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Analysis of Hydrolytic Products from Choline-Labeled Host Cell Phospholipids during Growth of *Rickettsia prowazekii*

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A phospholipase activity has been associated with the interaction of *Rickettsia prowazekii* with the surface of erythrocytes and competent host cells as well as during the growth of the rickettsiae within their host cells. Both fatty acid and lysophosphatides have been found in the interaction of rickettsiae with the surface of eucaryotic cells; this finding provided strong evidence for the activity of a phospholipase A. However, fatty acids, but not lysophosphatides, were found during the growth of rickettsiae within cells in which the phospholipids had been radiolabeled with oleic acid; this observation left the type of phospholipase activity in doubt. In this study, the water-soluble components of phospholipid hydrolysis by phospholipase A plus lysophospholipase and phospholipase C were determined following the growth of rickettsiae in host cells in which the phospholipids had been radiolabeled with choline. In infected cells relative to mock-infected cells, there was a loss of phosphatidylcholine with a corresponding increase not in lysophosphatidylcholine but in the water-soluble components. There was a large increase in glycerylphosphorylcholine (185%) and a smaller increase in phosphorylcholine (16%). These results indicate that both phospholipase A activity (plus a lysophospholipase activity) and phospholipase C were increased during infection by *R. prowazekii* and that the former was the predominant activity.

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate intracellular bacterium with a typical gram-negative bacterial morphology (10, 12). To the best of our knowledge, these organisms have no exotoxins that are important in the diseases that they cause. They are virulent because they grow to fill the cytoplasm and, in doing so, destroy their host cells. Rickettsiae are most unusual bacteria in that they can grow only within the cytoplasm of eucaryotic cells unbounded by either a phagosome or a phagolysosome. To enter its host cell, the rickettsia induces phagocytosis (8), but how does it get out of the phagosome into the cytoplasm? Then, after the parasites have filled the cytoplasm, how do they get out of that cell to begin a new cycle of infection? We have presented evidence that the answers to both questions may involve a phospholipase (13, 16).

Fatty acids and lysophosphatides, the products of phospholipase A activity, are formed when rickettsiae interact with erythrocytes or cytochalasin B-treated L929 cells: the rickettsiae can attach to the cell but remain on the eucaryotic cell membrane, unable to be internalized (11, 15, 16). Even without cytochalasin treatment, if the number of rickettsiae associated with the host cell membrane exceeds the capacity of the host cell to internalize them, host cells phospholipids are hydrolyzed with the formation of fatty acid (oleic acid) and lysophosphatides (16). There is no entry of rickettsiae, no cytotoxicity, no hemolysis, and no phospholipid hydrolysis when the rickettsiae are metabolically inactivated by a variety of conditions. To explain the entry of rickettsiae into the cytoplasm, we hypothesized (17) that following the adherence of a rickettsia to the host cell membrane, a phospholipase A activity ensues and continues as long as the rickettsia is associated with this membrane. The host cell responds to the degradation of the phospholipids of its plasma membrane by internalizing the damaged patch of membrane and internalizes, pari passu, the attached rickettsia. Since the phospholipase activity has continued during these phagocytic events, shortly after the phagosome is completed, the rickettsia escapes through the membrane lesion of the phagosome and is in the cytoplasm.

After the rickettsiae are internalized, phospholipase activity is again seen (13). The phospholipid hydrolysis caused by the exponentially growing intracytoplasmic rickettsiae eventually exceeds the capacity of the host cell for repair and leads to destruction of the host cell, a destruction that is again accompanied by the formation of radiolabeled oleic acid derived from the phospholipids of the host cell (13). This hydrolysis of phospholipid is certainly part of the cellular pathogenicity of the rickettsiae and may well be the mechanism of escape from the exhausted host cell. In this system in which normal rickettsial growth is taking place, the type of phospholipase activity responsible for the damage to the host cell has not been demonstrated. However, a phospholipase A was suggested since radiolabeled oleic acid was formed from radiolabeled host cell phospholipids. However, the suggestion has also been made, on the basis of Western blotting (immunoblotting) of rickettsial protein with antibodies to eucaryotic phospholipases, that rickettsiae have even more cross-reactive phospholipase C than phospholipase A_2 (4). In addition, we have never found an infection-associated increase in lysophospha-

TABLE 1. Distribution of radioactivity

Phase	Radioactivity (%) in:		*P* value
	Mock-infected culture	Infected culture	
Medium			
Aqueous	7 ± 2	13 ± 3	<0.02
Organic	7 ± 1	8 ± 1	<0.76
Monolayer			
Aqueous	3 ± 2	3 ± 2	<0.10
Organic	83 ± 2	76 ± 3	<0.02

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TABLE 2. Analysis of the monolayer organic phase

Compound	Radioactivity (10^3 cpm) in:		*P* value
	Mock-infected culture	Infected culture	
LPC	16 ± 5	19 ± 6	<0.082
Sphingomyelin	149 ± 47	131 ± 46	<0.109
PC	234 ± 49	183 ± 55	<0.016

tides in these host cells, cells in which we could clearly demonstrate an increased formation of fatty acid. These observations raised the interesting question of whether the breakdown of the host cell phospholipids during growth of the rickettsiae was due to phospholipase A or phospholipase C or both. In this study, the phospholipids of the host cell were radiolabeled with choline so that the formation of water-soluble products from phosphatides and lysophosphatides could be analyzed. From a phosphatidylcholine (PC) substrate, glycerylphosphorylcholine (GPC) would be the water-soluble product formed from the breakdown of lysophosphatidylcholine (LPC) that together with fatty acids are the products of phospholipase A activity, and phosphorylcholine (CP) would be formed along with diglycerides as the products of phospholipase C activity.

The rickettsial inoculum was prepared from *R. prowazekii* Madrid E (yolk sac passage 281) grown in the yolk sacs of antibiotic-free hen eggs (SPAFAS, Inc., Roanoke, Ill.) and was purified and stored as previously described (7). The numbers of viable rickettsiae were estimated by their hemolytic activity (9). The extent of the rickettsial infection was monitored by removing coverslips from the dishes and staining by the method of Gimenez (3). Typically in the initial infection, 95% of the cells were infected and there were five rickettsiae per infected cell.

Mouse L929 cells were cultured in Eagle minimum essential medium supplemented with 10% heat-treated (56°C for 30 min) newborn calf serum in a humidified 5% CO_2 incubator at 34°C. Cell suspensions were prepared and adjusted to a density of 2.5×10^5 cells per ml. [*methyl*-^{14}C]choline chloride (2 to 3 μCi; ICN, Irvine, Calif.) was added to 7 ml of the cell suspension, and 1-ml samples were plated into six 35-mm-diameter dishes. Samples of the unlabeled suspension were prepared for enumeration of the rickettsiae and determination of the cell viability. To allow labeling of the host cell's PC, the cells were incubated with radiolabel for 48 h, and the medium was removed and replaced for up to 24 h with 1 ml of Iscove modified Dulbecco medium, supplemented as previously described (14), per dish. The cells were mock infected or infected with rickettsiae at a ratio of 85 viable organisms per L929 cell as previously described (14). The infected and mock-infected cells were incubated for 30 to 48 h, at which time cell viability and rickettsial growth were determined, and the medium and monolayer were extracted by the method of Bligh and Dyer (1) to assess phospholipid hydrolysis. The radioactive water-soluble components (GPC, CP, and choline) were separated by Dowex-50 (200/400 mesh, H^+ form) column chromatography (2). The column separations were verified by thin-layer chromatography (5). The composition of the organic phases of the monolayers of mock-infected and infected samples was determined by thin-layer chromatography. The dried sample was redissolved in 55 μl of $CHCl_3$, 5 μl was counted, 25 μg of each standard (LPC, sphingomyelin, and PC) was added, and the mixture was spotted on an acetone-washed and air-dried silica gel plate. The mobile phase was $CHCl_3$-CH_3OH-CH_3COOH-H_2O (55:35:5:5). The spots were visualized in I_2 vapor, marked, scraped, and counted by liquid scintillation techniques.

After incubation of infected and mock-infected cultures that had been labeled with choline, the distribution of radioactivity into organic and aqueous phases from the medium and the monolayer was determined (Table 1). The majority of the labeled choline remained with the phospholipids in both cultures, but there was a significant decrease, from 83 to 76%, in these counts in the infected cultures. Accordingly, there was a significant increase in the radioactivity appearing in the aqueous phase from the infected cultures relative to the mock-infected cultures, 13 versus 7%. There was no significant difference between the infected and mock-infected cultures in the radioactivity present in the aqueous phase from the monolayer and the organic phase from the medium.

Analysis of the composition of the organic phase from the infected and mock-infected monolayers by thin-layer chromatography showed that radioactive PC, LPC, and sphingomyelin were present (Table 2). The decrease in radioactivity seen in this phase in the infected cultures was due to the loss of PC. There was no significant change in the other two components. The lack of a significant increase in LPC was supported by the inability to find an increase in lysophosphatides in infected cultures when the cultures were labeled with either oleic acid or P_i (data not shown). Furthermore, the loss of PC in these infected cultures was supported by a decrease in the total lipid phosphorus in infected cultures relative to mock-infected cultures in other experiments (data not shown).

The principal purpose of this study was to examine the water-soluble products of phospholipid hydrolysis. Table 3 shows the results from four experiments, with each experiment having triplicate independent determinations for mock-infected and infected cultures. In all four experiments, there was a significant increase in the GPC appearing in the aqueous phase of the extracted medium, ranging from 86 to 272%. The increase in CP in these extracts from the infected cultures was more modest, ranging from 8 to 33%, and in only one of the four experiments was the increase significant at the $P = 0.05$ level. However, when all 12 determinations were analyzed in a

TABLE 3. Analysis of the medium aqueous phase

Expt	GPC				CP			
	Mock infected (10^3 cpm)	Infected (10^3 cpm)	% Increase	*P* value	Mock infected (10^3 cpm)	Infected (10^3 cpm)	% Increase	*P* value
1	5.6 ± 0.3	10.5 ± 0.6	86	<0.001	46.7 ± 0.9	53.0 ± 10.9	14	<0.370
2	9.6 ± 1.0	27.1 ± 8.5	182	<0.024	35.8 ± 5.0	41.9 ± 6.6	17	<0.067
3	5.4 ± 1.9	19.9 ± 1.3	272	<0.001	15.3 ± 2.4	20.2 ± 1.6	33	<0.038
4	13.5 ± 1.2	43.0 ± 3.9	218	<0.001	30.1 ± 1.4	32.5 ± 1.3	8	<0.149
All	8.1 ± 3.5	23.0 ± 11.7	185	<0.001	32.1 ± 12.8	37.3 ± 14.9	16	<0.034

pairwise comparison, a significant increase of 16% in the formation of CP was seen. Although the percent increase in the formation of CP is small relative to the increase in GPC, because of the high level of CP formation in the mock-infected cultures, the increase in the amount of CP formed is one-third of that of GPC.

These results extend the role of phospholipase A activity from the association of *R. prowazekii* with host cells and erythrocytes (11, 15, 16), a role that appears to be involved in the entrance of rickettsiae, to the growth of rickettsiae within their host cells, a role that appears to be involved in the pathogenesis of rickettsial infections and in the exit of rickettsiae from their host cells (13). When host cell phospholipids are labeled with oleic acid, increased oleic acid, but not increased lysophosphatides, is found as a product of hydrolysis in infected cultures relative to mock-infected control cultures. When host cell PC is labeled with choline, increased GPC is found as a product of hydrolysis in infected cultures. These results indicate that during rickettsial growth within their host cells, lysophosphatides are indeed formed concomitantly with fatty acid, but that there is a lysophospholipase, of either rickettsial or host cell origin, that hydrolyzes the lysophosphatide to fatty acid and GPC. Silverman et al. (6) have shown that pretreatment of *R. rickettsii*, but not of host cells, with bromophenacyl bromide (an inhibitor of phospholipase A_2) and antiserum to venom phospholipase A_2 will reduce the uptake of this organism and its ability to cause plaque formation. This finding suggests that the phospholipase A involved in rickettsia-host cell interactions is of rickettsial origin rather than a latent host cell phospholipase that is activated by rickettsiae. Whether the modest percent increase in phospholipase C activity indicated by the increased formation of CP in our study is due to a rickettsial phospholipase C or to the activation of the considerable phospholipase C activity seen in the uninfected cells remains to be determined.

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