagosomal compartment. Additionally, the presence of lysosome-associated membrane glycoprotein 1 (LAMP1), which is delivered via biosynthetic pathway through the *trans*-Golgi network, and immature proforms of lysosomal hydrolases such as procathepsin D has been confirmed in early phagosomes. Another molecule, coronin I, is an actin-binding protein that associates with early phagosomes only transiently and its stable connections with the vacuole is suggested to inhibit its further fusions and so prevent organelle maturation [2].

For mature phagosome the absence of Rab5 and the presence of organelle markers such as Rab7, mannose 6-phosphate receptor, cathepsin D and vacuolar ATPase are typical [2]. Rab5 and Rab7 allow phagosomes to interact with early and late endosomes, respectively. Together with other 60 predicted Rabs they belong to the Ras superfamily of small GTPases and participate in regulation of vesicular transport. From other members of the family also Rab14 seems to be associated with phagosome and in future it could be a possible candidate for efficient isolation of this compartment [3], [4]. Mycobacterium containing vacuole bears on its surface markers of early endosome- Rab5, but not Rab7, and coronin I. The processing of cathepsin D to its active, two chains form is probably caused by rapid increase in numbers of vacuolar ATPases and a marked drop in pH mediated by interferon gamma. Mannose 6-phosphate receptor takes part in lysosomal sorting of soluble hydrolases [2].

Our laboratory group is interested in characterization of host-intracellular pathogen interaction using *Francisella tularensis* LVS (live vaccine strain) infection of murine macrophages. For this purpose we attempted to isolate and characterize vacuole containing *F. tularensis* LVS from mouse macrophage cell line J774.2. The linear gradient centrifugation on 20–60 % w/v sucrose density column or step gradient separation on 20/35/50 % w/v sucrose column were used for subcellular fractionation. For easier detection of bacterium in isolated fractions we took advantage of *Francisella tularensis* LVS harboring green–fluorescence protein (LVS-GFP), which was kindly provided by Anders Sjöstedt (Umea University, Umea, Sweden).

Briefly, cells were grown in high glucose DMEM with glutamax I (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (Gibco BRL) at 37 °C and 5% CO_2 in a humid environment for 48 hrs before infection. Macrophages were infected at a multiplicity of infection (MOI) of 500 for 1 – 2hrs, free bacteria were washed out by ice cold PBS and surface associated bacteria were internalized during following 30 min incubation period.

Separation of vacuole containing bacteria on linear sucrose gradient was performed as described by Pasquali et al. [5] and Fialka et al. [11] with slight modifications. Briefly, cells were homogenized by multiple passages through a 27-gauge needle in homogenization buffer (HB; 250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM HEPES, 0.05% gelatine, pH 6.8, protease inhibitor cocktail). The nuclei together with unbroken cells and some larger subcellular components were removed by repeated centrifugation step at 300x g (postnuclear supernatant; PNS). Continuous 20–60% sucrose

gradients (in HB) were prepared in Open-Top Centrifuge Tubes Polyclear™ (Beckman Coulter, Inc., Fullerton, USA) using the BioComp gradient master (BioComp Instruments Inc., Fredericton, N.B., Canada) and membrane fraction (m.f.) obtained as sediment after ultracentrifugation of PNS at 100 000x g for 30 min was loaded on top of the gradient column. After centrifugation on Beckman Optima™ MAX ultracentrifuge (Beckman Coulter, Inc., Fullerton, USA) (18 000x g, 4 °C, 16 hrs), about 20 fractions were collected with the Piston gradient fractionator (BioComp Instruments Inc., Fredericton, N.B., Canada). Separation on sucrose step gradient was performed as described by Chakraborty [6] with slight modification. PNS was subjected to velocity sedimentation (3000x g, 45 min, 4 °C) through the 20/35/50 % w/v sucrose step gradient. Fractions of 0.5 ml were collected from the top of the gradient.

Amount of bacteria in the fractions detected as colony forming units per milliliter (CFU/ml) was slightly increasing over the linear gradient, but accumulated namely in the very dense fractions no.15–20 (50–60% sucrose). In case of velocity sedimentation the major part of bacteria-containing fractions were collected from interface of 20/35% sucrose as detected by anti-GFP immunodetection of GFP-LVS.

Separated fractions were subjected to the analysis of organelle-specific enzymatic activities of β- hexoseaminidase [7], rotenone- resistant NADH- cytochrome c- reductase [8] and alkaline phosphodiesterase [9] and Western blot analysis for the presence of established marker proteins (LAMP1, calnexin, cathepsin D, Rab5 and Rab7). We found that β- hexoseaminidase, the marker of lysosomes, was distributed in fractions no. 11–20 (42–60% sucrose) from both, infected and control cells. Rough endoplasmic reticulum (ER), expressed as enzymatic activity of the rotenone- resistant NADH- cytochrome c- reductase, was found in fractions 13–15 (46–50% sucrose) and appears to be comparable in both, infected and control cells.

Proteins from collected gradient fractions were precipitated by chloroform-methanol [10], dissolved in SDS-sample buffer and equal amount of protein (20 μg) from each fraction was separated by 12% SDS-PAGE followed by electrophoretic transfer onto PVDF membrane. We found significant difference in expression of LAMP1 protein in fractions 11–18 (42–56% sucrose) in infected cells as compared to non-infected in which the high accumulation of bacteria was shifted to fractions 15–20. When we followed calnexin, organelle marker of endoplasmic reticulum, we detected its distribution all over the gradient. This finding is in agreement with observation of Fialka et al. [11] and Garin et al. [12] who also observed presence of proteins specifically residing in ER in broad spectrum of organellar fractions from density gradient. Although this might represent contamination, it is also possible that ER components could interact directly with some organelles, including phagosome [12]. Immature form of cathepsin D probably delivered directly from the *trans*-Golgi network was observed in fractions 1–2 in both infected and non-infected cells and in fractions 11–13 and 17–18 in control cells. Low amount of the lysosomal form of cathepsin D (46 kDa) was detected in fraction 14–16 in both infected and control cells. Rab5 protein, the marker of early endosomes, was detected in fractions 7–17 in infected cells and 11–17 in control cells. Rab7 protein, the marker of late endosomes, was detected over the all gradient in infected cells but only in fractions 4–6 and 11–20 in control non-infected cells.

Using step density gradient and velocity sedimentation most of the bacteria were found in fraction I6, e.g. on interface between 20 and 35% of sucrose, at the same location where LAMP1, cathepsin D and rab7 were found.

Based on these findings, we suppose that bacteria containing vacuole from J774 macrophages is found in fractions together with LAMP1, rab7 and cathepsin D thus indicating the features of late endosomes/phagolysosomes. However, we are aware of the need of electron microscopic study to control purity of fractions, support and verify this conclusion.

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