INFECTION AND IMMUNITY, Oct. 1982, p. 109–113
0019-9567/82/100109-05$02.00/0

Vol. 38, No. 1

Phospholipase A and the Interaction of *Rickettsia prowazekii* and Mouse Fibroblasts (L-929 Cells)

HERBERT H. WINKLER* AND ELIZABETH T. MILLER

Laboratory of Molecular Biology, Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, Alabama 36688

Received 26 March 1982/Accepted 23 June 1982

L-929 cells were killed when approximately 50 viable *Rickettsia prowazekii* organisms per L-cell were centrifuged onto a monolayer. The glycerophospholipids of the L-cell were hydrolyzed to lysophosphatides and free fatty acids. Concomitantly, there was a loss of membrane integrity as shown by release of lactate dehydrogenase and ^{86}Rb and permeability to trypan blue dye. No glycerophospholipid hydrolysis or cytotoxicity occurred when the rickettsiae were inactivated by heat, UV irradiation, *N*-ethylmaleimide, or metabolic inhibitors before their addition to the L-929 cells. On the other hand, treatment of the L-929 cells with the cytoskeleton agents colchicine or cytochalasin B or with *N*-ethylmaleimide inhibited neither the phospholipase A activity nor the loss of membrane integrity. Cytochalasin B-treated cells could be damaged by even small numbers of rickettsiae. We suggest that this phospholipase A activity is used by the rickettsiae to escape from the phagosomes into the cytoplasm of host cells.

The genus *Rickettsia* differs from most obligate and facultative intracellular parasites in that rickettsiae grow in the cytoplasm of their hosts unbounded by phagosomal or phagolysosomal membranes. Walker and Winkler (13) demonstrated that successful entry of *Rickettsia prowazekii* into L-cells requires that the rickettsiae be metabolically active and that the host cells by capable of phagocytosis, even though they are not professional phagocytes. Similar findings were observed much earlier by Cohn et al. (4) working with *Rickettsia tsutsugamushi*. These observations suggest that rickettsiae induce their own phagocytosis by the host cells, but they leave unexplained the mechanism by which the rickettsiae escape from the phagosomes to the cytoplasm. The hemolysis of erythrocytes by rickettsiae has suggested a model in which hemolysis is an abortive attempt by the rickettsiae to parasitize eucaryotic cells that cannot be induced to phagocytize (18). Recently, we demonstrated (20) that the lysis of the erythrocyte membranes is due to the activity of a phospholipase A which hydrolyzes the phospholipids of the erythrocytes to the corresponding lysophosphatides and free fatty acids (FFA). Based on this model, small amounts of phospholipid hydrolysis would be expected during the normal entry of rickettsiae, and large amounts would be expected if phagocytosis is blocked or if supraoptimal ratios of rickettsiae to host cells are used. In this study and in a preliminary communication (21), we show that the last two predictions are borne out.

MATERIALS AND METHODS

Rickettsia preparation and growth. *R. prowazekii* Madrid E strain was propagated in embryonated, antibiotic-free hen eggs by inoculation with 0.2 ml of a seed pool (yolk sac passage no. 274 and 275). Rickettsial suspensions were prepared from heavily infected yolk sacs by a modification of the methods of Bovarnick and Snyder (3) and Wissemen et al. (24) as previously described (7). Only fresh unfrozen rickettsiae were used. The medium for the rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate solution originally devised by Bovarnick et al. (2).

Cytotoxicity methods. L-929 cells were grown in 35-mm dishes to be confluent on the day of the experiment. The culture medium was Eagle minimum essential medium supplemented with 10% calf serum without any antibiotics. L-929 cells were labeled with [1-^{14}C]oleic acid (0.25 μCi per dish) for 8 h on the day before the experiment and then were reincubated without the label overnight. Rickettsiae were added to the dishes in 1.5 ml of sucrose-phosphate-glutamate containing 10 mM $MgCl_2$ and 0.1% glucose. Subsequent incubations were also carried out in this solution. Multiplicities of infection were based on metabolically active (viable) rickettsiae as determined by the antibody-hemolysis method of Walker and Winkler (14). The dishes were centrifuged 15 min at 400 × *g* at 37 or 2°C to bring the rickettsiae into association with the L-929 cells. L-929 cell damage was assessed by the release of lactate dehydrogenase (LDH) from the cells, by increased permeability to trypan blue dye, or by the

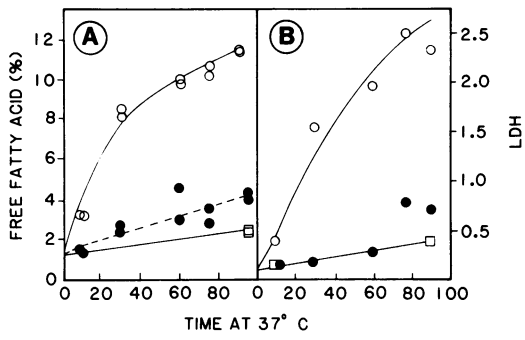

FIG. 1. Time courses for release of FFA (A) and LDH (B) at 37°C. Rickettsiae were centrifuged onto L-929 cells at 2°C at multiplicities of 0 (□), 10 (●), or 100 (○). LDH is measured in μmol of NADH per min per 10^6 L-929 cells; FFA is measured in percentage of total labeled phospholipid hydrolyzed.

release of ^{86}Rb from the cells. Fatty acid hydrolysis was measured by determining the percentage of radioactive FFA relative to the total labeled phospholipids after separation of these species by thin-layer chromatography.

Lipid methodology. Lipids were extracted by essentially the method of Bligh and Dyer (1) as previously described (19). To each 1 ml of aqueous sample to be extracted were added 0.3 ml of EDTA (100 mM, pH 4.3), 1.6 ml of chloroform, and 3.2 ml of methanol. This monophasic system was gassed with nitrogen, and the extraction was continued overnight at 4°C. The lipids were separated from the water-soluble material by adding 1.6 ml each of chloroform and water per ml of the original suspension and centrifuging it to separate the phases. The chloroform layer was evaporated to dryness with a vacuum evaporator, dissolved in chloroform-methanol (2:1), and stored at −20°C under nitrogen.

A two-dimensional chromatographic separation of phospholipids was performed with Silica Gel SG-60 thin-layer plates heated for 1 h at 110°C. The solvents used by Rouser et al. (11) were employed: solvent 1 consisted of chloroform–methanol–28% ammonia (65:25:5), and solvent 2 consisted of chloroform–acetone–methanol–acetic acid-water (6:8:2:2:1). FFA were separated from the other components of the extract by one-dimensional thin-layer chromatography. The solvent was petroleum ether-diethyl ether-acetic acid (80:20:1). Localization was accomplished with rhodamine 6G (0.012% in water). The phospholipid and fatty acid spots were scraped from the plate and counted by liquid scintillation techniques.

RESULTS

Time course of hydrolysis of phospholipids and cell lysis. Centrifugation of *R. prowazekii* onto monolayers of L-929 cells which had had their phospholipids labeled with [1-^{14}C]oleic acid resulted in the hydrolysis of these phospholipids and the formation of labeled free oleic acid (Fig. 1A). In these experiments, rickettsiae were centrifuged onto the monolayers at 2°C, and then the medium was removed and replaced with medium at 37°C. A time course could be generated by this temperature shift method without the uncertainty about when hydrolysis started that existed when the rickettsiae were centrifuged onto the monolayer at 37°C. There was little formation of [^{14}C]oleic acid from the labeled phospholipids if rickettsiae were absent or if the temperature was maintained at 2°C throughout the incubation period.

The release of LDH into the medium indicated marked damage to the membranes of the L-929 cells (Fig. 1B). Further confirmation of cytotoxicity was established by observing an increase in the percentage of cells which were stained by trypan blue dye and by observing a rapid loss of ^{86}Rb from cells labeled with this potassium analog (data not shown).

Centrifugation of the rickettsiae onto the L-cell monolayer was not necessary for hydrolysis of the L-cell phospholipids and release of LDH into the medium to occur. These phenomena occurred both in monolayers without centrifugation (Table 1) and in a suspension system (data not shown), but in these cases many more rickettsiae were required for FFA formation and cytotoxicity to be observed.

As with hemolysis (20), a phospholipase A activity was responsible for the lysis of the host cell membrane phospholipids as evidenced by the formation of both FFA and lysophosphatides. Rickettsial lysis of $^{32}PO_4$-labeled L-929 cells resulted in about a 25% conversion of both labeled phosphatidylcholine and phosphatidylethanolamine to their lysophosphatide derivatives (Table 2).

TABLE 1. Phospholipase A activity and cytotoxicity without centrifugation

Multiplicity of infection[a]	Release of:	
	LDH activity[b]	FFA[c]
0	0.1 ± 0.1	0.3 ± 0.0
50	0.2 ± 0.1	0.4 ± 0.1
100	0.3 ± 0.1	0.4 ± 0.1
250	0.6 ± 0.1	0.6 ± 0.1
500	1.2 ± 0.2	0.9 ± 0.0
1,000	3.0 ± 0.3	2.2 ± 0.1
2,000	6.4 ± 0.7	5.1 ± 0.5

[a] The indicated multiplicity of rickettsiae in a total volume of 1.5 ml was added to a confluent monolayer of L-929 cells in a 35-mm dish and incubated at 37°C for 60 min.

[b] Measured in μmol of NADH per min per 10^6 cells. Values show mean ± standard deviation; $n = 3$.

[c] [^{14}C]oleic acid released from labeled phospholipid as percentage of total lipid radioactivity. Values show mean ± standard deviation; $n = 3$.

TABLE 2. Formation of lysophosphatides in interaction of rickettsiae with L-cells

Expt[a]	PE + LPE[b]	% LPE[c]	PC + LPC[d]	% LPC[c]
Sham	9.5 ± 1.5	1 ± 1	7.7 ± 1.2	4 ± 2
Lytic	7.6 ± 0.7	24 ± 3	6.6 ± 1.0	27 ± 5

[a] Rickettsiae were centrifuged onto a monolayer of ^{32}P-labeled L-929 cells at a ratio of 100:1 and incubated at 37°C for 1 h. Phospholipids were extracted and separated by thin-layer chromatography, and the individual spots were scraped and counted. In the sham experiment, the rickettsiae were added to the extracted L-929 cells. The mean and standard deviations for three experiments are shown in all cases.

[b] The sum of the radioactivities in the spots for phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) was normalized to the radioactivity in the sphingomyelin spot, which was set equal to 1.

[c] Percentage of the radioactivity in the sum contributed by the lysophosphatide.

[d] Sum of radioactivities of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) computed as in footnote *b* above.

In the absence of defatted serum albumin or whole serum in the medium, the labeled FFA which was formed was extractable from the monolayer. However, when 1% albumin or 10% whole normal rabbit serum was present during the lysis period, the majority of the labeled FFA was now in the medium, presumably complexed to the albumin (data not shown). The labeling protocol resulted in the incorporation of oleic acid not only into phospholipids but also into di- and triglycerides. The di- and triglyceride regions of the chromatograms each had about 4% of the total oleic acid incorporated. No hydrolysis of fatty acid from either of these neutral lipids was observed after the interaction with rickettsiae.

Effects of inhibitors. Treatments that inactivate the rickettsiae with respect to hemolysis (9), induced phagocytosis (13), or lysine uptake (12) also prevented lysis of the cells of the monolayer and hydrolysis of labeled phospholipids (Table 3). The effects of the alkylating agent *N*-ethylmaleimide, heat, and UV irradiation could be assigned unambiguously to the rickettsiae since the L-929 cells never were exposed to these treatments. The reversibility of the effects of NaF and KCN required that these compounds remain in the incubation mixture. Thus, both the rickettsiae and L-929 cells were possible targets for these inhibitors. However, under the conditons employed, the target of these compounds was more likely to be the rickettsiae. KCN at 1 mM has little effect on L-929 cellular metabolism but a pronounced effect on rickettsial metabolism (13). NaF treatment at 2 mM instead of 40 mM (as in Table 3) resulted in submaximal inhibition. Although preincubation of L-929 cells for 15 min with NaF (2 mM) did not increase the inhibition, preincubation of the rickettsiae with NaF at this concentration did markedly increase the inhibition of both FFA formation and LDH release (data not shown).

In contrast to the inhibitory effects of agents directed against the rickettsiae, treatment of L-929 cells with *N*-ethylmaleimide or cytochalasin B (CB) stimulated both LDH and FFA release in the presence of rickettsiae. Colchicine was without significant effect. None of these compounds had any effect in the absence of rickettsiae. We have not yet found a treatment of L-929 cells that inhibits lysis without destroying the monolayers.

Effect of the ratio of rickettsiae to L-929 cells. There was a highly reproducible sigmoidicity in the plot showing the effect of the number of rickettsiae interacting with an L-929 cell (Fig. 2). The hydrolysis of phospholipid (FFA formation) increased markedly as the ratio of rickettsiae to L-929 cells (multiplicity) was increased beyond 20. However, when CB-treated L-929 cells that are unable to phagocytize rickettsiae were used, the formation of labeled FFA increased at lower multiplicities so that the sigmoidicity of the curve was abolished. Other experiments demonstrated that the amounts of FFA formed from CB-treated and untreated cells became equivalent at higher multiplicities and that the maxi-

TABLE 3. Effect of inhibitors on cytotoxicity and phospholipase A activity

Inhibitor[a]	Treatment administered to:	LDH release[b]	FFA formation[b]
None		100	100
N-Ethylmaleimide	Rickettsiae	3	2
N-Ethylmaleimide	L-cells	285	268
Heat (56°C)	Rickettsiae	4	4
UV irradiation	Rickettsiae	4	2
CB	Both	261	290
KCN	Both	2	2
NaF	Both	1	3
Colchicine	Both	120	101

[a] *N*-Ethylmaleimide (1 mM) was added to either rickettsiae or L-cells for 10 min and was neutralized with β-mercaptoethanol (2 mM) before these cells were combined. Rickettsiae were heated at 56°C for 30 min or were exposed to UV light for 10 min at 12 cm. CB (10 μg/ml), KCN (1 mM), NaF (40 mM), or colchicine (5 μM) were added to both rickettsiae and L-cells.

[b] Figures show percentages of the uninhibited control. These data are from seven experiments in which control LDH was 3.0 ± 1.3 μmol of NADH per min per 10^6 cells and the control percentage [^{14}C]oleic acid hydrolyzed from labeled phospholipid was 9.3 ± 4.8.

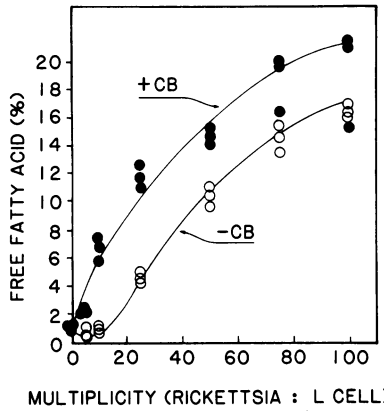

FIG. 2. Effect of rickettsial multiplicity on FFA release. Rickettsiae were centrifuged onto L-929 cells with (●) and without (○) CB and incubated an additional 45 min at 37°C. CB (10 μg/ml) was present throughout the incubation where indicated.

mum amount of hydrolysis of labeled phospholipids was about 25%.

DISCUSSION

R. prowazekii interacts with the cell membranes of eucaryotic cells in the processes of hemolysis, induction of phagocytosis, entry into cytoplasm from the phagosomes, and egress from host cells. The first of these processes (hemolysis) is the only one whose basic mechanisms are reasonably well understood (8–10, 16–18, 20, 22). This is in part because rickettsiae are so incredibly efficient at lysing erythrocytes: given enough erythrocytes, every viable rickettsia will lyse several cells. The comparative simplicity of erythrocytes and the technical ease of measuring hemoglobin release have also played a role in the elucidation of this process. However, hemolysis itself may have little or no role in the pathogenesis of rickettsial diseases or in the interactions of rickettsiae with potential hosts in vivo. Thus, the two cardinal aspects of the rickettsia-erythrocyte interaction (the essentially irreversible attachment step and the hydrolysis of the phospholipids of erythrocyte membranes by phospholipase A) could represent activities that the rickettsiae have evolved for other, relevant, purposes.

In this study, we have found that the phospholipase A activity can also be observed in the interaction of rickettsiae and competent host cells. We hypothesize that this activity may be the basis of one or more of the following: the induction of phagocytosis by rickettsiae, the lysis of and escape from phagosomes by rickettsiae, and the rupture of the host cell membrane at the termination of infection of one cell, allowing the egress of rickettsiae to infect other cells. The phenomenon of mouse toxicity, in which the injection of large concentrations of metabolically active typhus rickettsiae rapidly causes the death of mice without infection by or growth of these rickettsiae (25), had been suggested over a decade ago to be related to hemolysis and cell penetration (6, 23). The phospholipase activity may represent the common denominator. Similarly, the unexplained toxicity of large numbers of rickettsiae for rabbit polymorphonuclear leukocytes (15) could also be due to this phospholipase A activity since the products of glycerophospholipid hydrolysis have been identified in this system.

Although a direct causal relationship between the hydrolysis of membrane phospholipid and cytotoxicity is impossible to prove in a system in which the interaction of intact cells is studied, such a relationship is highly likely. Within the limits of our precision, the time courses were similar, and the effects of both inhibitors and stimulators were identical on both FFA formation and LDH release. Nevertheless, one cannot definitely refute arguments in favor of a more complex system in which the rickettsiae caused either unexplored perturbations of the cells, which led to activation of a phospholipase (and perhaps other effects), or simultaneous increases in permeability and FFA formation effected by different mechanisms.

When relatively few (<10) rickettsiae impinged upon on an untreated L-929 cell, there was no demonstrable cytotoxicity; no FFA formation or LDH release could be detected. Presumably, the damage caused by the internalization of these rickettsiae was nominal and could be repaired rapidly. On the other hand, L-929 cells which were unable to internalize rickettsiae after treatment with CB were damaged by small numbers of rickettsiae which presumably remained on the outside of the membrane and were actively attempting to enter these cells. Thus, the interaction of rickettsiae with these inhibited cells is very similar to that with erythrocytes, in which rickettsiae cannot be internalized and a single rickettsia can lyse the cell. For cytotoxicity to occur in a fully competent host cell, the phagocytic capacity of that cell must be overwhelmed by the large number of rickettsiae attempting to enter it.

The immediate toxic effect on L-cells of the obligate intracellular parasitic bacterium *Chlamydia psittaci* as described by Moulder et al. (5), differs markedly from the toxicity seen with *R. prowazekii* in that internalization of the chlamydiae is necessary for toxicity to occur. This difference could be due to the different niches occupied by these two intracellular parasites. The chlamydiae remain within their phagosomes

and modify these membranes so that lysosomal fusion does not occur, whereas the rickettsiae must escape from their phagosomes. Thus, the chlamydiae have no reason to express a membranolytic phospholipase A activity during their induction of phagocytosis.

The rickettsiae must be metabolically active for cytotoxicity to occur. A variety of agents rendered the organisms unable to damage the host cell membrane. Since the reaction catalyzed by phospholipase A is exergonic, this metabolic requirement may involve the attachment of the rickettsiae to the host cell membrane or the presentation or activation of the enzyme. This requirement for rickettsial metabolic integrity makes fractionation of rickettsial lysates and characterization of the phospholipase A activity difficult. The question remains open whether rickettsiae actually have their own phospholipase A or activate a latent host cell enzyme. Both alternatives appear theoretically reasonable and can be reconciled with the existing data.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-15035 from the National Institute of Allergy and Infectious Diseases.

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