

Effect of Gamma Interferon on Phospholipid Hydrolysis and Fatty Acid Incorporation in L929 Cells Infected with *Rickettsia prowazekii*

HERBERT H. WINKLER,* LUZ DAY, ROBIN DAUGHERTY, AND JENIFER TURCO

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

Received 17 February 1993/Accepted 27 May 1993

Treatment of *Rickettsia prowazekii*-infected L929 cells with gamma interferon (IFN- γ) immediately after infection altered the lipid metabolism of the host cells as determined by measurement of phospholipid hydrolysis and oleic acid incorporation into phospholipids and neutral lipids. At 48 h postinfection, there was increased phospholipid hydrolysis in infected cultures relative to mock-infected cultures and a further increase in radiolabeled phospholipid hydrolysis in IFN- γ -treated infected cultures. Oleic acid, the radiolabeled product of hydrolysis, was found in both the free fatty acid and neutral lipid fractions. None of the mock-infected cultures demonstrated increased hydrolysis of their radiolabeled phospholipids in response to treatment with IFN- γ . Most of the radiolabeled oleic acid incorporated into cultures in the interval between 24 and 48 h after infection and IFN- γ treatment was present in the phospholipid fraction. However, the neutral lipid fraction from the infected cultures that had been IFN- γ treated was labeled to a greater extent than that from the untreated cultures. Thin-layer chromatographic analysis of the neutral lipid fractions from both the hydrolysis and incorporation experiments demonstrated that most of the radiolabel was in triglycerides. The infected cultures at 30 h were intact as assessed by the exclusion of trypan blue, but at 48 h postinfection in the IFN- γ -treated infected cultures more than half of the cells were unable to exclude trypan blue. In no case did the mock-infected cells show substantial damage as a result of IFN- γ treatment.

Growth of the etiologic agent of epidemic typhus, the obligate intracytoplasmic bacterium *Rickettsia prowazekii*, is inhibited by gamma interferon (IFN- γ) in fibroblastic host cells (16, 18, 27). In addition, the combination of rickettsial infection and treatment with IFN- γ causes a cytotoxic response in host cells (13, 17). This cytotoxic response is very dramatic in RAW264 cells, macrophage-like cells, in which essentially all of the IFN- γ -treated cells, when infected with *R. prowazekii*, die within 4 h. In murine fibroblastic L929 cells, the cytotoxic response is less dramatic, with death being proportional to the load of rickettsiae and requiring days of rickettsial growth (19).

Phospholipase activity has been implicated in damage to, and ultimate destruction of, eucaryotic cells by *R. prowazekii* (24, 26). Erythrocytes from a variety of species, cells that cannot serve as competent host cells for rickettsiae, are rapidly lysed following attachment of *R. prowazekii* (23). This hemolysis is accompanied by the formation of fatty acids and lysophosphatides derived from host cell phospholipids, the products and substrate of phospholipase A activity (25). A similar phenomenon occurs when approximately 25 or more rickettsiae interact with cytochalasin B-treated L929 cells: a condition under which rickettsiae can attach to host cells but remain on the host cell membrane unable to be internalized (26). Even without cytochalasin treatment, L929 cells are rapidly lysed if a large number of rickettsiae associate with the host cell membrane and exceed the capacity of the host cells to internalize them (26). The membrane damage that occurs in these host cells, whether they are cytochalasin treated or not, is accompanied by hydrolysis of fatty acids from the host cells phospholipids.

Once the rickettsiae are internalized, growth of the organisms also leads to destruction of the host cells, a destruction that is accompanied by hydrolysis of fatty acids from the phospholipids of the host cells (24). Thus, the phospholipids of the host cells are a major target of damage caused by *R. prowazekii*.

IFN- α , IFN- β , and IFN- γ have been shown to have significant lipolytic and lipogenic actions when added to eucaryotic cells (2, 6-8, 11, 12, 28). These actions often have been characterized in specialized cells and are often transient, occurring in the first few hours after their addition and therefore are outside of the time frame necessary for the effects of rickettsiae plus IFN- γ on fibroblasts.

In this study we examined the effects of IFN- γ on the lipids of *R. prowazekii*-infected cells. IFN- γ treatment of the host cells augments the normal formation of fatty acids that results from the phospholipase activity associated with a rickettsial infection and, in addition, leads to an increase in the incorporation of these free fatty acids into triglycerides. In addition, greater host cell damage was observed in IFN- γ -treated infected cultures.

MATERIALS AND METHODS

Rickettsiae. The rickettsial inoculum was prepared from *R. prowazekii* Madrid E (yolk sac passage 281) grown in the yolk sacs of antibiotic-free chicken eggs (Truslow Farms, Chestertown, Md.) and purified and stored as previously described (15). The numbers of viable rickettsiae were estimated by their hemolytic activity (22). The extent of the rickettsial infection at zero time was monitored by removing coverslips from the dishes and staining by the method of Gimenez (9). Typically, 95% of the cells were infected and there were five rickettsiae per infected cell.

* Corresponding author.

Cell cultures. Mouse L929 cells were cultured in Eagle minimal essential medium supplemented with 10% heat-treated (56°C for 30 min) newborn bovine serum. The cells were grown as monolayers in a humidified atmosphere of 3% CO₂ in air at 34°C. To radiolabel host cell phospholipids, the cell suspension was adjusted to a density of 5.5×10^5 cells per ml, 0.25 μ Ci of [9,10-³H]oleic acid per ml was added to part of the cell suspension, and 1-ml samples were plated into 35-mm-diameter dishes. One-milliliter samples of the remaining suspension were aliquoted into 35-mm dishes either with sterile 13-mm glass coverslips to allow enumeration of the rickettsiae or without coverslips to allow determination of the viability of cells at the end of the experiment. After the cells were incubated for 6 h, the medium used was removed from each dish and replaced for overnight incubation with 1 ml of Iscove modified Dulbecco medium supplemented with 2 mM L-glutamine, 110 mg of sodium pyruvate per liter, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 4 g of glucose per liter, 10 mg of insulin per liter, 10 mg of human transferrin per liter, 10 μ M 2-aminoethanol, 50 μ M 2-mercaptoethanol, 0.01 μ M sodium selenite, 2 g of fatty acid-free bovine serum albumin per liter, and 4 mg of linoleic acid per liter (IMDM medium). The remainder of the experiment was conducted with this medium.

On the next day, the cells were mock infected or infected with rickettsiae at a ratio of 85 viable organisms per L929 cell in Hanks' balanced salt solution with 0.1% gelatin and 5 mM monopotassium glutamate (HBSSGG). After 1 h of incubation, the monolayers were washed three times with HBSSGG, 1.5 ml of IMDM medium was added to each dish, and the cells were incubated for an additional 2 h. At the end of the second hour, the media from the dishes that were to be treated with IFN- γ were pooled, 80 U of IFN- γ per ml was added, and this medium was redistributed at 1.5 ml per dish. Recombinant murine IFN- γ derived from *Escherichia coli* (1.9×10^7 U/mg) was generously provided by Genentech, Inc. (South San Francisco, Calif.). The IFN- γ was assayed to determine antiviral units as previously described (14, 20). Coverslips for 0 h (postinfection) were removed at this stage.

At the conclusion of the experiment, cells from the medium and trypsinized monolayers were combined and stained with trypan blue and both viable and dead cells were enumerated.

Lipid determinations. Three dishes of cells were used in every experiment, and their mean values were determined. To determine the extent of hydrolysis of phospholipids to free fatty acids, the lipids (from both the media and monolayers of the radioactive cultures) were extracted by the method of Bligh and Dyer (1). The resulting monophasic was gassed with N₂ gas and kept refrigerated overnight. The lipids were separated from the water-soluble materials by adding water and chloroform to the monophasic and centrifuging the mixture to separate the phases. The chloroform layer was evaporated to dryness under N₂ gas. This material was then redissolved in 200 μ l of chloroform, and the lipids were separated by elution from a 3-ml column of aminopropylsilane-bonded silica gel (J. T. Baker, Inc., Phillipsburg, N.J.). After the column was activated with 4 ml of hexane, the sample was applied and the neutral lipids were eluted with 4 ml of chloroform-2-propanol (2:1), the fatty acids were eluted with 4 ml of 2% acetic acid in diethyl ether, and the phospholipids were eluted with 4 ml of methanol. The eluents were collected in 20-ml glass scintillation vials, evaporated to dryness, and counted by liquid scintillation techniques.

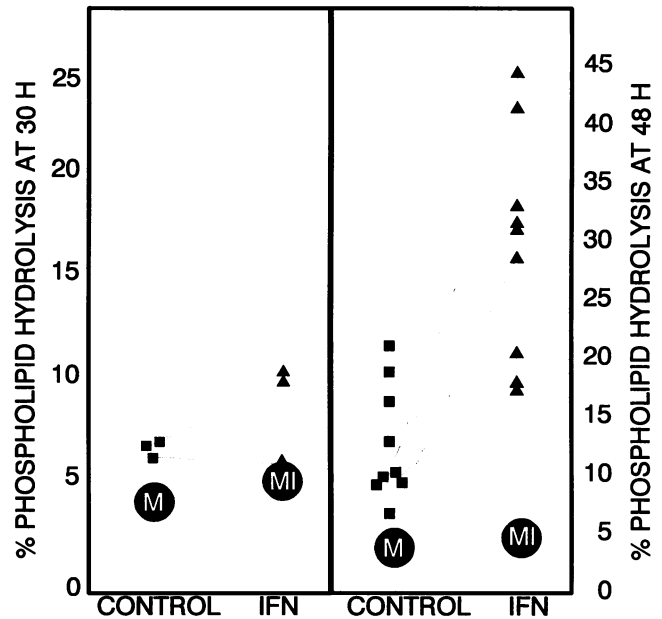


FIG. 1. Hydrolysis of phospholipids that had been radiolabeled with oleic acid. The hydrolysis of phospholipids 30 (left panel) and 48 (right panel) h postinfection for control (■) or IFN- γ -treated (▲) infected cultures. Each point represents the mean of three determinations for each experiment, and the mean values from the same experiment are connected by dotted lines. The averages of the mock-infected cultures are indicated by the circles labeled M and MI.

To determine the postinfection incorporation of radioactive oleic acid, the cells were plated, infected, and treated as described above except that no radioactive oleic acid was added prior to infection. At 24 h postinfection, the medium was removed and replaced with IMDM medium containing 0.25 μ Ci of [³H]oleic acid per ml; with or without IFN- γ , as appropriate. The cells were incubated for the indicated time, the medium was discarded and replaced with 1.5 ml of buffered saline, and extraction was conducted as described above.

Thin-layer chromatography of the neutral lipid fraction. The composition of the neutral lipid fraction was determined by thin-layer chromatography in some experiments. The sample was redissolved in 50 μ l of chloroform, 2.5 μ l of each standard at 10 mg/ml was added, and the mixture was spotted onto an acetone-washed and air-dried silica gel G plate. The standards used were tripalmitin, dipalmitin, oleic acid, and cholesterol oleate. The mobile phase was hexane-diethyl ether-formic acid (70:30:1). The plate was dried and sprayed with 0.1% rhodamine in water to visualize the spots. The spots were marked, scraped into vials, and counted by liquid scintillation techniques.

RESULTS

Hydrolysis of oleic acid-radiolabeled phospholipids. Hydrolysis of host cell phospholipids that had been radiolabeled before infection with the monounsaturated fatty acid oleic acid was measured in cultures at 30 and 48 h postinfection or post-mock infection (Fig. 1). In these experiments, IFN- γ , when added, was added 2 h after infection. If IFN- γ was added 24 h prior to infection, the growth of the rickettsiae was inhibited to such a large extent that no increase in

TABLE 1. Phospholipid hydrolysis at 48 h postinfection^a

Culture	Mean % radioactivity \pm SE	
	Fatty acid	Neutral lipid
Mock infected	0.9 \pm 0.3	3.2 \pm 0.2
Mock infected + IFN	1.0 \pm 0.2	3.7 \pm 0.2
Rickettsiae	4.7 \pm 0.9 ^b	7.3 \pm 1.0 ^b
Rickettsiae + IFN	13.0 \pm 1.6 ^b	16.2 \pm 2.3 ^b

^a L929 cell phospholipids were labeled with oleic acid before infection and IFN- γ treatment, and the extracted radioactivity present in the fatty acid and neutral lipid fractions was measured.

^b Significantly different at the $P < 0.001$ level by Student's t test.

phospholipid hydrolysis was seen (data not shown). In cells assayed at 30 h postinfection, there was a low level of hydrolysis but two of three experiments showed increased hydrolysis of phospholipids after IFN- γ treatment.

At 48 h postinfection, there was increased phospholipid hydrolysis in infected cultures relative to the mock-infected cultures and a very dramatic further increase was apparent in cells that had been treated with IFN- γ (Fig. 1). In these nine experiments, there was a more-than-twofold increase in radiolabeled phospholipid hydrolysis in IFN- γ -treated infected cultures at 48 h relative to that in untreated infected cultures. The oleic acid derived from the labeled phospholipid in these experiments was found both as free oleic acid and as oleic acid in neutral lipid (Table 1). This IFN- γ -mediated increase represented a 277% increase of the radiolabel in the fatty acid fraction and a 222% increase in the neutral lipid fraction. Mock-infected cultures did not demonstrate increased hydrolysis of their radiolabeled phospholipids in response to treatment with IFN- γ .

Identification of radiolabeled phospholipid-derived neutral lipid. The neutral lipid fraction, in which the radiolabel present was derived from hydrolysis of oleic acid-labeled phospholipids, was obtained by column chromatography of extracts of 48-h IFN- γ -treated, infected cultures and further analyzed by thin-layer chromatography. As shown in Table 2, most of the radioactivity comigrated with the triglyceride standard. This was true whether or not the cultures had been treated with IFN- γ . This indicated that a portion of the radiolabeled oleic acid formed from the hydrolysis of the labeled phospholipids was reincorporated into triglyceride.

Incorporation of oleic acid into phospholipids and neutral lipids. In contrast to the protocol for the hydrolysis experiments, in which the host cells were radiolabeled with oleic acid before infection and IFN- γ treatment, in these experiments the host cells remained unlabeled until 24 h after infection (and, where indicated, IFN- γ treatment), at which time the radiolabeled oleic acid was added and the incorporation of this fatty acid over the next 24 h was determined. As shown in Table 3, most of the incorporated oleic acid was

TABLE 2. Composition of neutral lipid fraction

Neutral lipids	Mean % of total \pm SD ^a	
	Hydrolysis experiments	Incorporation experiments
Diglycerides	8 \pm 2	10 \pm 3
Triglycerides	72 \pm 13	70 \pm 7
Cholesterol esters	2 \pm 1	3 \pm 2

^a Data from thin-layer chromatographic analyses of seven incorporation and five hydrolysis experiments are shown.

TABLE 3. Incorporation of oleic acid into infected-cell lipids^a

Culture	Mean oleic acid incorporation (dpm, 10 ³) \pm SD into:	
	Phospholipid	Neutral lipid
Mock infected	451 \pm 105	19 \pm 4
Mock infected + IFN	451 \pm 105	24 \pm 9
Rickettsiae	364 \pm 58 ^b	40 \pm 8 ^c
Rickettsiae + IFN	216 \pm 32 ^b	153 \pm 36 ^c

^a Incorporation of oleic acid into the lipids of infected cultures was determined during the interval between 24 and 48 h postinfection.

^b Significantly different at the $P < 0.03$ level by Student's t test.

^c Significantly different at the $P < 0.09$ level by Student's t test.

present in the phospholipid fraction from both infected and mock-infected cultures whether the cultures were IFN- γ treated or untreated. The most striking finding was that the neutral lipid fraction from the infected cultures that had been IFN- γ treated was labeled to a greater extent than the neutral lipid fraction from the untreated cultures. This increased incorporation of radiolabeled oleic acid into the neutral lipid fraction did not occur in mock-infected cultures. Thin-layer chromatographic analysis of the neutral lipid fraction demonstrated that the incorporation was principally into triglycerides (Table 2).

Cytotoxicity. Host cell viability was evaluated by assessing the ability of cells to exclude trypan blue. Cell viability at 48 h postinfection, as a percentage of mock-infected cell viability (100%), was as follows: mock infection plus IFN, 97% \pm 4%; rickettsiae, 46% \pm 12%; rickettsiae plus IFN, 28% \pm 2%. Analysis of paired samples of IFN- γ -treated and untreated infected cultures showed that the former had only 62% of the viability of the latter. This is significantly different at the $P < 0.03$ level by Student's t test. The infected cultures at 30 h were greater than 99% intact by trypan blue exclusion. In no case did the mock-infected cells show substantial damage as a result of IFN- γ treatment.

DISCUSSION

Although a variety of interferons can stimulate phospholipid hydrolysis in some systems, generally fibroblasts and endothelial cells (2, 6-8, 11, 12, 28), and inhibit phospholipid hydrolysis in other systems, generally mononuclear leukocytes and macrophages (3-5, 10, 21), under our conditions there was no effect of IFN- γ on phospholipid hydrolysis or synthesis of radiolabeled triglycerides in uninfected cultures. It is likely that the time course used in our experiments was too extended for these effects to be evident; for example, in the study of Ponzoni et al. (11) the stimulation caused by IFN- γ had returned to the baseline value by 60 min after addition. Likewise in our experiments, IFN- γ caused no cytotoxicity when added to mock-infected cells. On the other hand, in contrast to the mock-infected cultures, the effects of infection or the combination of infection and IFN- γ on phospholipid hydrolysis and the synthesis of radiolabeled triglycerides were dramatic. Infection of L929 cells with *R. prowazekii* resulted in increased cytotoxicity at 48 h and increased hydrolysis of labeled phospholipids at both 30 and 48 h relative to the uninfected controls. Furthermore, IFN- γ treatment further stimulated the hydrolysis of labeled phospholipids and increased the incorporation of radiolabeled oleic acid (either derived from phospholipid hydrolysis or added exogenously 24 h postinfection) into triglycerides.

The damage to infected cells treated with IFN- γ was significantly greater than that to untreated infected cells, and we speculate that this increased damage was caused by the increased phospholipid hydrolysis.

The phospholipase previously associated with rickettsial infections, phospholipase A, would hydrolyze radiolabeled oleic acid from the labeled phospholipids that, in the simple situation, would be recovered in the fatty acid fraction. However, if the free oleic acid formed from phospholipid hydrolysis were incorporated into a neutral lipid, the radiolabel would be recovered in the neutral lipid fraction instead. (Very little oleic acid was incorporated into neutral lipid relative to phospholipid during the preinfection labeling period.) In our experiments, the radiolabeled oleic acid moiety was recovered as both free fatty acid and triglyceride and only a minor amount of diglycerides was evident on the chromatograms. Therefore, it is likely that phospholipase A activity was responsible for the phospholipid hydrolysis observed. However, the possibilities that diglycerides were formed from the radiolabeled phospholipids and deacylated to free fatty acids and that these free fatty acids were reesterified into triglycerides cannot be eliminated.

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