Host cell-derived sphingolipids are required for the intracellular growth of *Chlamydia trachomatis*

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Summary

*Chlamydia trachomatis*, an important cause of human disease, is an obligate intracellular bacterial pathogen that relies on the eukaryotic host cell for its replication. Recent reports have revealed that the *C. trachomatis* vacuole receives host-derived sphingolipids by fusing with trans-Golgi network (TGN)-derived secretory vesicles. Here, it is shown that these lipids are required for the growth of the bacteria. *C. trachomatis* was unable to replicate at 39°C in the Chinese hamster ovary (CHO)-derived cell line SPB-1, a cell line incapable of synthesizing sphingolipids at this temperature because of a temperature-sensitive mutation in the serine palmitoyltransferase (SPT) gene. Complementation with the wild-type SPT gene or addition of exogenous cell-permeable sphingolipid precursors to the mutant cells restored their ability to support chlamydial replication. L-cycloserine (L-CS) and fumonisin B1 (FB1), inhibitors of sphingolipid biosynthesis, decreased the proliferation of the bacteria in eukaryotic cells at concentrations that also decreased host cell sphingolipid synthesis. In the case of FB1, the vacuoles appeared aberrant; the addition of sphingolipid precursors was able to reverse the altered morphology of the FB1-treated vacuoles. Collectively, these data strongly suggest that the growth and replication of chlamydiae is dependent on synthesis of sphingolipids by the eukaryotic host cell and may contribute to this organism’s obligate intracellular parasitism.

Introduction

*Chlamydia trachomatis* is the major cause of non-congenital blindness in the Third World and is the leading cause of sexually transmitted diseases in the western hemisphere (Schachter and Dawson, 1990; Braunwald, 1998). *Chlamydia* ssp. are obligate intracellular parasites that replicate through a unique biphasic life cycle (Moulder, 1991). They alternate between an extracellular, metabolically inactive form, the elementary body (EB), and an intracellular form, the metabolically active, but non-infectious, reticulate body (RB). EBs enter the eukaryotic host, presumably through receptor-mediated endocytosis, and subsequently reside within a membrane-bound compartment called the vacuole or inclusion. Within this vacuole, the EB transforms into the RB, and bacterial replication ensues. After multiple division cycles, the RBs convert back to EBs, the host cell is lysed and infectious progeny are released.

Understanding the complex interplay between an obligate parasite and its host cell may lead to novel therapeutic approaches, as well as to new insights into host cell biology. It has long been postulated that it is the inability to produce high-energy metabolites such as ATP and GTP that makes chlamydiae dependent on a eukaryotic host (Moulder, 1962), but the recent sequencing of the *C. trachomatis* genome reveals that the organism is potentially capable of generating at least low levels of these compounds (Stephens et al., 1998). Thus, the parasitic lifestyle may reflect a requirement for more complex nutrients from the host cell, such as lipids, for growth and expansion of the inclusion membrane. Although the genome sequence indicates that the bacteria have the capacity to synthesize fatty acids, phosphatidylglycerol and phosphatidylethanolamine de novo, the biosynthetic machinery for sphingomyelin, which comprises 4% of chlamydial lipids (Newhall, 1988), is absent (Stephens et al., 1998). Consistent with this, recent work has demonstrated that endogenous host-derived sphingolipids, as well as exogenously added C6-NBD-ceramide, are incorporated into both the vacuolar membrane and the bacteria themselves (Hackstadt et al., 1995; 1996; Scidmore et al., 1996). The acquisition of

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these lipids by the bacteria suggests an essential role for lipids in the life cycle. However, inhibition of sphingolipid transport from the trans-Golgi network (TGN) to the vacuole by brefeldin A (BFA) did not decrease the amount of viable progeny. Thus, the role of these lipids remains unclear.

In this report, we have investigated further the requirement of *C. trachomatis* for host cell sphingolipids. Using a temperature-sensitive CHO mutant cell line deficient in the synthesis of sphingolipids at non-permissive temperatures, we demonstrate that host cell sphingolipids are required for bacterial growth and the production of infectious progeny. Furthermore, pharmacological inhibitors of sphingomyelin biosynthesis decreased the amount of viable bacterial progeny produced in CHO cells. Thus, host-derived sphingolipids play a crucial role in the replication of *C. trachomatis* within the host cell.

**Results**

*C. trachomatis does not grow in cells deficient in serine palmitoyltransferase activity*

Synthesis of sphingolipids begins in the endoplasmic reticulum with the condensation of serine with palmitoyl-CoA to 3-keto-sphinganine by the enzyme serine palmitoyltransferase (SPT; Fig. 1). Further modifications to produce mature sphingolipids occur in the endoplasmic reticulum and the Golgi complex. A CHO-K1 mutant cell line, SPB-1, encoding a thermolabile SPT has been isolated (Hanada et al., 1990; 1990). At the semi-permissive temperature (32°C), the cell line possesses only 4–8% of wild-type levels of SPT activity. Nevertheless, the cells are viable when grown in lipid-deficient media at this temperature. After incubation at non-permissive temperatures (39°C) for 4 days, virtually no SPT activity is detectable, the *de novo* synthesis of sphingomyelin decreases to less than 4% of the parental strain, the total amount of sphingomyelin is decreased by approximately two-thirds and cell growth ceases (Hanada et al., 1997). Sphingolipid synthesis and cell viability at the non-permissive temperature are restored by stable transfection of cDNA encoding a subunit of the wild-type SPT into the SPB-1 cell line; this complemented cell line is named SPB-1/cLCB-1 (Hanada et al., 1997).

To investigate the role of sphingolipids in the growth of *C. trachomatis*, CHO-K1, SPB-1 and SPB-1/cLCB-1 cells were grown for 3 days in lipid-deficient media at either the semi-permissive or the non-permissive temperature under conditions previously demonstrated to prevent *de novo* synthesis of sphingolipids (see Hanada et al., 1997; Experimental procedures). Subsequently, the cells were infected with the L2 serovar of *C. trachomatis* and incubated further at either 32°C or 39°C. The formation of inclusions was assayed by quantifying the fraction of cells containing vacuoles at 24 h post infection (hpi). The relative yield of infectious progeny was determined by harvesting infected cells at 44 hpi, isolating EBs and subsequently titrating the progeny on HeLa cells. As shown in Fig. 2A, at the semi-permissive temperature (32°C), the fraction of SPB-1 cells harbouring inclusions and the amount of infectious progeny was reduced fivefold compared with CHO-K1 cells grown at the same temperature. This finding is consistent with the 4–8% residual SPT activity in the SPB-1 cells. At 39°C, the temperature-sensitive cells exhibited a much more striking defect in their ability to support the replication and production of infectious *C. trachomatis*. The fraction of

![Fig. 1. The biosynthetic pathway of sphingolipids. The sites of action of L-CS and FB1 are shown. The SPB-1 cells encode a temperature-sensitive serine palmitoyltransferase (SPT), the first enzyme in the pathway.](image-url)
cells containing inclusions was reduced 400-fold in the SPB-1 cells compared with CHO-K1 cells grown at 39°C, and the amount of infectious progeny was similarly decreased 150-fold compared with CHO-K1 cells grown at 39°C. Again, this reduction in growth and replication of C. trachomatis correlates with the almost complete absence of detectable SPT activity in the SPB-1 cells grown at non-permissive temperatures. The complemented cell line exhibited close to wild-type levels of infected cells and infectious progeny at both temperatures. Together, these results suggest that the lack of growth in the SPB-1 cells at the non-permissive temperature is related to the absence of SPT activity.

The size of the chlamydial inclusions did not differ between the CHO-K1, SPB-1 and SPB-1/cLCB-1 cell lines grown at 32°C (Fig. 2B), but, as we have reported previously (van Ooij et al., 1998), vacuole fusion was inhibited in all cell lines grown at this temperature, resulting in the appearance of multiple, small vacuoles in some cells.

Exogenous sphingolipids can restore growth of C. trachomatis in SPB-1 cells

It has been demonstrated previously that the addition of exogenous sphingosine, dihydrosphingosine and sphingomyelin can restore the growth defect of the SPB-1 cells grown at non-permissive temperatures in lipid-deficient media (Hanada et al., 1992). To ascertain further that the decreased ability of C. trachomatis to replicate in SPB-1 cells at 32°C or 39°C was a result of the lack of sphingolipids and not an indirect consequence of the lack of SPT activity, SPB-1 cells were incubated for 3 days at 32°C or 39°C in lipid-deficient media with daily addition of 2 μM sphingosine, 2 μM dihydroceramide, 50 μM sphingomyelin or carrier (ethanol). On day 4, the cells were infected with C. trachomatis for 1 h and incubated further in lipid-deficient medium containing ethanol, 2 μM sphingosine, 2 μM dihydroceramide or 2 μM sphingomyelin at 32°C or 39°C for 24 h. The formation of vacuoles and the relative yield of infectious progeny were determined as described above. At 32°C or 39°C, the addition of sphingosine and dihydroceramide to SPB-1 cells partially restored the production of vacuoles and fully restored the production of infectious progeny compared with the untreated SPB-1 cells (Fig. 3; data not shown). The discrepancy between the fraction of cells containing vacuoles and the production of infectious progeny upon addition of dihydroceramide and sphingosine to the SPB-1 cells has not been investigated further.

In contrast, the addition of sphingomyelin to the SPB-1 cells failed to restore the growth of the bacteria (data not shown), probably because it is not membrane permeable and does not enter the lipid biosynthetic pathway. As a
control to test whether the sphingomyelin add-back experiment was successful, we tested whether sphingomyelin addition could prevent cell death in SPB-1 cells that were cultured for 3 days under non-permissive conditions. Trypan blue staining for cell viability demonstrated that daily addition of sphingomyelin resulted in similar to previously published data (Hanada et al., 1992). Thus, the failure of C. trachomatis to proliferate in the SPB-1 cells is not a result of the abnormal growth state of the host cells. However, the results of the sphingosine and dihydroceramide add-back experiments clearly demonstrate that the impaired ability of the bacteria to replicate in SPB-1 cells at 32°C or 39°C is because of the absence of de novo synthesized sphingolipids. That the bacterial growth defect is more pronounced at 39°C than at 32°C correlates well with the more severe deficiency in sphingolipid biosynthesis at the non-permissive temperature compared with the semi-permissive temperature.

C. trachomatis can bind and enter cells in the absence of sphingolipids

The lack of bacterial proliferation in the SPB-1 cells at non-permissive temperatures could be attributed to a requirement for de novo synthesized sphingolipids during growth. Alternatively, it may result from the loss of a receptor on the surface of the host cell or from the inability of the bacteria to enter the host cell in the absence of sphingolipids. To evaluate these possibilities, two experimental approaches were used. First, binding assays were performed to determine whether C. trachomatis could attach to host cells in the absence of sphingolipid synthesis. [35S]-labelled bacteria were added at 4°C for 1 h to CHO-K1, SPB-1 and SPB-1/cLCB-1 cells previously incubated at the non-permissive temperature for 3 days. Bacterial adhesion was measured by determining the amount of radiolabelled EBs that bound to the cells after extensive washing. This was compared with the adhesion of the radiolabelled bacteria that bound in the presence of heparin, which blocks specific binding of the L2 serovar of C. trachomatis to the host cell (Zhang and Stephens, 1992). The difference between the number of counts recovered in the absence and presence of heparin corresponds to the specific binding of bacteria. At either 32°C (data not shown) or 39°C (Table 1), the specific binding of the bacteria to the mutant cells was similar to that observed for the parental CHO-K1 or the complemented SPB-1/cLCB-1 cells.

Secondly, the ability of chlamydiae to enter the host cell in the absence of sphingolipid synthesis was also tested (Fig. 4). We reasoned that, if de novo synthesis of sphingolipids is required for the binding or uptake process, no bacteria should be able to enter the SPB-1 cells incubated at 39°C before the addition of sphingosine. On the other hand, if sphingolipids are only required at a step subsequent to entry, bacteria should be able to enter the host cells even in the absence of exogenous sphingosine. For these experiments, SPB-1 cells were grown at 39°C for 3 days in lipid-deficient media. The cells were then infected for 1 h and subsequently placed in media containing heparin (which prevents further bacterial

Table 1. Binding of C. trachomatis to SPT-deficient cells is not decreased.

| Cell line | Heparin | CPM bound | SD | Specific binding |
|-----------|---------|-----------|----|-----------------|
| CHO-K1    | −       | 250       | 49 | 113             |
|           | +       | 137       | 67 |                 |
| SPB-1     | −       | 244       | 58 | 159             |
|           | +       | 85        | 24 |                 |
| cLCB-1    | −       | 201       | 74 | 146             |
|           | +       | 55        | 55 |                 |

CHO-K1, SPB-1 and SPB-1/cLCB-1 cells were grown at 39°C for 3 days, and [35S]-labelled bacteria were added for 1 h. Radioactivity bound to the host cells was determined as described in Experimental procedures. The amount of specific binding was determined by subtracting the amount of radioactive bacteria that bound to the cells in the presence of heparin from the amount of radioactive bacteria that bound to the cells in the absence of heparin.
Inhibitors of sphingolipid synthesis decrease the production of viable C. trachomatis progeny

The requirement of C. trachomatis for host cell sphingolipid synthesis should render its growth sensitive to inhibitors of sphingolipid synthesis and may allow the sphingolipid requirement of the bacteria to be examined in more detail. L-CS and FB₁ block the synthesis of all sphingolipids by inhibiting enzymatic functions early in the synthesis pathway (Fig. 1). L-CS inhibits serine palmitoyl transferase, the enzyme with the temperature-sensitive mutation in SPB-1 cells. FB₁ is a competitive inhibitor of sphinganine and sphingosine N-acetyltransferases and leads to a rapid inhibition of sphingomyelin biosynthesis, eventually resulting in a decrease in total sphingomyelin (Merrill et al., 1993; Schroeder et al., 1994; Badiani et al., 1996). We tested whether these drugs abrogated intracellular growth of C. trachomatis.

CHO-K1 cells were infected with C. trachomatis and, after removal of the inoculum, the infected cells were incubated at 37°C in the presence of the indicated concentrations of drug. Vacuole formation was assayed at 20 hpi, and production of viable progeny was quantified at 44 hpi. The inhibitory effects of the drug on sphingomyelin biosynthesis were determined by exposing uninfected CHO-K1 cells to the drug for 2 h, then adding [³H]-serine for an additional 24 h in the continued presence of the drug. The resulting [³H]-sphingolipids were resolved by thin-layer chromatography (TLC), and inhibition of specific sphingolipid biosynthesis was assessed by computer-assisted densitometry of the autoradiographs.
To rule out the possibility that these drugs were directly toxic to the bacteria, we attempted to restore intracellular growth of the bacteria by the addition of appropriate sphingolipid precursors that should bypass the drug-induced blockade of sphingolipid biosynthesis. CHO-K1 cells were infected with *C. trachomatis* for 1 h at 37°C, and the inoculum was replaced with medium containing l-CS (A) or FB1 (C) at the indicated concentrations. The fraction of infected cells at 20 hpi (stippled bars) and the relative amounts of infectious progeny produced at 44 hpi (solid black bars) were measured as described in *Experimental procedures*. A minimum of 300 cells on triplicate coverslips was counted, and the mean ± standard error is shown. In some cases, the error bars are too small to be seen. The percentage of infected cells in untreated samples was set to 100%, and the percentage of infected cells in the presence of inhibitor was compared with untreated cells. The experiment was repeated at least twice with each inhibitor with similar results.

B and D. The effect of l-CS and FB1 on de novo sphingomyelin biosynthesis. CHO-K1 cells were exposed to the indicated concentration of l-CS (B) or FB1 (D) for 2 h, at which time [3H]-serine was added and the incubation continued for an additional 24 h in the presence of the drug. Sphingolipids were extracted and separated by TLC. The region of the TLC plate containing sphingomyelin is shown, which was identified by co-electrophoresis of a purified sphingomyelin standard. Both the sphingomyelin standard and the [3H]-serine containing in vivo synthesized sphingomyelin run as a doublet. The reason why one band of the doublet disappears at increasing concentrations of FB1 is unknown. The autoradiographs were quantified by computer-assisted densitometry, and the relative decrease in de novo sphingomyelin biosynthesis is shown below the autoradiograph.

To rule out the possibility that these drugs were directly toxic to the bacteria, we attempted to restore intracellular growth of the bacteria by the addition of appropriate sphingolipid precursors that should bypass the drug-induced blockade of sphingolipid biosynthesis. CHO-K1 cells were infected with *C. trachomatis*, incubated with 10 μg ml⁻¹ FB1 in the presence or absence of 2 μM dihydroceramide, and the number of infected cells and percentage of abnormal appearing vacuoles were determined. The addition of dihydroceramide failed to restore bacterial growth in FB1-treated infected cells (determined by the fraction of cells containing vacuoles; data not shown). However, there was a significant increase in the fraction of normal-staining vacuoles from 22% to 80% (scored independently by two different investigators). We found that sphingolipid biosynthesis was restored under these conditions, as demonstrated by the conversion of C6-NBD-ceramide into C6-NBD-sphingomyelin in the presence of FB1 (data not shown). It was not possible to restore growth or normalize vacuole morphology by the addition of sphingosine or dihydroceramide to *Chlamydia*-infected cells exposed to l-CS, despite the ability of l-CS-treated CHO-K1 cells to incorporate C6-NBD-ceramide into sphingolipids (data not shown). These findings suggest that the effects of these drugs may be complex and multifactorial. At least in the case of FB1, part of its detrimental effect on the intracellular growth of *C. trachomatis* results from its ability to inhibit de novo synthesis of host cell sphingolipids.

Discussion

Using genetic and pharmacological approaches to block de novo synthesis of sphingolipids, these studies show that host cell-derived sphingolipids are necessary for the growth of *C. trachomatis*. The SPB-1 cell line, a CHO-K1 mutant cell line that is incapable of synthesizing sphingolipids at 39°C, could not support the growth of *C.
trachomatis when grown at a non-permissive temperature. Interestingly, under conditions that have previously been shown to reduce sphingomyelin content by only 66% (Hanada et al., 1992), this cell line could not support growth of C. trachomatis. As de novo synthesis is almost entirely abolished, it appears that the bacteria require newly synthesized sphingolipids.

Restoration of SPT enzyme activity by complementation with the wild-type subunit allowed the SPB-1 cells to support C. trachomatis replication. Bacterial growth could also be restored by the addition of the exogenous cell-permeable sphingolipid precursors, sphingosine and dihydroceramide, either before or after bacterial binding. These two compounds have distinct additional activities: sphingosine is an inhibitor of protein kinase C (Tolan et al., 1997), whereas dihydroceramide is an inhibitor of acyl-CoA:cholesterol acyltransferase (Ridgway, 1995). However, as both compounds restore bacterial growth, it is probable that bacterial growth in the SPB-1 cells at non-permissive temperatures is a direct result of the restoration of sphingolipid synthesis. The finding that growth of C. trachomatis could be rescued by the addition of sphingolipids after binding suggests that inhibition of de novo host sphingolipid synthesis does not prevent synthesis or presentation of the receptor. Host cell sphingolipids may be required for efficient uptake or, more probably, for incorporation into the C. trachomatis bacterial or inclusion membrane.

Sphingomyelin was unable to restore the growth of C. trachomatis in SPB-1 cells, probably because it is not membrane permeable and does not enter the lipid biosynthetic pathway. In support of this, others have shown that exogenously added fluorescent sphingomyelin is not incorporated into the chlamydial vacuole, although it is inserted into the outer leaflet of the host cell plasma membrane (Hackstadt et al., 1996). Nevertheless, the restoration of bacterial growth by the cloned SPT gene and by exogenously added cell-permeable sphingolipid precursors, even after binding and possibly internalization of the bacteria, demonstrates that the absence of growth in the mutant cell line is clearly as a result of the lack of sphingolipid synthesis.

Several pieces of evidence suggest that it is not simply the altered growth state of the SPB-1 cells that prevents proliferation of C. trachomatis. Most convincingly, daily addition of sphingomyelin reversed the growth defects of the SPB-1 cell line (Hanada et al., 1992; this work), but failed to rescue the ability of the bacteria to replicate. Next, in the experiments in which sphingosine or dihydroceramide was added after binding and uptake of the bacteria, C. trachomatis vacuoles and progeny production were nonetheless restored despite the fact that the SPB-1 cells may not have resumed proliferation. Other conditions that inhibit host cell proliferation, including host cell enucleation or inhibition of eukaryotic protein synthesis, do not affect intracellular growth of the bacteria (Perera et al., 1990; Moulder, 1991). The requirement of C. trachomatis for host-derived sphingolipids makes these organisms susceptible to pharmacological inhibitors of sphingolipid biosynthesis. Treatment of infected CHO-K1 cells with two different inhibitors of sphingolipid synthesis reduced bacterial growth, resulting in a decrease in both the number of infected cells and the production of viable progeny. As the drugs were added after the cells were infected, it is probable that they prevented bacterial growth at a step subsequent to entry. These findings suggest that the failure of C. trachomatis to proliferate in L-CS- and FB1-treated cells may be a result of the lack of host cell sphingomyelin incorporation into the vacuolar and/or bacterial membrane.

We note two potential limitations to the interpretation of these experiments. First, it is possible that the effects of sphingolipid synthesis inhibitors result from the accumulation of precursors (Wang et al., 1991; Yoo et al., 1992; Rosenwald and Pagano, 1993; Chen et al., 1995). As both inhibitors show identical effects on the growth of the bacteria, but probably cause the accumulation of different sphingolipid precursors, it is improbable that it is the build-up of precursors that leads to the cessation of bacterial growth.

Secondly, each of the sphingolipid inhibitors could have a direct toxic effect on C. trachomatis development. Interestingly, Hatch and McClarty have reported that FB1 treatment of L929 cells decreased the incorporation of [14C]-serine into sphingomyelin in both uninfected and C. trachomatis-infected cells, but did not inhibit bacterial replication, as evidenced by unchanged incorporation of [3H]-adenine into bacterial DNA (Hatch and McClarty, 1998). This finding would suggest that FB1 is not directly inhibitory to C. trachomatis replication, but perhaps affects development by limiting the receipt of sphingomyelin to the vacuole. Consistent with this hypothesis, we noted aberrant-appearing vacuoles in the presence of inhibitory concentrations of the drug. The RBs appeared to stain irregularly with an anti-MOMP antibody compared with the uniform staining observed in C. trachomatis-infected cells not exposed to the drug. Hackstadt et al. (1995) reported that treatment of Chlamydia-infected cells with BFA, which inhibits the anterograde transport from the Golgi apparatus, resulted in smaller, more compact vacuoles. The changes in vacuolar morphology detected in BFA-treated cells may be a result of inhibition of sphingolipids acquired from Golgi-derived vesicles. However, as the bacteria are still capable of growing in BFA-treated cells, we postulate that the vacuole must be able to acquire sphingolipids by fusion with other sphingolipid-containing host cell compartments or by other lipid
transport pathways. Indeed, there is precedence for an additional, brefeldin-insensitive sphingolipid transport pathway in at least some cultured cell lines (van Helvoort et al., 1996; Raggers et al., 1999). Alternatively, BFA may be directly toxic to the bacteria, resulting in growth inhibition and smaller vacuoles.

Chlamydia is unique among bacteria in its requirement for sphingolipids. Biochemical fractionation has shown that the chlamydial membrane consists of 4% sphingolipids (Newhall, 1988). Although this is a relatively small fraction of total lipids, it appears that host-derived sphingolipids are nonetheless necessary for the completion of the C. trachomatis life cycle. While it is formally possible that exposure to sphingolipids in the vacuole constitutes the signal that initiates the conversion of EBs to RBs, this hypothesis would not explain the decrease in inclusion number observed when sphingolipid biosynthesis was blocked. It would thus seem improbable that sphingolipids serve as the signal for EB to RB conversion. As sphingolipids are incorporated into the bacteria themselves to a larger extent than the vacuolar membrane, they probably play a direct role in the growth of the bacteria.

The pathway(s) by which C. trachomatis acquires sphingolipids from the host cell are of great interest. So far, the vacuole has been found to be devoid of host cell proteins, as determined by fluorescence and electron microscopy of eukaryotic proteins destined for the basolateral membrane (reviewed by Hackstadt et al., 1997). Recently, a novel sphingolipid transport pathway has been proposed for MDCK cells in which sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins form microdomains in the exoplasmic leaflet of the Golgi membrane. It is hypothesized that this allows these molecules to be sorted and directed to the apical surface (Simons and Ikonen, 1997). It is intriguing to speculate that the C. trachomatis vacuole intersects with this pathway.

To the best of our knowledge, this is the first example of an intracellular organism that requires host cell sphingolipids for intracellular growth and development and may, in part, explain its parasitic life cycle. The dependence of the bacteria on host cell lipids may lead to new avenues for the treatment of chlamydial infections, as well as providing further insights into the intracellular trafficking of sphingolipids.

Experimental procedures

Chemicals

Sphingosine (â-erythro-sphingosine), sphingomyelin and dihydroceramide (â-erythro-â-hydro-ceramide) were obtained from Matreya. C6-NBD-ceramide [6-[(N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]sphingosine] was obtained from Molecular Probes. Lipids were suspended in methanol or ethanol and stored in small aliquots at ~0°C. Sphingosine or dihydroceramide (2 mM in ethanol) was diluted 1:10 into 0.2 mM bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and added directly to the medium to a final concentration of 2 µM. Sphingomyelin was dried, resuspended at a concentration of 1 mM in sterile H2O and sonicated for 15 min. This solution was diluted further in medium to 50 µM before addition. Fumonisins B1 (FB1) was obtained from Alexis Biochemicals. Lipid standards were obtained from Matreya. All other chemicals were obtained from the Sigma Chemical Company unless otherwise specified.

Cell culture

HeLa cells (ATCC CCL2) were maintained in Dulbecco's modified Eagle medium H 21 (DMEM H21) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and grown at 37°C in an atmosphere of 5% CO2. CHO-K1, SPB-1 and SPB-1/clCB-1 cells were maintained in Ham's F-12 medium supplemented with 10% FBS at 37°C plus 5% CO2 (CHO-K1 cells) or 32°C plus 5% CO2 (SPB-1 and SPB-1/clCB-1 cells). For the induction of the temperature-sensitive phenotype, cells were seeded onto 12 mm glass coverslips or 12-well plates and incubated at 32°C for 16–24 h. The medium was then replaced with Ham's F-12 medium supplemented with 0.1% FBS, 1% Nutridoma-SP (Boehringer Mannheim) and 250 µM oleic acid/5% fatty acid free BSA (Sigma) (low-serum medium), and the cells incubated at 39°C for 3 days. This medium contains less than 1 µM sphingomyelin (Hanada et al., 1992). Sphingosine (2 µM) or dihydroceramide (2 µM) was added daily as indicated. Cell viability was quantified by counting the fraction of cells that were stained with trypan blue. Unless otherwise indicated, all media were purchased from the UCSF Cell Culture Facility.

Inhibitors

The effect of L-CS and FB1 on sphingolipid synthesis was tested by labelling CHO-K1 cells with L-[^3H]serine (4 µCi ml⁻¹; New England Nuclear) in the presence of various concentrations of L-CS and FB1 for 24 h. Lipids were extracted and analyzed as described previously (Bligh and Dyer, 1959) and then separated by TLC on Silica 60 TLC plates (EM Science) in chloroform–methanol–NH₃–H₂O (35:15:2:0.5). Lipid standards (Matreya) were visualized by staining the TLC plate with 0.05% Brilliant blue R250 in 0.1 NaCl in 25% methanol followed by destaining in 0.1 M NaCl in 25% ethanol (Nakamura and Handa, 1984). Autoradiographs were performed using En³Hance (New England Nuclear). Autoradiographs were scanned in using a UMAX scanner and the bands of interest quantified using the IPLAB gel program.

To determine whether C6-NBD-ceramide could be incorporated into newly synthesized sphingomyelin in the presence of FB1 or L-CS, CHO K1 cells were grown for 2 h at 37°C in media with no drug or with 1.0 mM of L-CS, 1.5 mM L-
After 3 days, these cells were infected for 1 h at the same sphingomyelin in 12-well dishes or on 12 mm coverslips. m serum media (see above) with or without the daily addition of m with 10% FBS, vancomycin hydrochloride (60 oij et al used. Bacteria were propagated as described previously (van Q 2000 Blackwell Science Ltd, CHO-K1or SPB-1 cells were grown at 33

Quantification of viable C. trachomatis progeny
CHO-K1or SPB-1 cells were grown at 33°C or 39°C in low-serum media (see above) with or without the daily addition of 2 μM sphingosine, 2 μM dihydroceramide or 50 μM sphingomyelin in 12-well dishes or on 12 mm coverslips. After 3 days, these cells were infected for 1 h at the same temperature with C. trachomatis serovar L2 suspended in

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Growth and propagation of C. trachomatis
For all experiments, the L2 serovar of C. trachomatis was used. Bacteria were propagated as described previously (van Ooij et al., 1997). Briefly, L-929 cells (ATCC CCL1) maintained in spinner flasks in RPMI-1640 supplemented with 10% FBS, vancomycin hydrochloride (60 μg ml⁻¹; Abbott Laboratories) and gentamicin sulphate (10 μg ml⁻¹) were infected with L2 for 36 h. Cells were pelleted, resuspended in 35 ml of PBS and sonicated three times for 30 s each using a Branson sonifier 450. Cell debris was pelleted at 4°C at 1500 g in a Sorval RT600B centrifuge. The supernatant was transferred to Oakridge tubes and centrifuged at 4°C at 17 000 g for 1 h in a Sorval SS-34 centrifuge. The resulting pellet was resuspended in 5 ml of SPG (10 mM NaH₂PO₄, 5 mM L-glutamic acid and 250 mM sucrose, pH 7.2) and stored at −80°C in small aliquots.

For the production of 35S-labelled bacteria, L-929 cells propagated in 150 cm² tissue culture flasks (Corning Glass Works) were infected for 1 h at 37°C. Medium was removed and replaced with RPMI-1640. At 10 hpi, the medium was replaced with methionine-free medium supplemented with 10% dialysed serum, and 100 μg ml⁻¹ cycloheximide (Sigma) was added. At 12 hpi, 100 μCi [35S]-Translabel (ICN) was added. At 36–44 hpi, the cells were scraped from the flask, pelleted at 4000 g at 4°C for 15 min and sonicated for 3 × 15 s. The lysate was centrifuged at 3000 r.p.m. for 10 min at 4°C in a Sorval RT600B, and the supernatant was then pelleted at 23 300 g. The pellet was resuspended in 800 μl of SPG and layered on top of 1.5 ml of 30% Renografin (Squibb Diagnostics) in SPG. This was centrifuged at 37 250 g in a Beckman SW55Ti rotor for 1 h at 4°C. The pellet was again resuspended in 800 μl of SPG and layered on a step gradient consisting of 300 μl of 54% renografin, 450 μl of 45% renografin and 750 μl of 40% renografin in SPG. After centrifugation at 64 250 g for 1 h at 4°C, the pellet was resuspended in 1 ml of SPG and stored at −80°C in small aliquots.

Evaluation and quantification of abnormal vacuole morphology
CHO-K1 cells were infected with C. trachomatis for 1 h, and non-adherent bacteria were removed by washing. Media containing heparin (0.5 mg ml⁻¹) and either Cs or FB1 was added to slips and wells. After 24 h, cells on coverslips were stained with the Chlamydia detection kit. For all experiments, the number of progeny are expressed relative to that of untreated cells. All experiments were performed in triplicate, and a minimum of 300 cells was counted per sample.

Quantification of progeny from inhibitor titrations
CHO-K1, SPB-1 and SPB-1/cLCB-1 cells were incubated at 32°C or 39°C for 3 days in low-serum medium. The cells were washed three times with cold, sterile PBS. 35S-labelled bacteria diluted in 200 μl of SPG were added, and the cells were incubated at 4°C for 1 h with slow agitation to allow the bacteria to bind to the cells. To measure non-specific binding, heparin (0.5 mg ml⁻¹) was added to parallel samples (Zhang and Stephens, 1992). Cells were washed three times with cold PBS to remove unattached bacteria and then lysed with 1% SDS. The lysate was transferred to vials containing scintillation fluid, and the wells were washed once with lysis buffer. The wash was added to the lysate, and the amount of 35S in the samples was determined using a Beckman L-1801 scintillation counter.

C. trachomatis binding assay
CHO-K1, SPB-1 and SPB-1/cLCB-1 cells were incubated at 32°C or 39°C for 3 days in low-serum medium. The cells were washed three times with cold, sterile PBS. 35S-labelled bacteria diluted in 200 μl of SPG were added, and the cells were incubated at 4°C for 1 h with slow agitation to allow the bacteria to bind to the cells. To measure non-specific binding, heparin (0.5 mg ml⁻¹) was added to parallel samples (Zhang and Stephens, 1992). Cells were washed three times with cold PBS to remove unattached bacteria and then lysed with 1% SDS. The lysate was transferred to vials containing scintillation fluid, and the wells were washed once with lysis buffer. The wash was added to the lysate, and the amount of 35S in the samples was determined using a Beckman L-1801 scintillation counter.

One of the investigators was blinded with regard to the identity of the sample. The ratio of abnormal to normal
vacuoles did not differ by more than 10% between the two investigators.

Statistical analysis

Statistical significance was determined by the Student's two-tailed t-test. Experiments were carried out in triplicate, and the means ± SEM are shown.

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References

Badiani, K., Byers, D.M., Cook, H.W., and Ridgway, N.D. (1996) Effect of fumonisin B1 on phosphatidylethanolamine biosynthesis in Chinese hamster ovary cells. Biochim Biophys Acta 1304: 190–196.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can J Biochem Biophys 37: 911–917.

Braunwald, E. (1998) Harrison's Principals of Internal Medicine, 14th edn. Braunwald, E. (ed.). New York: McGraw-Hill Book Company.

Brenchley, M., and Clapham, D.E. (1999) Ceramide as a modulator of endocytosis. J Biol Chem 270: 13291–13297.

Hackstadt, T., Scidmore, M., and Rockey, D. (1995) Lipid metabolism in Chlamydia trachomatis-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. Proc Natl Acad Sci USA 92: 4877–4881.

Hatch, G.M., and McClarty, G. (1998) Phospholipid composition of purified Chlamydia trachomatis mimics that of the eukaryotic cell. Inf. Immun. 66: 3727–3735.

van Helvoort, A., Smith, A.J., Spong, H., Fritzsche, I., Schinkel, A.H.P., and van Meer, G. (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87: 507–517.

Merrill, A.H., Wang, E., Gilchrist, D.G., and Riley, R.T. (1993) Fumonisins and other inhibitors of de novo sphingolipid biosynthesis. Adv Lipid Res 26: 215–234.

Moulder, J.W. (1962) The Biochemistry of Intracellular Parasitism. Moulder, J.W. (ed.). Chicago: The University of Chicago Press, pp. 48–70.

van Ooij, C., Apodaca, G., and Engel, J. (1997) Characterization of the Chlamydia trachomatis vacuole and its interaction with the host endocytic pathway in HeLa cells. Infect Immun 65: 758–766.

van Ooij, C., Homola, E., Kincaid, E., and Engel, J. (1998) Fusion of vacuoles containing Chlamydia trachomatis is inhibited at low temperature and requires bacterial protein synthesis. Infect Immun 66: 5364–5371.

Perera, E., Yen, T.S., and Ganem, D. (1990) Growth of Chlamydia trachomatis in enucleated cells. Infect Immun 58: 3816–3818.

Raggers, R.J., van Helvoort, A., Evers, R., and van Meer, G. (1999) The human multidrug resistance protein MRPI translocates sphingolipid analogs across the plasma. J Cell Sci 112: 415–422.

Ridgway, N.D. (1995) Inhibition of acyl-CoA: cholesterol acyltransferase in Chinese hamster ovary (CHO) cells by short-chain ceramide and dihydroceramide. Biochim Biophys Acta 1256: 39–46.

Rosenwald, A.G., and Pagano, R.E. (1993) Inhibition of acyl-CoA: cholesterol acyltransferase in Chinese hamster ovary (CHO) cells by short-chain ceramide and dihydroceramide. Biochim Biophys Acta 1256: 39–46.

van Ooij, C., Homola, E., Kincaid, E., and Engel, J. (1998) Fusion of vacuoles containing Chlamydia trachomatis is inhibited at low temperature and requires bacterial protein synthesis. Infect Immun 66: 5364–5371.

Perera, E., Yen, T.S., and Ganem, D. (1990) Growth of Chlamydia trachomatis in enucleated cells. Infect Immun 58: 3816–3818.

Raggers, R.J., van Helvoort, A., Evers, R., and van Meer, G. (1999) The human multidrug resistance protein MRPI translates sphingolipid analogs across the plasma. J Cell Sci 112: 415–422.

Ridgway, N.D. (1995) Inhibition of acyl-CoA: cholesterol acyltransferase in Chinese hamster ovary (CHO) cells by short-chain ceramide and dihydroceramide. Biochim Biophys Acta 1256: 39–46.

Rosenwald, A.G., and Pagano, R.E. (1993) Inhibition of acyl-CoA: cholesterol acyltransferase in Chinese hamster ovary (CHO) cells by short-chain ceramide and dihydroceramide. Biochim Biophys Acta 1256: 39–46.

Schachter, J., and Dawson, C.R. (1990) The epidemiology of trachoma predicts more blindness in the future. Scand J Infect Dis 69: 55–62.

Schroeder, J.J., Crane, H.M., Xia, J., Liotta, D.C., and Merrill, A.H.J. (1994) Disruption of sphingolipid metabolism and stimulation of DNA synthesis by fumonisin B1: a molecular mechanism for carcinogenesis associated with Fusarium moniliforme. J Biol Chem 269: 3475–3481.

Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (1996) Sphingolipids and glycoproteins are differentially trafficked to the Chlamydia trachomatis inclusion. J Cell Biol 134: 363–374.

Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. Nature 387: 569–572.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., et al. (1998) Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282: 754–759.

Tolan, D., Conway, A.M., Pyne, N.J., and Pyne, S. (1997) Sphingosine prevents diacylglycerol signaling to mitogen-
activated protein kinase in airway smooth muscle. *Am J Physiol* **273** (3 Pt 1): C928–C936.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., and Merrill, A.H. (1991) Inhibition of sphingolipid biosynthesis by fumonisins: implications for diseases associated with *Fusarium moniliforme*. *J Biol Chem* **266**: 14486–14490.

Yoo, H.-S., Norred, W.P., Wang, E., Merrill, A.H., and Riley, R.T. (1992) Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity are correlated in LLC-PK1 cells. *Toxicol Appl Pharm* **114**: 9–15.

Zhang, J.P., and Stephens, R.S. (1992) Mechanism of *Chlamydia trachomatis* attachment to eukaryotic host cells. *Cell* **69**: 861–869.