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Serum albumin disrupts *Cryptococcus neoformans* and *Bacillus anthracis* extracellular vesicles

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Summary

For both pathogenic fungi and bacteria, extracellular vesicles have been shown to contain many microbial components associated with virulence, suggesting a role in pathogenesis. However, there are many unresolved issues regarding vesicle synthesis and stability, including the fact that vesicular packaging for extracellular factors involved in virulence must also have a mechanism for vesicle unloading. Consequently, we studied the kinetics of vesicle production and stability using [1-^{14}C] palmitic acid metabolic labelling and dynamic light scattering techniques. *Cryptococcus neoformans* vesicles were produced throughout all stages of fungal culture growth and they were stable once isolated. Density gradient analysis revealed that only a portion of the vesicle population carried cryptococcal polysaccharide, implying heterogeneity in vesicular cargo. Vesicle incubation with macrophages resulted in rapid vesicle instability, a phenomenon that was ultimately associated with serum albumin. Additionally, albumin, along with mouse serum and murine immunoglobulin destabilized *Bacillus anthracis* vesicles, but the effect was not observed with ovalbumin or keyhole limpet haemocyanin, demonstrating that this phenomenon is neither host-, microbe- nor protein-specific. Our findings strongly suggest that cryptococcal vesicles are short-lived *in vivo* and vesicle destabilization is mediated by albumin. The ability of albumin to promote vesicular offload through destabilization indicates a new activity for this abundant serum protein.

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Introduction

There is now convincing evidence that microbes as diverse as Gram-negative bacteria, Gram-positive bacteria, mycobacteria, fungi and protozoa release extracellular vesicles that carry microbial products into the extracellular space (Rodrigues *et al*., 2007; Kulp and Kuehn, 2010; Rivera *et al*., 2010; Prados-Rosales *et al*., 2011; Vallejo *et al*., 2011). For several pathogenic microbes, these vesicles have been shown to contain virulence factors, suggesting that vesicular transport provides a mechanism for their concentrated delivery to host cells and tissues. If delivery of concentrated virulence factor cargo is an important role for secreted vesicles, then microbial vesicles must be able to release their intraluminal contents. Thus, vesicles must be either inherently instable or be actively lysed through exposure to the host environment. Previous studies have demonstrated that Gram-negative bacterial outer membrane vesicles are stable over months, depending on temperature (Arigita *et al*., 2004). Hence the mechanism of vesicular cargo release remains an enigmatic and important question.

In this study, we investigated the stability of vesicles from two phylogenetically distant organisms, the fungus *Cryptococcus neoformans* and the Gram-positive bacterium *Bacillus anthracis*. The first organism, *C. neoformans*, is the leading cause of meningitis in HIV-affected population (Pfaller and Diekema, 2010). Despite advancements in antifungal therapy, cryptococcosis remains a disease that is difficult to treat and has high morbidity and mortality. A key aspect of cryptococcal pathogenesis is the ability of this fungus to establish chronic infections that resist immunological clearance. *C. neoformans* is thought to undermine immune function through numerous mechanisms, which include the release of polysaccharide-containing vesicles. Cryptococcal vesicles also contain proteins, lipids and RNA (Rodrigues *et al*., 2007; 2008; Eisenman *et al*., 2009; Nicola *et al*., 2009), which may all modulate *C. neoformans*–host cell interactions. Macrophage cells incubated with cryptococcal vesicles exhibit enhanced phagocytosis and fungicidal activity, and manifested different cytokine profiles (Oliveira *et al*., 2010), establishing that vesicles have biological effects on host cell responses. The second organism studied, *B. anthracis*, is the causative agent of anthrax. *B. anthracis* is a

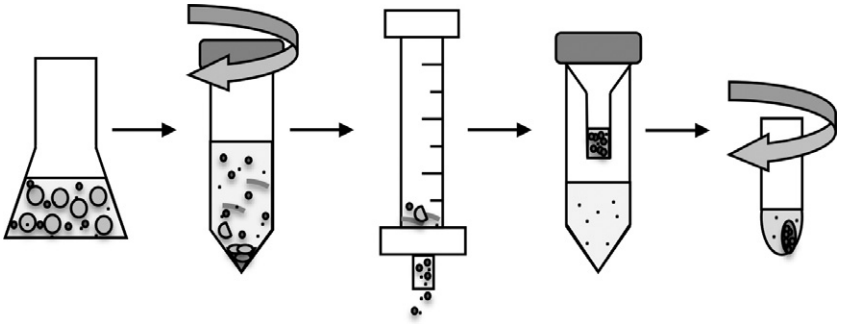

Fig. 1. Schematic representation of radioactive vesicle recovery assay. (i) Mid-log cultures were labelled using [1-^{14}C] palmitic acid and incubated 4–72 h. After incubation, cultures consist of cells (large grey circles), vesicles (small dark circles) and soluble secretions (black dots). To purify radiolabelled vesicles, (ii) cultures were centrifuged to remove the cell pellet, (iii) supernatant was filtered to remove any residual cells or cell fragments, (iv) the cell-free supernatant was then concentrated in an Amicon centrifugal concentration unit (100 kDa cut-off), and (v) vesicles were further purified from the concentrate by ultracentrifugation. The radioactivity of the cell pellet, flow-though and concentrate were measured to calculate total radioactivity, and per cent radioactivity refers to per cent of total radioactivity taken up by the cells.

toxigenic microbe that releases various toxin components packaged in vesicles that must be disrupted for toxin release (Rivera *et al.*, 2010).

The stability of microbial vesicles was studied using metabolic labelling and light scattering techniques. Our studies show that vesicles are stable in saline solutions but are rapidly disrupted by mammalian serum. The major serum component that mediated vesicle disruption was serum albumin, which disrupted both fungal and bacterial vesicles. Our findings provide a mechanism for vesicular content release *in vivo* and suggest a new activity for albumin in host–microbial interactions.

Results

Vesicle isolation from C. neoformans

Prior studies had generated vesicles from relatively large culture volumes ranging from 0.5 to 1 l (Rodrigues *et al.*, 2007; 2008; Oliveira *et al.*, 2010). Large volumes are impractical for carrying out kinetic studies involving metabolic labelling given radioactive waste considerations and the need for assaying numerous experimental conditions. Consequently, our first goal was to ascertain whether vesicles could be recovered from small cryptococcal cultures in both exponential and stationary growth phases. We hypothesized that vesicles were produced throughout both the exponential and stationary phases, with increasing vesicle concentration over 3 days. To validate or refute this hypothesis, we performed a pulse-chase experiment with [1-^{14}C] palmitic acid, and surveyed radioactivity released into the supernatant that could be recovered following a vesicle preparation (Fig. 1). Palmitic acid was chosen because it is a precursor for many other membrane lipids yet is less likely to be metabolized into CO_2 than a general precursor such as acetate or glycerol. The filtered supernatant, including vesicles, was concentrated with a 100 kDa cut-off filter, and the cell pellet, concentrate and flow-through (FT) were surveyed by scintillation counting. Concentrates from cells not pulsed with [1-^{14}C] palmitic acid were plated for cfu on Sabouraud agar plates, which confirmed that the concentrate was indeed cell-free (data not shown).

Using [1-^{14}C] palmitic acid metabolic labelling, we detected radioactivity in both the concentrate and flow-through in as little as 4 h, corresponding to approximately one replication for the fungal cells in culture (Fig. 2A).

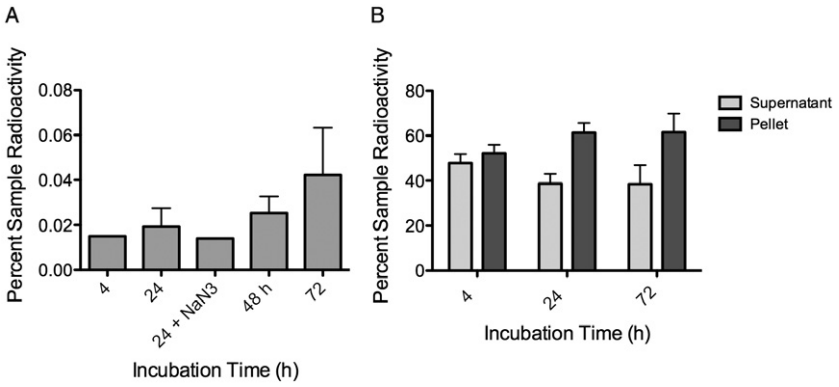

Fig. 2. Kinetics of vesicle production by *C. neoformans* cells.
A. Radioactive signal was followed in metabolically labelled cultures over 72 h. Vesicle accumulation as a function of time was inferred from the proportion of radioactivity in the concentrate fraction.
B. Concentrates from cultures labelled similarly to part (A) were ultracentrifuged, to determine the pelletable proportion of the concentrate. The per cent of pelletable concentrate radiation increases with time, indicating that the majority of radioactivity is vesicle associated.
Bars represent standard error.

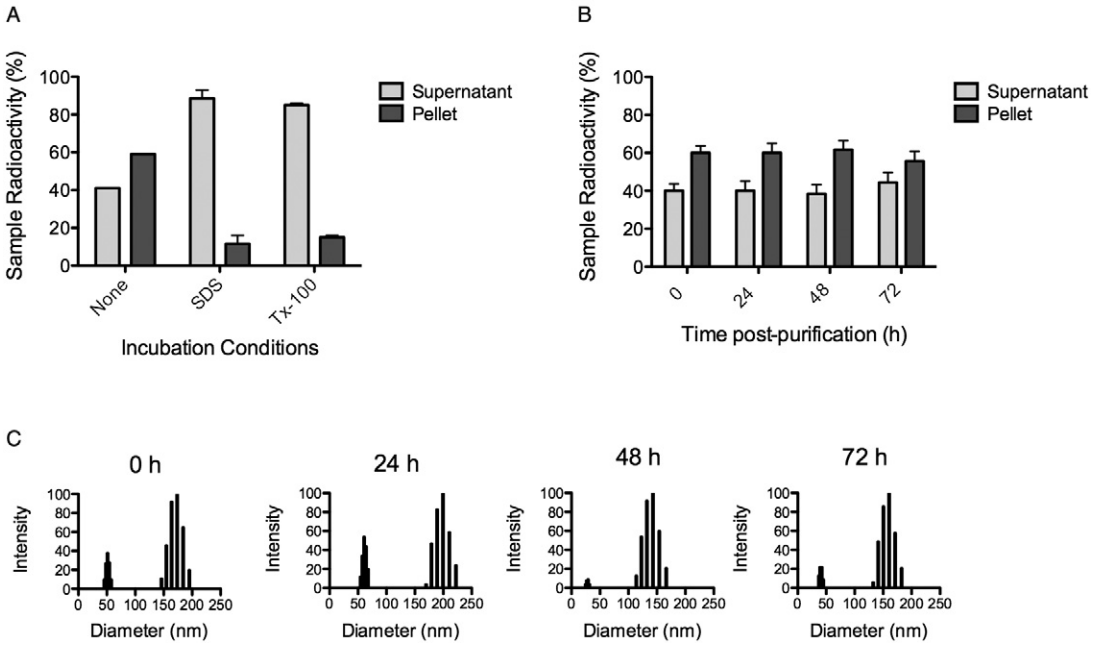

Fig. 3. Stability of vesicles as a function of time.
A. Vesicles were purified after 72 h post [1-^{14}C] palmitic acid addition and incubated in PBS, 10% SDS or 1% Triton X-100 for 1 h. The increase in supernatant radioactivity demonstrates vesicles are detergent sensitive.
B. Purified vesicles stored for 0, 24, 48 or 72 h at room temperature were centrifuged at high speed to separate pellet-associated from soluble (supernatant-associated) radioactivity. The absence of appreciable change in the radioactivity of the supernatant and pellet fractions indicates stability.
C. Vesicle size was analysed by dynamic light scattering at 0, 24, 48 and 72 h post purification. The similarity in the distribution of vesicle dimensions as a function of time is consistent with vesicular stability.
Bars represent standard error.

Secreted radioactivity signal increased over time (Fig. 2A and data not shown). Radioactive detection was not due to inherent interference, as CPM measured from media alone was not above background levels (data not shown). Almost all radioactivity added to the culture was tracked during the time-course, as the 4 h pulse produced a consistent uptake of $>95\%$ of the [1-^{14}C] palmitic acid by the fungal cells (data not shown). Pelletable radioactive signal required active cells, as the addition of NaN_3 or heat treatment at 65°C for 1 h after radioactivity exposure resulted in no pelletable radioactivity from the supernatant despite incorporation into the cellular component (Fig. 2A and data not shown). Based on these exploratory experiments, we concluded that the pulse-chase could be used to follow radioactive vesicle secretion, and that radioactive vesicles accumulated with culture age.

To further confirm that the pelletable radioactivity in the concentrate fraction corresponded to the vesicle fraction, we repeated the assay as above, with the additional step of a high-speed centrifugation that should pellet any vesicles, but not soluble secreted radioactivity. Indeed, we found that the majority of the concentrate radioactivity was pelletable, and that the radioactivity in the pelletable fraction increased over time (Fig. 2B). The radiation in the concentrate therefore corresponds to the presence of vesicles secreted by *C. neoformans*.

Vesicle stability

To explore vesicle stability, we harvested vesicles from 72 h cultures grown with [1-^{14}C] palmitic acid. First, we confirmed that the pelletable radioactivity represented vesicles by disrupting the samples with detergent and showing that this greatly reduced pelletable radioactivity. As expected, incubation of this fraction with SDS or Triton X-100 decreased pelletable radioactivity to less than 10% of the total sample radioactivity (Fig. 3A). Next, we investigated vesicle stability over time by incubating purified vesicles for 0, 24, 48 or 72 h in the concentrated, conditioned medium at room temperature and measuring the amount of radioactivity in pelletable and supernatant fractions. We found that the percentage of pelletable radioactivity within each sample remained constant over time, suggesting that cryptococcal vesicles are stable after isolation.

Although vesicle quantity remained stable over time, we hypothesized that the vesicle size may decrease after

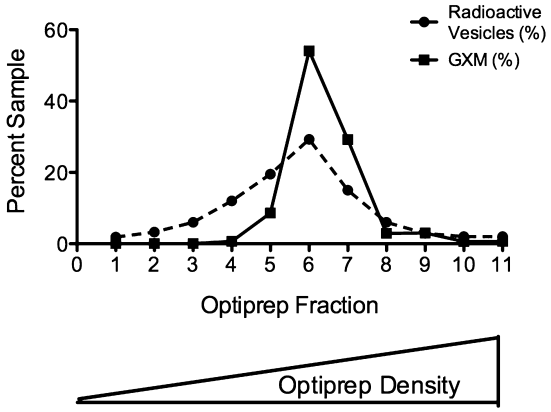

Fig. 4. Analysis of vesicle density sedimentation reveals location of radioactive lipid versus GXM in fractions. A 5% stepwise density gradient was run and investigated for either radiolabel signal (dashed line) or GXM concentration (black line). Values were normalized to 100%. A fraction of vesicles does not appear to contain GXM (fractions 2–4).

secretion. For example, a vesicle that loses membrane integrity may 'leak' its constituents into the supernatant, rather than break up, and still remain pelletable. To test this hypothesis, we investigated the size of vesicles purified from 72 h cultures by dynamic light scattering. By measuring the concentrate directly, we observed two population sizes: a smaller one between 40 and 60 nm and a larger one between 100 and 200 nm (Fig. 3B) (Eisenman *et al*., 2009). We measured the vesicle population size at 0, 24, 48 and 72 h, and observed that the larger population remained stable in solution. The smaller population remained stable for 24 h and subsequently decreased in size. This result suggests that vesicle preparations contain both inherently stable and unstable populations. The pellet likely represents both the intact, stable population and the lipid membranes of the smaller, unstable population.

Vesicular population distribution

The dynamic light scattering data (Fig. 3B) suggested that vesicles included differently sized subpopulations, and we hypothesized that subpopulations also differed in density. To test this, we evaluated the sedimentation of [1-^{14}C] palmitic acid-labelled vesicles on an iodixanol density gradient using 5% increments and assessed 1 ml fractions for radioactivity by scintillation counting. A majority of the radioactive signal was found in fractions 3–8, with the highest concentration in fraction 5–7 (Fig. 4). Thus, while vesicles occurred in discretely sized populations, vesicle density was relatively homogenous. To investigate whether the fractions constituted a homogenously distributed vesicle population with regards to cargo, we ran purified vesicles from non-pulsed cultures on an identical gradient and assessed for glucuronoxylomannan (GXM), the major polysaccharide component of the *C. neoformans* capsule by ELISA. GXM signal was distributed between fractions 5–7 with the highest concentration found in fraction 6 (Fig. 4). While the two distributions sedimented with similar curve tracings, we noted that the radioactive signal was slightly more widely spread than the GXM signal. Significantly, these analyses revealed detection of radioactive signal in fractions 3 and 4 with no detectable GXM signal (Fig. 4). Consequently, we conclude that vesicles are heterogeneous in cargo such that not all contain GXM.

Interaction of vesicles with macrophages

Prior studies have shown that macrophages are stimulated by cryptococcal vesicular preparations (Oliveira *et al*., 2010), implying that they can interact and respond to these fungal products. Similarly, *B. anthracis* vesicle populations can mediate toxicity for macrophages, implying that anthrax toxin components are released in some manner (Rivera *et al*., 2010). Consequently, we sought to determine if the phagocytic cells such as macrophages took up cryptococcal vesicles using metabolically labelled vesicles. We added [1-^{14}C]-radiolabelled vesicles to cultures of J774.1 macrophage-like cells and we then assayed the amount of radiation in the monolayer and supernatant to determine the amount of radioactivity taken up over time. To our surprise, we found that the uptake of radioactive signal did not change significantly over time (Fig. 5A). Furthermore, there was no significant difference in the amount of supernatant radioactivity in conditions where the solution was incubated with and without a macrophage monolayer. This result was initially interpreted as indicating that the macrophage cells do not take up vesicle-associated lipids over time. Similarly, we studied the interaction of *C. neoformans* with primary mouse macrophage cells and again found no significant increase in cell-associated radioactivity compared with vesicles incubated in the absence of cells (Fig. 5B).

In considering our results, we reasoned that another explanation for the absence of radioactivity in the cellular fraction was that the vesicles were being disrupted in the presence of macrophages, such that the labelled lipids remained in solution. Such a mechanism could be easily reconciled with the observation that vesicle addition to macrophages resulted in macrophage activation and cytokine secretion, since the cargo would have been able to interact with the cells directly. Consequently, we initially hypothesized that the vesicles were being lysed by a secreted macrophage substance, a medium-related substance, or both. To test this, we incubated radiolabelled vesicles in minimal medium, spent macrophage medium or unspent macrophage medium for 2 h at 37°C. After 2 h

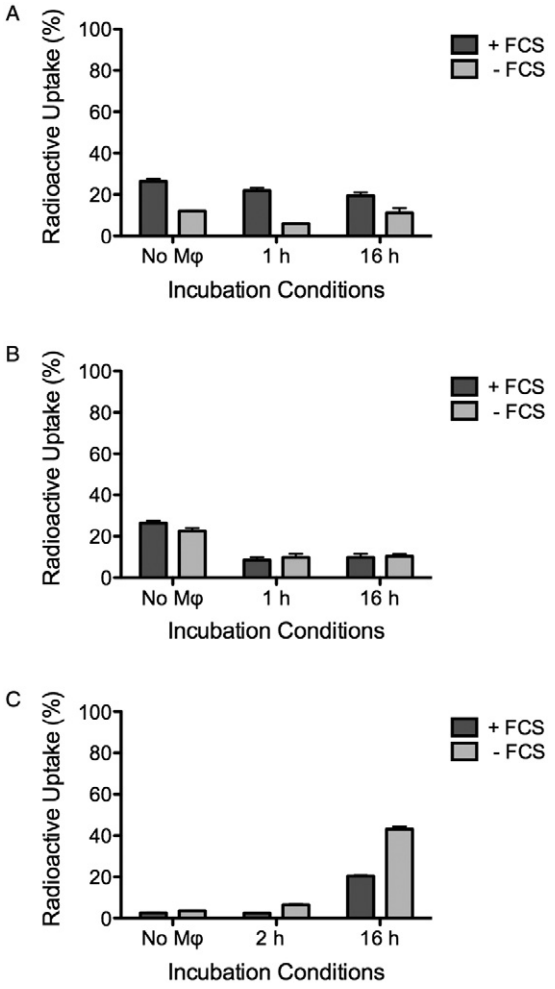

Fig. 5. Fate of vesicle radioactivity after incubation with phagocytic cells.
A. Purified cryptococcal vesicles were incubated with J774.1 macrophage-like cells with or without serum for 1 or 16 h. Supernatant- and cell-associated radioactivity were assessed and normalized to 100% for each individual sample.
B. Repeat of part (A) using bone marrow-derived macrophage cells.
C. Purified anthracis vesicles were incubated with J774.1 cells with or without serum for 2 or 16 h. Supernatant- and cell-associated radioactivity were assessed and normalized to 100% for each individual sample. Bars represent standard error in all panels.

in minimal medium, we found the majority of pelletable radioactivity remained pelletable (Fig. 6A). However, after 2 h in fresh macrophage medium, over 50% of the sample radioactivity was found in the supernatant, with spent macrophage medium increasing supernatant-associated radioactivity to over 75% of total sample radioactivity. We further determined that the vesicle destabilizing substance originated from the serum component of macrophage medium (Fig. 6A). These results suggested that vesicle destabilization was mediated by a serum-derived substance, although a macrophage-secreted substance may also contribute to vesicle destabilization.

To investigate the nature of the vesicle destabilizing substance, we tested the effect of proteinase K-treated and delipidated sera on vesicular stability. We found that proteinase K treatment, but not delipidation, increased vesicle stability slightly in serum (Fig. 6B). Treatment of the serum for 1 h at 65°C prior to addition to vesicles did not prevent vesicle disruption, a finding that combined with the proteinase K results suggested that a heat-stable protein or peptide was the destabilizing factor. To test whether protease activity destabilized vesicles, we incubated serum and radiolabelled vesicles with a general protease inhibitor cocktail but this had no effect on vesicle stability (Fig. 6B).

We initially hypothesized that the vesicle destabilization activity in serum resulted from enzymes that damaged lipid bilayers such as lipase and/or phospholipase. To test the role of phospholipase activity, we included Compound O, Compound Ax and Compound P in incubations with serum and radiolabelled vesicles. Compound O inhibits lysophospholipase and lysophospholipase transacetylase activity, while Compound Ax and Compound P inhibit phospholipase A and B activity respectively. However, none of the inhibitors tested resulted in vesicle stability in serum (Fig. 6C). We also tested the ability of phospholipases to directly damage vesicles by incubating vesicles with either phospholipase A2 or phospholipase C in PBS. Neither of these enzymes affected vesicle stability (Fig. 6C), arguing against phospholipase activity as the source of destabilization. Further studies demonstrated that the pancreatic lipase was also not responsible for vesicle destabilization (data not shown).

Next, we attempted to identify the vesicle destabilizing activity in serum by fractionating its components by molecular mass using a S200 HPLC column, which revealed five major protein peaks that we labelled A–E (Fig. 7A). We pooled and concentrated fractions containing these peaks, and then incubated the concentrated fractions with radiolabelled vesicles. Incubation of labelled vesicles with fractions D and E did not increase supernatant-associated radioactivity, but incubation with both B and C resulted in 70–80% supernatant-associated radioactivity (Fig. 7B). Since these peaks were dominated by bovine serum albumin (BSA) (Fig. 7A), we considered that BSA was destabilizing the vesicles. Incubation of radiolabelled vesicles with 300 mM BSA resulted in $>80\%$ supernatant-associated radioactivity, demonstrating that BSA was likely the destabilizing substance in serum.

Given that the albumin destabilized cryptococcal vesicles, we investigated if this effect applied to other microbial vesicles. Consequently, we generated and purified radiolabelled vesicles from 24 h *B. anthracis* cultures and tested their stability in 10 mM glucose with and without 300 mM BSA. To help stabilize the *B. anthracis*

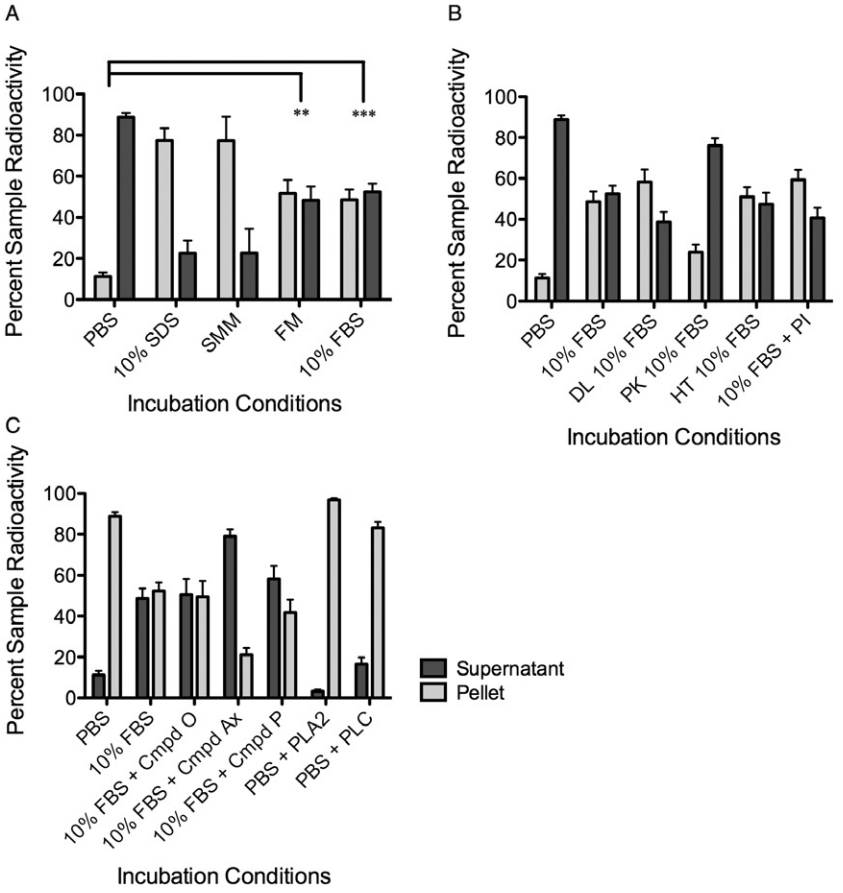

Fig. 6. Vesicle stability in various conditions.
A. Purified radiolabelled vesicles were resuspended in PBS, 10% SDS, spent macrophage medium (SMM), fresh medium (FM) or 10% serum (FBS). All samples were incubated at 37°C for 1 h, after which supernatant and pellet radioactivity were assessed and normalized to 100% radioactivity for each individual sample.
B. Radiolabelled vesicles were incubated with delipidated (DL) FBS, proteinase K-treated (PK) FBS, heat-treated (HT) FBS or FBS including a protease inhibitor cocktail (PI). Samples were incubated and processed as in part (A).
C. Radiolabelled vesicles were incubated in serum containing Compound O, Compound Ax or Compound P, or in PBS containing phospholipase A2 (PLA2) or phospholipase C (PLC). Samples were incubated and processed as in part (A).
Bars represent standard error.
$^{**}P$-value < 0.05; $^{***}P$-value < 0.001.

vesicles, 10 mM glucose was added, as incubation of *B. anthracis* vesicles in PBS consistently resulted in higher supernatant radioactive counts than was observed with cryptococcal vesicles in PBS, consistent with the reduced stability in buffer. However, the proportion of supernatant radioactivity was significantly increased in the presence of both SDS and BSA, demonstrating that albumin destabilizes *B. anthracis* vesicles as well as cryptococcal vesicles (Fig. 7C and data not shown). From these data, we concluded that albumin had a broad ability to disrupt microbial vesicles from multiple organisms.

Having identified albumin as the destabilizing substance, we re-evaluated macrophage interactions with radiolabelled vesicles in the absence of serum. We repeated the stability assays as above, but instead used a serum-free medium to evaluate the interaction of vesicles with macrophages. The absence of serum did not promote uptake of radioactive signal by either J774.1 or BMDM cells (Fig. 5A and B). We concluded that cryptococcal vesicles were not taken up by macrophage, and hypothesized that *B. anthracis* vesicles may be more stimulatory than cryptococcal vesicles on account of the fact that the former is an accidental pathogen of mammals whereas anthrax is a mammalian disease. Consequently, we repeated the uptake experiment using radiolabelled anthracis vesicles and observed J774.1 macrophage uptake of radioactive signal in the presence and absence of serum, with more uptake in serum-free conditions (Fig. 5C). We observed similar results with BMDM (data not shown). These results demonstrate that vesicle uptake is greater in the absence of serum.

We investigated whether other sources of serum would also destabilize vesicles, based on the high conservation of albumin between species, by suspending vesicles in mouse serum or 300 mM BSA. Either solution resulted in a major decrease in vesicle size as measured by dynamic light scattering, indicative of vesicular disruption (Fig. 8A). Similar changes in vesicle population size distribution were observed with *B. anthracis* vesicles, confirming the general activity of serum against microbial vesicles (Fig. 8B). When the mouse serum was heat-inactivated at 56°C for 1 h, fewer microbial vesicles were destroyed (data not shown), suggesting either that the murine albumin effect is partially heat-labile and/or that a separate, heat-labile component of serum also contributes to vesicle destruction.

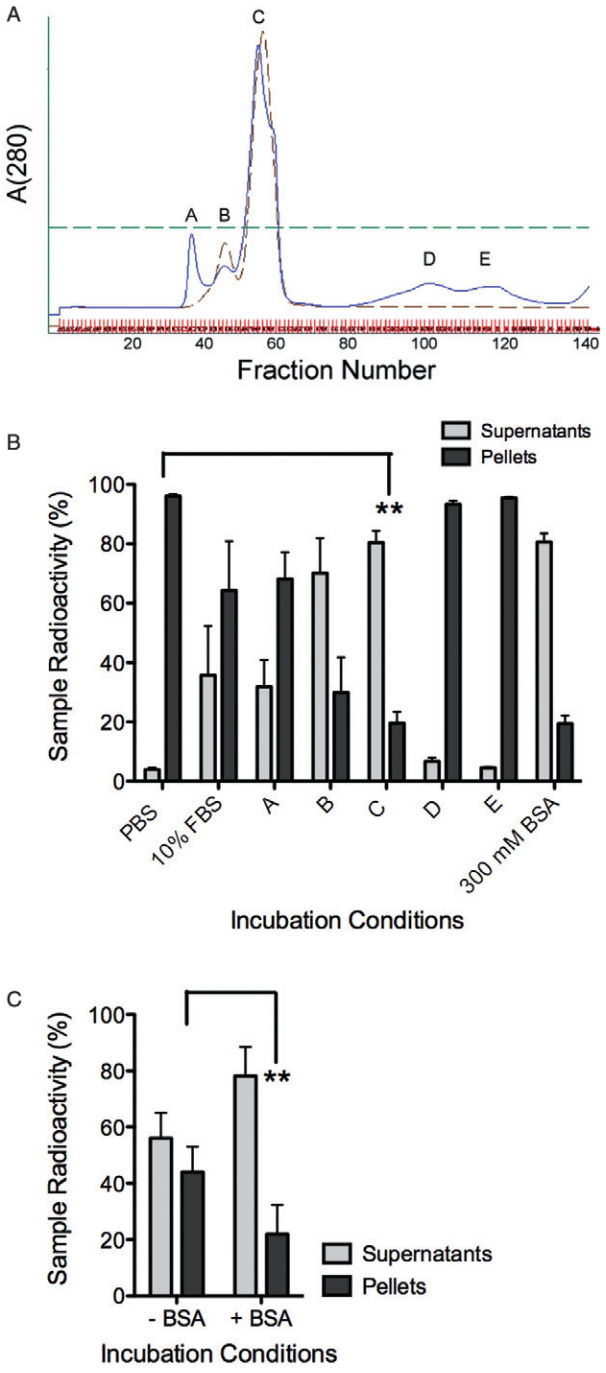

Fig. 7. Serum albumin disrupts microbial vesicles.
A. Serum was run on an S200 HPLC column, revealing five major peak fractions (A–E). Peaks were collected, pooled and concentrated.
B. Radiolabelled vesicles were incubated with fractions A-E or 2% (300 mM) BSA overnight at 37°C, after which supernatant and pellet radioactivity were assessed and normalized to 100% radioactivity for each individual sample. Bars represent standard error. ** P-value < 0.05.
C. Radiolabelled anthracis vesicles were incubated in 10% glucose with or without 300 mM BSA, after which supernatant and pellet radioactivity were assessed and normalized to 100% radioactivity for each sample. ** P-value < 0.05.

A dose response study of vesicular stability as a function of BSA concentration revealed that protein concentrations > 12 mM were associated with vesicular disruption as manifested by an increase in supernatant-associated radioactivity (Fig. 8C). We found that this disruption was albumin-specific, as ovalbumin and keyhole limpet haemocyanin (KLH) at either 30 or 300 mM concentrations did not cause comparable effects on vesicles (data not shown). We did observe vesicle disruption after incubation with IgG2b against *B. anthracis* lethal factor, but the increased destruction levels by antibody and albumin compared with ovalbumin and KLH suggest that protein-mediated vesicular disruption is a property of only certain proteins.

Our next goal was to determine the rapidity by which albumin disrupted vesicles. Consequently, we incubated radiolabelled vesicles with albumin at 37°C for various time intervals. After incubation, samples were ultracentrifuged to pellet vesicles and the proportion of supernatant-associated radioactivity was measured as an indicator of disrupted vesicles. The majority of vesicle disruption occurred in less than 1 min (Fig. 9A). To confirm this result by another technique, we used dynamic light scattering to observe the average vesicular size over ten 30 s measurements. Albumin was added after the first 30 s interval, and the average size of the vesicle population dropped from 400 nm to below the limits of detection (approximately 30 nm) within 60 s (Fig. 9B). Addition of mAb 18B7 to purified vesicles did not reduce the average vesicular size (data not shown). This confirms that albumin acts within 1 min to disrupt cryptococcal vesicles.

Discussion

After validating metabolic labelling as a method to investigate production of extracellular vesicles, we focused on the question of vesicle stability. Purified cryptococcal vesicles were remarkably stable, which we found surprising given that one of the proposed functions of vesicle secretion in *C. neoformans* is the transport of large polysaccharide oligomers to the outside of the cell (Rodrigues *et al.*, 2007). However, this stability is comparable to that described in other systems. Membrane vesicles from other microbes, such as *Neisseria meningitidis*, are stable for up to 1 year when stored at 4°C, although stability decreases when stored at 37°C or 56°C (Arigita *et al.*, 2004). In contrast, *B. anthracis* vesicles were considerably less stable than cryptococcal vesicles, suggesting that differences in their lipid compositions (Rodrigues *et al.*, 2007; Rivera *et al.*, 2010) translated into differences in stability.

The finding of a stable population of polysaccharide-containing secreted vesicles leads to the questions: why

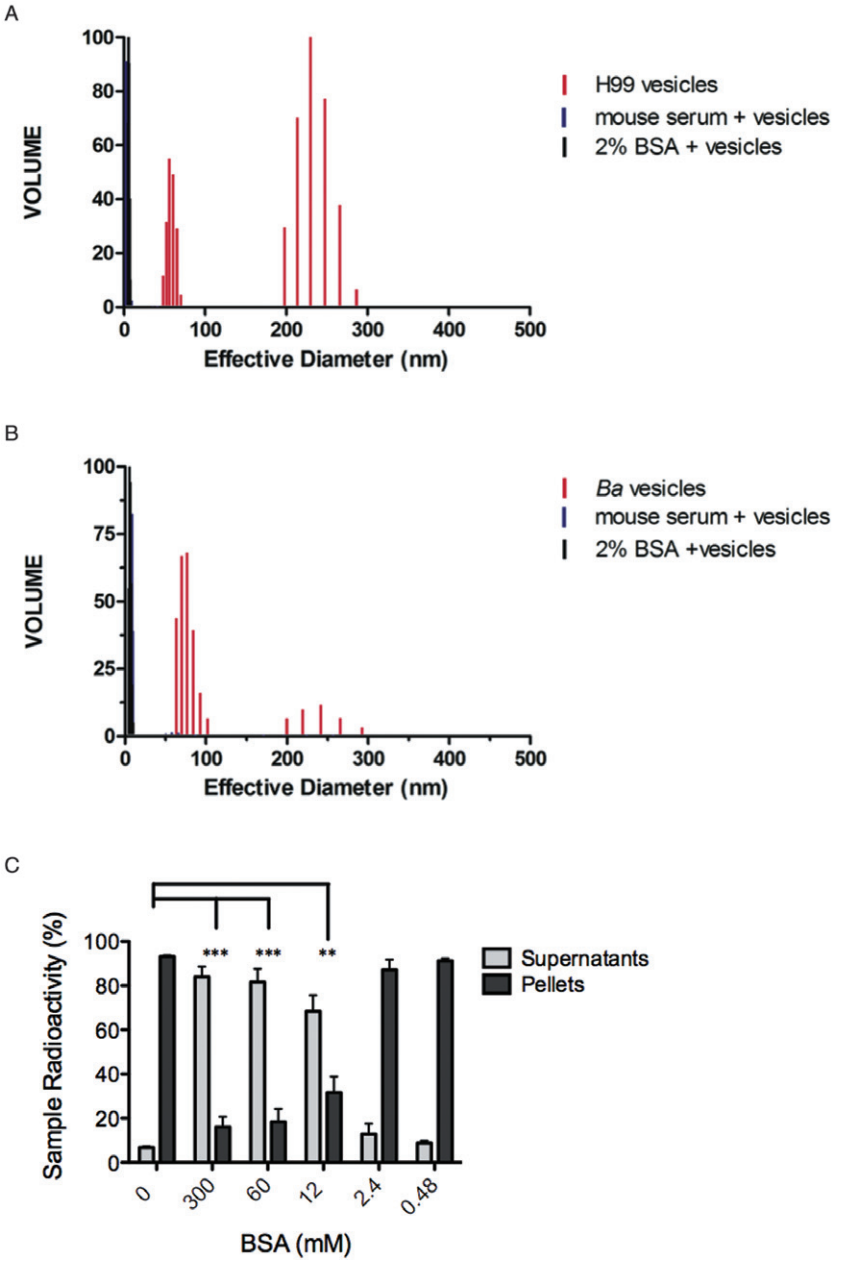

Fig. 8. Mouse serum decreases microbial vesicle size.
A. Cryptococcal vesicles were incubated with PBS, 2% (300 mM) BSA or 2% mouse serum for 1 h at 37°C and size was monitored using dynamic light scattering.
B. Bacillus vesicles were incubated with PBS, 2% (300 mM) BSA or 2% mouse serum for 1 h at 37°C and size was monitored using dynamic light scattering.
C. Radiolabelled vesicles were incubated with fivefold serial dilutions of BSA, starting with 300 mM. Samples were incubated 1 h at 37°C, followed by ultracentrifugation to determine supernatant and pellet radioactivity, which were assessed and normalized to 100%. Bars represent standard error. ** *P*-value < 0.05; *** *P*-value < 0.001.

do vesicles contain GXM polymers? One possibility was that they represent a minor proportion of vesicles that are defective in some manner and thus fail to disgorge their contents in external spaces, an explanation that could account for their paucity relative to the numbers expected from electron micrographs. Another possibility was that they are intended for export and represent a mechanism for delivery of fungal products in a concentrated manner. From the viewpoint of pathogenesis, vesicles are interesting because their content has been associated with virulence for both *C. neoformans* and *B. anthracis* (Rodrigues *et al*., 2007; 2008; Rivera *et al*., 2010). Cryptococcal vesicles contain GXM, which is a potent immunomodulator (Vecchiarelli, 2007) while *B. anthracis* vesicles contain several toxin components, which together result in cytotoxicity (Rivera *et al*., 2010). The finding of vesicular stability also left open the question of cargo delivery to host cells.

Previously, the observations that anthrax toxin components were released in vesicles (Rivera *et al*., 2010) made it difficult to understand how these proteins could interact with cellular receptors (Collier, 2009; Walter *et al*., 2009) and neutralizing antibodies. Our finding that both the fungal and bacterial vesicle stability decreased in serum provides a mechanism for the release of their virulence-associated cargo into tissue spaces. However, our finding

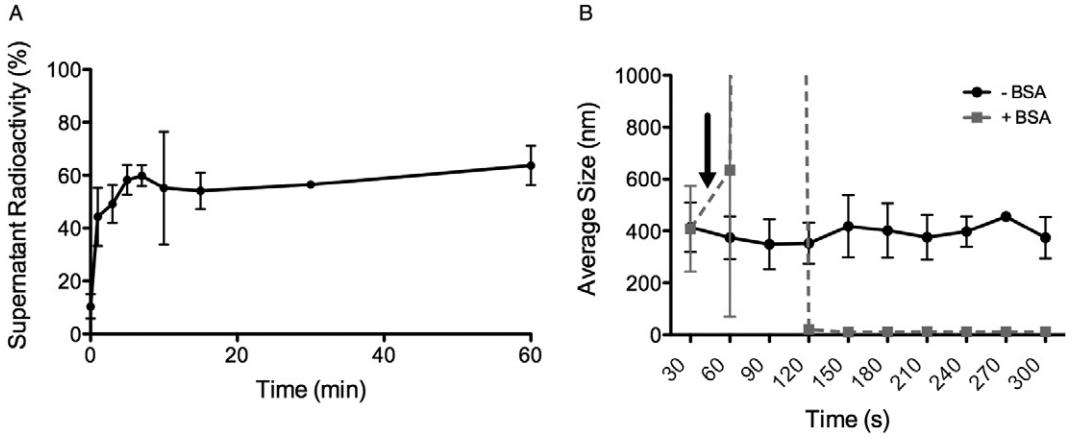

Fig. 9. Temporal kinetics of albumin-mediated vesicle disruption.
A. Radiolabelled cryptococcal vesicles were incubated with 300 mM BSA for the indicated time at 37°C, after which samples were ultracentrifuged to pellet vesicles. Radioactivity of the supernatant and pellet were measured to calculate total sample radioactivity, and supernatant radioactivity was used as a measure of vesicle destruction.
B. Vesicle preparations were measured by dynamic light scattering over ten 30 s intervals to read the average size of the population. The addition of albumin initially leads to instability in the signal, a phenomenon that presumably reflects equilibration of protein with the aqueous buffer, since the same effect was observed by just mixing buffer and albumin. However after 120 s there is no signal from the vesicles, consistent with their complete disruption. The same sample was read first without and then with the presence of 300 mM BSA, which was added between the 30 s and 60 s intervals (arrow).

that *B. anthracis* vesicles are also taken up by cells in the absence of serum suggests the vesicles can deliver cargo directly to macrophage, likely through phagocytosis or fusion with the plasma membrane, as has been observed by electron microscopy (Rivera *et al*., 2010). However, the instability of these vesicles in the presence of albumin implies that they are short-lived *in vivo*, and the release of their contents into body fluids thus makes vesicular transport fully compatible with current views on toxin entry (Young and Collier, 2007; van der Goot and Young, 2009) and antibody-mediated toxin neutralization, which posit components interacting directly with cellular receptors and/or specific antibody.

The instability of cryptococcal vesicles in serum is consistent with the notion that their cargo, including GXM, is released upon secretion. The abundance of albumin, which constitutes approximately two-thirds of serum protein (Meierhofer *et al*., 2010), would suggest that secreted fungal and bacterial vesicles are rapidly destabilized once secreted from their respective cells into the extracellular host environment. Incubation time-course studies confirmed that vesicle lysis occurs within seconds, which in turn suggests that vesicles are disrupted shortly after being released from the cell. Thus, cryptococcal vesicles are potential sources of both exo- and capsular polysaccharide.

Since the membrane composition varies between these microbial vesicles (Rodrigues *et al*., 2007; Rivera *et al*., 2010), one possibility is that albumin-destabilization proceeds through common pathways that destabilize the lipid membranes. In this regard, human plasma was reported to mediate lysis of liposomes, such as those administered for drug delivery (Mui *et al*., 1994). Albumin has recently been revealed to mediate this lysis through binding of cholesterol on the liposomes (Meierhofer *et al*., 2010). However, neither fungal nor bacterial vesicles contain cholesterol, suggesting that albumin-mediated vesicle instability must have a more generic mechanism. For fungal vesicles, albumin might bind to the ergosterol, resulting in destabilization of the vesicle membrane. However, albumin is also known to bind fatty acids (Ascenzi and Fasano, 2009), which could compromise vesicular membrane integrity.

In summary, our studies establish the usefulness of metabolic labelling as a method for studying vesicular secretion, stability and fate in small volume culture preparations. Our work shows that vesicles are unstable in serum and that this effect is mediated by serum proteins, most notably albumin. Hence, protein-mediated vesicle instability provides an answer to the question of how cargo is delivered from the intraluminal space to solution where it can mediate noxious effects on the host. The role of albumin in this process can be described as ambiguous with regards to host protection, since it destabilizes vesicles but also assists in unloading their cargo. Albumin has been reported to inhibit the growth of the fungus *Blastomyces dermatitidis in vitro* (Giles and Czuprynski, 2003), and it is conceivable that effects on fungal vesicles may contribute to this effect. The results indicate the need to consider effects of non-immune host components such as albumin on microbial structures when investigating mechanisms of virulence.

Experimental procedures

Strains

All *C. neoformans* experiments were conducted with the clinical isolate H99 strain (serotype A). Fungal cultures were grown in minimal medium (15 mM dextrose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine and 3 μM thiamine-HCl). All *B. anthracis* experiments were conducted with the Sterne strain 34F2 (pXO1+, pXO2–), which was obtained from Dr Alex Hoffmaster at the Center for Disease Control (Atlanta, GA). Bacterial cultures were grown from frozen stock in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C for 18 h with shaking.

Vesicle isolation

For *C. neoformans* vesicle isolation from 15 ml of cultures, cells were first removed by two subsequent centrifugations for 10 min each, 2500 *g*, at room temperature. Supernatants were then filtered through a 0.8 μm syringe tip filter. Cell-free supernatants were concentrated in Amicon Ultra-15 centrifugal filter devices with a 100 kDa cut-off by spinning 5 min, 850 *g*, at room temperature. In some experiments, the concentrate was spun 1 h, 100 000 *g*, at 4°C, after which the supernatant was removed and the pellet was resuspended in 250 μl of phosphate-buffered saline (PBS). *B. anthracis* vesicles were isolated from culture supernatants similarly, but the final resuspension was in 10 mM glucose in PBS.

Pulse-chase with radioactive palmitic acid

For *C. neoformans* cultures, an overnight culture of H99 strain was diluted to approximately 5×10^4 cells ml^{-1} in 200 ml of minimal medium and grown to exponential phase (approximately 5×10^6 cells ml^{-1}). Aliquots of 15 ml were grown in 30 ml PETG flasks (Thermo Scientific) and 2 μCi of [1-^{14}C] palmitic acid was added to each aliquot, followed by incubation at 30°C, 150 r.p.m. After 4 h, cells were harvested by centrifuging for 10 min, 2500 *g*, at room temperature, and washed once with deionized water. The cell pellets were then suspended in 15 ml of cold minimal medium, and incubated for an additional 4, 24 or 72 h at 30°C with shaking. Controls included samples incubated without [1-^{14}C] palmitic acid (mock-pulsed cultures), without *C. neoformans* cells, or incubated with cold minimal medium containing 10 mM NaN_3. At the end of all incubations, samples were processed as described above. Where noted, the cell pellet was resuspended in 1 ml of deionized water and 0.1 ml was used for scintillation counting. The entire concentrate and a 1 ml of flow-through (FT) were taken for scintillation counting after noting the volumes of each. When ultracentrifugation was used, the high-speed supernatant was saved for scintillation counting, and the pellet was washed in 1 ml of TBS, spun again at 195 000 *g* for 1 h, and resuspended in 250 μl of PBS for scintillation counting. To pulse *B. anthracis* cultures, a saturated overnight culture was diluted to OD_{600} 0.1. Aliquots of 15 ml of culture were pulsed with 2 μCi of [1-^{14}C] palmitic acid for 4 h, after which cells were pelleted, washed and resuspended in cold BHI medium. Vesicles were collected as described above after 24 h incubation at 37°C. Scintillation counting was done in a Packard Bioscience Tricarb A2900 liquid scintillation counter.

Vesicle stability studies

To study vesicle stability, vesicles were harvested after 72 h incubation as described above and then incubated at room temperature in the spent cryptococcal medium (containing only particles > 100 kDa). At 0, 24, 48 and 72 h, the suspension was centrifuged for 1 h at 100 000 *g* to recover intact vesicles and the proportion of radiation in the pellet and supernatant was determined by scintillation counting as described above.

To assess the effect of media on vesicle stability, the pellet recovered from the last centrifugation step was resuspended in 1 ml of minimal medium, macrophage medium, spent macrophage medium or 10% FBS. Spent macrophage medium was generated by the removal of macrophage cells through centrifugation. Heat-treated samples were incubated for 1 h in a 65°C water bath. Phospholipase A2 and Phospholipase C were added to a final concentration of 1 μg ml^{-1}. Compound O (Sigma), Compound Ax (Sigma) and Compound P (Sigma) were used at 50, 25 and 25 μM respectively. Samples were incubated at 37°C, 5% CO_2 for 1 h before being centrifuged 1 h, 195 000 *g*, at 4°C as described above. The pellet was washed in 1 ml of TBS, spun at high speed again and resuspended in 250 μl for scintillation counting. The supernatant was also read for radioactive counts, and the per cent of CPM in each portion calculated based on total CPM of pellet and supernatant.

Dynamic light scattering

Vesicle size was investigated by light scattering as described (Eisenman *et al.*, 2009). Vesicles in minimal medium were measured in a 90Plus/BI-MAS Multi Angle Particle Sizing analyser (Brookhaven Instruments). The sample was illuminated with laser monochromatic light and the fluctuating signal generated by the Brownian motion of the vesicles was analysed by the autocorrelation function $C(t)$: $C(t) = Ae^{2\Gamma t} + B$, where t is time delay, A and B are optical constants determined by the instrument design, and Γ is related to the relaxation of the fluctuations by $\Gamma = Dq^2$. The parameter q is derived from the scattering angle Θ, the laser light wavelength λ_0 and the solvent refractive index (n) from the equation $q = (2\pi n/\lambda_0)2\sin(\Theta/2)$. D is derived from $D = (K_B T)/3\pi\eta(t)d$, which assumes a spherical shape for the scattering particle, where K_B is Boltzmann's constant (1.38054E-23 J deg^{-1}), T is the temperature in K (303 K), $\eta(t)$ is the viscosity of the liquid in which the particles are moving, and d is the particle diameter. In most experiments, data are expressed as the average of 10 runs, but in the time-course experiment, the mean of each run is plotted against time.

Density gradient centrifugation

Vesicles were harvested 72 h after pulsing with [1-^{14}C] palmitic acid as described above. Mock-pulsed vesicles were simultaneously harvested for capture ELISA studies. The pellet containing vesicles was resuspended in 3 ml of 35% Optiprep Density Gradient Medium (Sigma). A density gradient was generated by layering 3 ml of 30%, 2 ml of 25%, 2 ml of 20%, 2 ml of 15% and 2 ml of 10% Optiprep solution on top of each other. Gradients were centrifuged at 140 000 *g*, 4°C, for 15 h. The gradients were analysed by taking 1 ml fractions that were then analysed by radioactive counts or analysed for capsular polysaccharide by capture ELISA.

Capture ELISA to determine GXM

GXM-capture ELISA was used to detect GXM in isolated vesicles as previously described (Casadevall and Scharff, 1991; Casadevall *et al*., 1992). Briefly, 96-well ELISA plates were coated with 10 μg ml^{-1} 2D10 IgM antibody in PBS and then blocked in blocking buffer (2% bovine serum albumin in PBS) overnight at 4°C. Plates were incubated sequentially with 1:100 optiprep fractions, 20 μg ml^{-1} 18B7 IgG1 antibody and 20 μg ml^{-1} goat anti-mouse IgG-alkaline phosphatase conjugate for 1 h each at 37°C. Plates were washed between incubations with 0.5% Tween-20 in TBS. Plates were developed with addition of p-nitrophenyl phosphate (Sigma), and absorbance measured at 405 nm.

Vesicle uptake by macrophage

Microtitre (96-well) tissue culture plate wells were seeded with 4×10^4 cells of J774.16 mouse macrophage cell line or bone marrow-derived macrophage from BALB/c mice. Macrophage were grown in DME medium (Invitrogen) supplemented with 10% (v/v) FBS (Atlantic Biologicals), 10% NCTC (Invitrogen), 1% non-essential amino acids (Invitrogen) and 1% penicillin (Invitrogen). Cryptococcal vesicles were harvested 72 h after pulsing with [1-^{14}C] palmitic acid as described above. *B. anthracis* vesicles were harvested 24 h after pulsing with [1-^{14}C] palmitic acid as described above. The vesicle-containing pellet was resuspended in 200 μl of TBS and 100 μl of the vesicle solution was added to 1.8 ml of feeding medium with or without 10% serum. A volume of 200 μl of vesicle-containing medium was added to each well. Controls included vesicle-containing medium alone and cells treated with cold medium. After 2 or 24 h incubation at 37°C in 9.5% CO_2 supernatants were collected for scintillation counting. Cells were washed three times in PBS, lysed in 200 μl of 25 mM deoxycholate, and collected for scintillation counting. Vesicle uptake was estimated by calculating the per cent radioactivity taken up by the cells.

Serum partitioning by FPLC

A 2.5 ml sample of FBS was run on an S200 column using FPLC. Fractions (1.2 ml) were collected and protein concentration of the eluting solution was determined by reading the absorbance at 280 nm of the eluting solution. Peaks with A_{280} above background were pooled and concentrated using Millipore Centrifugal Concentrators with a 10 kDa molecular weight cut-off.

Statistical analyses

Significance was assessed using GraphPad Prism software. Results were analysed using a one-way ANOVA followed by Dunn's multiple comparison test and the unpaired *t*-test.

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