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Potential for Free Radical-Induced Lipid Peroxidation as a Cause of Endothelial Cell Injury in Rocky Mountain Spotted Fever

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Cells infected by *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, display unusual intracellular morphological changes characterized by dilatation of the membranes of the endoplasmic reticulum and outer nuclear envelope. These changes are consistent with those that might be expected to occur following peroxidation of membrane lipids initiated by oxygen radical species, such as the hydroxyl radical or a variety of organic radicals. Using a fluorescent probe, we have found significantly increased levels of peroxides in human endothelial cells infected by *R. rickettsii*. Studies with desferrioxamine, an iron chelator effective in preventing formation of the hydroxyl radical from hydrogen peroxide and the superoxide free radical, reduced peroxide levels in infected cells to those found in uninfected cells. This observation suggests that the increased peroxides in infected cells may be lipid peroxides, degradation products of free radical attack on polyenoic fatty acids. The potential for lipid peroxidation as an important mechanism in endothelial cell injury caused by *R. rickettsii* is discussed.

Compared with other members of the genus *Rickettsia*, *Rickettsia rickettsii* appears to have a unique mechanism for causing injury to cells (21, 22). Previous studies in our laboratory (18, 21, 22) and that of Walker (25) have shown that cells infected by this organism undergo rather striking changes in the organizational framework of the membranes that make up the endoplasmic reticulum. Initially, these changes constitute a mild dilatation of the reticular membranes, with slight distention of the cisternal space. This is followed by a progressively increased level of intracisternal swelling, which becomes so extensive that it is visible not only by conventional electron microscope techniques but also by the light microscope staining techniques routinely used to study these intracellular parasites. This type of cell injury, to our knowledge, has not been described for other rickettsial species or for other intracellular bacteria. *Rickettsia prowazekii*-infected cells, for example, show virtually no sign of terminal cell injury even when the cytoplasm of afflicted cells is packed with organisms (22). Although there is perhaps some intervening enzymatic or other biochemical deterioration of the plasma membrane that is responsible for eventual lysis of the cells, this has not been evident following extensive electron microscope studies. The cells just seem to burst as the burden of large numbers of rickettsial organisms becomes both physically and physiologically intolerable. *R. rickettsii*, on the other hand, does not accumulate in the cytoplasm of cells to the same extent as do other rickettsiae (31). With its capacity for bidirectional movement through the plasma membrane, it rarely, if ever, achieves the intracellular levels seen with other rickettsial species, except perhaps other members of the spotted fever group. Yet injury to cells infected by this organism is dramatic and can be detected in cells relatively early after infection. This observation suggested to us that a different and ostensibly more specific mechanism of injury might be involved in cells infected by *R. rickettsii*.

Because of the dramatic alteration in internal membranes in cells infected by this organism, we focused our attention on possible mechanisms which might have the potential for disrupting the normal physical integrity of membrane structure. One such mechanism is peroxidation of membrane lipids triggered by oxygen radicals or organic radicals that are generated by the metabolism of compounds present in the culture medium (5). These radicals are produced during normal cellular metabolism, but under most circumstances, they are kept in check by scavenger systems within the cell which prevent them from causing permanent cellular damage. The generation of free radicals in sufficiently large quantities, however, may preclude neutralization by host scavenger mechanisms, rendering these free radical species toxic to cells. The morphological changes that we have observed in cells infected by *R. rickettsii* are consistent with those expected to occur following peroxidation of membrane lipids (5, 23, 24). For this reason, we decided to explore the potential relationship between free radicals and cell injury caused by this organism. Some oxygen radicals, namely, the hydroxyl radical, may be generated from superoxide anion and hydrogen peroxide by the iron-catalyzed Haber-Weiss reaction (13). The results of our current studies show that there are significantly higher peroxide levels in cells infected by *R. rickettsii* than there are in uninfected cells. Furthermore, the levels of peroxides in cells infected by *R. rickettsii* can be reduced to levels comparable with those found in uninfected cells when the iron chelator desferrioxamine, which inhibits the formation of the hydroxyl free radical and which is a known effective modulator of lipid peroxidation, is added to the culture medium (13).

MATERIALS AND METHODS

Rickettsiae. A plaque-purified isolate of the Sheila Smith strain of *R. rickettsii*, propagated in irradiated Vero cells, was used in these studies. Infectivity of the seed was determined by plaque titration in Vero cells (30), and the seed was found to have a PFU titer of 10^7/ml. Growth of the Vero cells for propagation of the rickettsiae was carried out in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO). The cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

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Isolation and culture of endothelial cells from human umbilical vein. Human endothelial cells were isolated from the veins of freshly acquired umbilical cords according to the method of Gimbrone (8). The veins were cannulated with pyrogen-free Intramedic polyethylene tubing (Becton Dickinson and Co., Parsippany, N.J.), flushed with Dulbecco phosphate-buffered saline (PBS; GIBCO), and infused with a PBS solution containing 0.1% collagenase (type CLS; Organon Teknika, Malvern, Pa.) prewarmed to 37°C. Following incubation at room temperature for 15 to 20 min, the contents of the vein containing the detached endothelial cells were collected and the vein was perfused with Hanks balanced salt solution (GIBCO) to remove remaining cells. The combined perfusates were centrifuged at 1,050 × *g* for 5 min, and the pellet was suspended in medium 199 with Earle salts (GIBCO) containing 20% heat-inactivated human serum collected from single donors known not to have had a clinical case of Rocky Mountain spotted fever. The cells were grown in 35-mm Primaria tissue culture dishes (Becton Dickinson and Co., Lincoln Park, N.J.) at 37°C in an atmosphere of 5% CO_2 and 95% air. First- or second-passage cells were used in all studies. Cells were passaged by using a solution containing 0.25% trypsin and 0.01% EDTA. Split ratios generally were not greater than one to three, and the growth medium was changed three times per week.

Infection of endothelial cells by *R. rickettsii*. For studies on the growth of rickettsiae, endothelial cells were seeded onto 13-mm Thermanox plastic coverslips (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) inside 24-well tissue culture plates and allowed to grow for 24 to 48 h. A dilution of the rickettisal seed stock containing 10^5 PFU per ml prepared in medium 199 containing 10% human serum was added to each well. The coverslips were incubated for 2 h at 37°C in an atmosphere of 5% CO_2 and 95% air. The medium was removed after 2 h and replaced with fresh medium 199 with 20% human serum containing the appropriate concentrations of desferrioxamine. Coverslips were removed at 24, 48, and 72 h, washed free of extracellular rickettsiae with Hanks balanced salt solution, allowed to air dry, and stained by the method of Giménez (9) for enumeration of rickettsiae. A minimum of 150 cells were examined on each coverslip by light microscopy to determine the number of rickettsiae per cell and the percentage of cells infected.

For the peroxide studies, endothelial cells were seeded into 60-mm Primaria dishes and onto 13-mm coverslips. The cells were grown in medium 199 containing 20% human serum and infected as above. After 2 h of exposure to the inoculum, the medium was removed from all dishes and wells, and fresh medium with or without desferrioxamine was added to the cells. At 24-h intervals, the coverslips were removed to determine the progression of the infection and the plates were prepared for analysis of peroxides as described below.

Determination of intracellular peroxide levels. Endothelial cells grown in the 60-mm dishes were assayed for intracellular peroxide levels by a modification of the method of Cathcart et al. (4) over a 72-h period. At 24-h intervals, the medium was removed from the dishes and the cells were washed three times with 3 ml of PBS. After the third rinse was removed, 2 ml of PBS was added to each dish, and 5 μl of a stock solution (0.4 mM) of the probe 5- (and 6-) carboxy-2',7'-dichlorofluorescin diacetate (Molecular Probes, Eugene, Oreg.) in absolute ethanol was added. The dishes were swirled gently and incubated at room temperature for 10 min. The cells were again rinsed three times with 3 ml of PBS. After the final rinse was aspirated, 1.2 ml of distilled water was added to each dish, followed by 10 μl of an aqueous solution of 10% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to lyse the cells. The dishes were swirled again; the cells were scraped with a rubber policeman, and the contents were decanted into disposable Ultravu cuvettes (American Scientific Products, Inc., McGaw Park, Ill.) and placed in an ice bath. The samples were allowed to equilibrate to room temperature for 10 to 15 min before sample fluorescence was measured on a fluorescence spectrophotometer (MPF 66; The Perkin-Elmer Corp., Norwalk, Conn.) by using an emission wavelength of 535 nm and an excitation wavelength of 505 nm. Background fluorescence was determined in the absence of added probe and found to be insignificant in both infected and uninfected cell populations. Protein concentrations of each sample were determined by the method of Lowry et al. (16), and peroxide levels were expressed as fluorescent units per milligram of protein.

Since the conversion of dichlorofluorescin, the deacetylated form of the probe, to the fluorescent compound dichlorofluorescein requires the presence of both peroxide and a peroxidase (3), we examined the effect of adding horseradish peroxidase (3 U/ml; Sigma) to the cell extracts prior to measuring the fluorescence of the samples. The addition of this enzyme to cell extracts increased fluorescence approximately sevenfold compared with fluorescence in samples to which the enzyme was not added, thus indicating the specificity of the reaction.

To determine whether time and/or temperature of incubation affected the extent of hydrolysis of dichlorofluorescin diacetate to dichlorofluorescein in our assay system, we extended the incubation time from 10 to 30 min and raised the temperature from ambient to 37°C. These alterations had no effect on the rate of hydrolysis of the probe.

Transmission electron microscopy of infected endothelial cells. Culture dishes were removed from the incubator at 24-h intervals. The medium was decanted, and the cells were washed once with Hanks balanced salt solution. The cells were fixed overnight in situ at 4°C in a solution of 2% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer, pH 7.3. Following glutaraldehyde fixation, the cells were washed three times in cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h at room temperature, dehydrated in an ascending ethanol series, and embedded in Poly-Bed 812 (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were cut on an ultramicrotome (MT-5000; Ivan Sorvall, Inc., Norwalk, Conn.), picked up on carbon-coated collodion copper grids, stained with uranyl acetate and lead citrate, and viewed in a Siemens IA electron microscope operating at 80 kv.

Statistical analyses. For experiments in which intracellular peroxide levels were measured, the highest value for each group was designated as 100% and all subsequent values were normalized to that value. In each of the graphs, all of the normalized values have been combined and a mean and standard error of the mean for each set of experimental conditions are presented.

RESULTS

Transmission electron microscopy of *R. rickettsii*-infected human endothelial cells. Cells infected by *R. rickettsii* undergo striking intracellular changes which are usually apparent within 48 h postinfection. A transmission electron micrograph showing a typical human umbilical vein endothelial

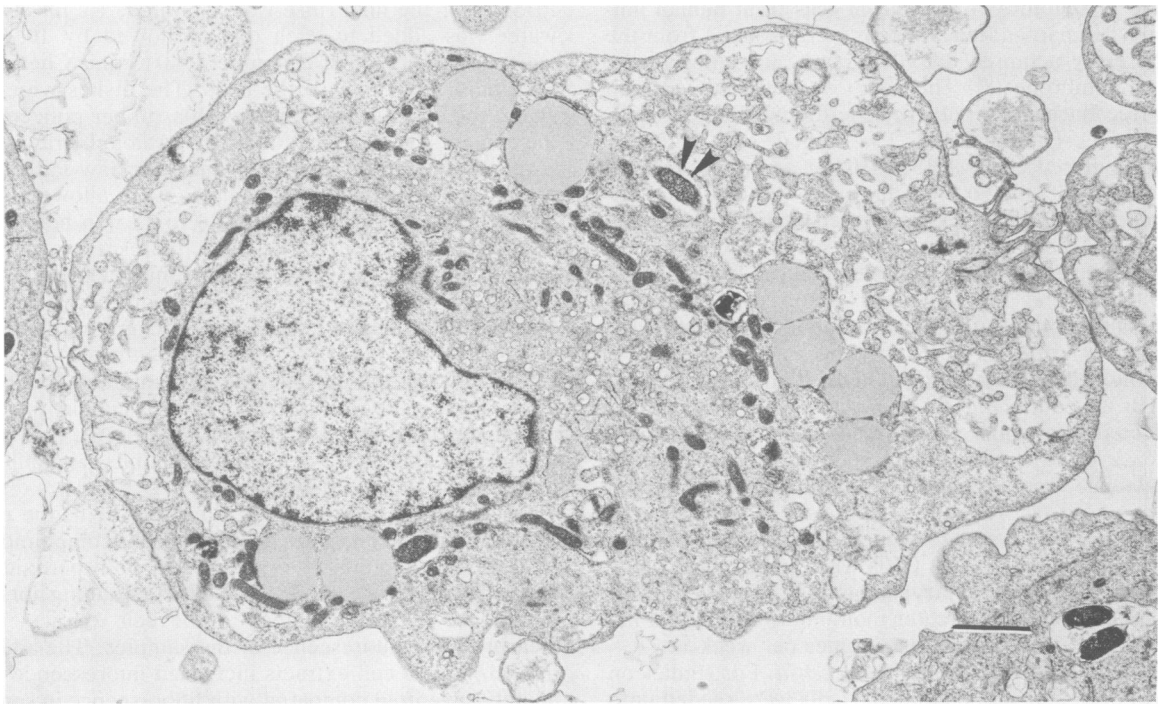

FIG. 1. Transmission electron micrograph of a *R. rickettsii*-infected human endothelial cell from a culture 96 h postinfection showing extensive dilatation of membranes of the endoplasmic reticulum and the presence of cytoplasmic rickettsiae (arrowheads). Bar, 1 μm.

cell infected by this organism is shown in Fig. 1. There are two distinct features of cells infected by *R. rickettsii* that are shown in this micrograph which distinguish them from cells infected by most other members of the genus *Rickettsia*. First is the comparatively small number of rickettsial organisms found within the cells as late as 72 to 96 h after infection. Second, there are distinct intracellular morphological changes eventually manifested in all infected cells that consist of rather extensive dilatation of the membranes that constitute the endoplasmic reticulum-outer nuclear envelope complex.

Measurement of intracellular peroxides within infected and uninfected endothelial cells. The molecular probe 5- (and 6-) carboxy-2′,7′-dichlorofluorescin diacetate was used to determine the relative levels of intracellular peroxide (measured as either hydrogen peroxide or lipid peroxide) in infected and uninfected endothelial cells. This compound becomes deacetylated upon passage through the plasma membrane of cells, and upon reaction with either hydrogen peroxide or lipid peroxides, it is oxidized to the fluorescent compound 5- (and 6-) carboxy-2′,7′-dichlorofluorescein (4). Figure 2 represents a composite of several experiments which shows that endothelial cells infected by *R. rickettsii* have statistically significant higher levels of intracellular peroxides than do uninfected control cells at 24, 48, and 72 h postinfection. Within a given experiment, the differences in the peroxide levels between infected and uninfected cells ranged from 1.5-fold to nearly 5-fold. In general, while intracellular peroxide levels in an infected cell population always were significantly higher than the peroxide levels in parallel uninfected cultures, the levels of peroxides in infected cells at the different times tested were not always different from one another.

Effect of desferrioxamine on peroxide formation in endothelial cells. The iron chelator desferrioxamine prevents the formation of the hydroxyl free radical from hydrogen peroxide and superoxide by the iron-catalyzed Haber-Weiss reaction. Since the hydroxyl radical is considered one of the more biologically active free radical species and a known initiator of lipid peroxidation, we decided to examine whether the addition of desferrioxamine to *R. rickettsii*-infected cell cultures might be capable of modulating intracellular peroxide levels. The capacity of this compound to reduce the levels of intracellular peroxides in rickettsia-infected cells over a 72-h period is shown in Fig. 3. At 24 h,

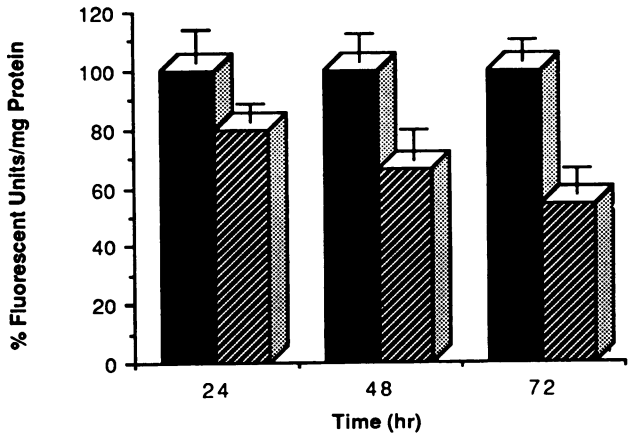

FIG. 2. Intracellular peroxide levels in human endothelial cells infected by *R. rickettsii* measured at 24, 48, and 72 h postinfection. Symbols: ■, infected cell population; ▨, uninfected control cell population. The data represent the mean values ± the standard error of at least 15 determinations at each time point.

peroxide levels in infected cultures treated with desferrioxamine were reduced to levels equal to or less than those of uninfected cultures and the peroxide levels in the treated infected cell populations were reduced in most instances by nearly 50% compared with those of untreated infected populations. Similar results were achieved at 48 and 72 h postinfection. Two other concentrations of desferrioxamine, 1 and 100 μg/ml, also were evaluated. The lower concentration had no detectable effect in reducing peroxide levels, and the higher concentration showed some inhibition of rickettsial growth (see below). On examination of the relative levels of peroxides in the uninfected cell populations with time, there appeared to be an overall decrease from 24 to 72 h. However, there were no significant differences in levels between the 24-h intervals.

To assure that desferrioxamine did not influence our results by inhibiting the oxidation of dichlorofluorescin to dichlorofluorescein, parallel experiments were carried out in which the drug was added just prior to or simultaneously with the probe. The results (data not shown) suggested that desferrioxamine had no effect on the oxidation of the probe.

Effect of desferrioxamine on the growth of *R. rickettsii* in endothelial cells. To confirm that the reduction in the levels of intracellular peroxide in cells treated with desferrioxamine could be attributed to actual inhibition of peroxide formation and not to antirickettsial activity of the drug, we examined the effects of desferrioxamine on the growth of *R. rickettsii* over a 72-h period. Initially, four concentrations of desferrioxamine were used, 1, 10, 50, and 100 μg/ml. However, as indicated above, 1 μg/ml had no detectable effect in reducing peroxide levels (and also, no observable inhibitory effect on rickettsial growth). On the other hand, 100 μg/ml, while showing a considerable capacity for lowering intracellular peroxide levels, also displayed some antirickettsial activity. For these reasons, both the 1 and 100 μg/ml concentrations were excluded from subsequent experiments. The concentrations of 10 and 50 μg/ml, which showed no antirickettsial activity as determined by standard rickettsial counting techniques, were used, and the results were compared with those of untreated controls cells. Figure 4 represents a composite of several experiments which shows the effects of these two concentrations of desferrioxamine on the growth of *R. rickettsii* in endothelial cells. The apparent cessation of the growth of the rickettsiae after 48 h in both treated and untreated cells probably reflects the inability to accurately measure the growth of the organism beyond this time point. A certain percentage of heavily infected cells typically begins to detach from the surface of the culture vessel by 72 h, and the cells which were previously uninfected become infected, tending to skew the data used to calculate the average number of rickettsiae per infected cell toward a lower number.

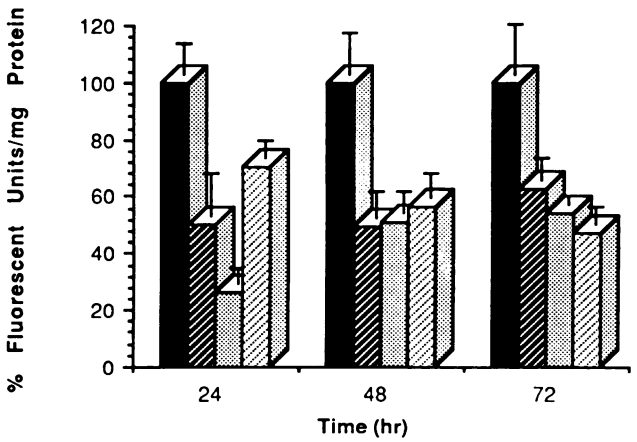

FIG. 3. Effect of desferrioxamine on intracellular peroxide levels in human endothelial cells infected by *R. rickettsii*. Peroxide levels were measured at 24, 48, and 72 h postinfection. The first three bars of each group represent infected cell populations, treated with 0, 10, and 50 μg of desferrioxamine per ml, respectively. The fourth bar in each group represents uninfected control cultures that were not treated with desferrioxamine. The data represent the mean values ± the standard error of at least six determinations.

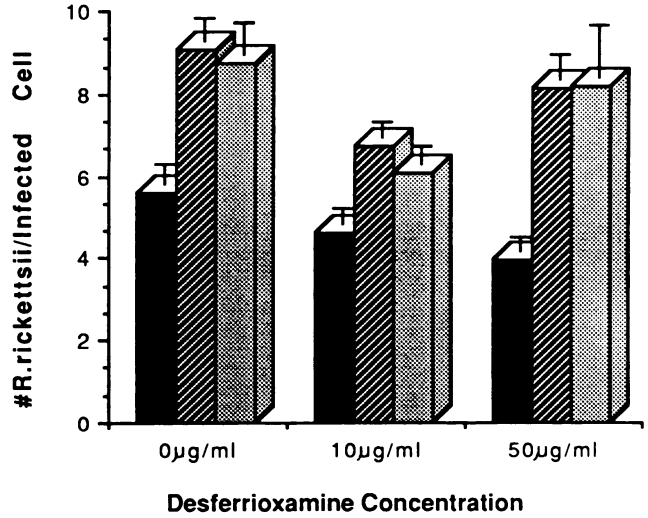

FIG. 4. Growth of *R. rickettsii* in human endothelial cells in the presence of desferrioxamine at 24 h (■), 48 h (▨), and 72 h (▩) postinfection. The data represent the mean values ± the standard error of at least three experiments.

Studies were also carried out to determine whether desferrioxamine affected the rate of growth of endothelial cells in culture. Our studies showed that in subconfluent cultures, there was no detectable effect on the rate of endothelial cell growth when cells were exposed to concentrations of 10 and 50 μg/ml of the drug for periods of up to 72 h.

DISCUSSION

Rocky Mountain spotted fever is the most severe of the rickettsial diseases of humans, causing widespread vasculitis and involvement of many of the major organ systems of the body (1, 10, 17, 25, 27, 28). The endothelial cells of the smaller blood vessels are generally regarded as the target cells in human infection, although smooth muscle cells may become involved in more severe forms of the disease. We have been using endothelial cells from the human umbilical vein to study the interaction between *R. rickettsii* and cells which are of the same species and ontogenic type as those which are thought to be principally parasitized in vivo. Previous studies in our laboratory have focused on the kinetics of infection by this organism and the manifestations of endothelial cell injury as seen by transmission electron microscopy (18, 20). We have also described an increased propensity for platelets to adhere to cells infected by *R. rickettsii* (19), an observation of special importance with respect to the coagulopathy and thrombocytopenia commonly seen as clinical manifestations of the disease (14, 15). Our observations by electron microscopy helped us to focus

on a possible mechanism for cell injury caused by *R. rickettsii*. The extensive dilatation of the internal membranes of cells infected by this organism appeared similar to that reported for other systems in which peroxidation of membrane lipids was shown to cause marked distortion of internal membranes accompanied by increased membrane permeability (2).

Lipid peroxidation, mediated by oxygen radicals, has been associated with numerous clinical conditions, including hepatitis B vasculitis, malaria, and endotoxin liver injury (13). Free radicals and hydrogen peroxide, generated during normal oxidative metabolism, are capable of directly exerting damaging effects on cells (6). However, the highly reactive hydroxyl radical formed from the superoxide anion and hydrogen peroxide is considered to be a good candidate for inducing cell injury because of its extreme toxicity (12, 13).

The first step in our attempt to establish that lipid peroxidation is a potential cause of endothelial cell injury in Rocky Mountain spotted fever was to examine *R. rickettsii*-infected endothelial cells for evidence of increased peroxidation of lipids. A popular method for demonstrating lipid peroxidation in cells and tissues is to measure the levels of malonaldehye, a degradation product of unstable hydroperoxides (5). While our experiments showed a 2.5-fold greater difference in infected cells than in uninfected cells, the test apparently has been found to be unreliable and prone to misinterpretation (11). As a result, we sought a more reliable method for our study. We subsequently used a technique that utilized a fluorescent probe capable of measuring intracellular peroxide levels. Although this probe does not necessarily distinguish between organic lipid peroxides and hydrogen peroxide, it does indicate whether there are increased levels of total intracellular peroxide in infected cells. This appeared to be an acceptable approach for two reasons. (i) Increased levels of hydrogen peroxide within infected cells can lead to increased levels of the hydroxyl radical through interaction with the superoxide anion, thereby initiating lipid peroxidation. (ii) The hydroxyl radical through its interaction with methylene carbons separating double bonds in polyenoic fatty acids eventually can lead to the formation of lipid hydroperoxides from which additional organic radicals and malonaldehyde are generated (5).

Our studies have shown that human endothelial cells infected by *R. rickettsii* have significantly higher levels of intracellular peroxides than do uninfected control cells. These increased peroxide levels were apparent by 24 h postinfection and remained elevated through 72 h, when the experiments were terminated because of the onset of some cell detachment in the infected-cell populations. Perhaps the most exciting potential ramification of this observation is that previous studies in our laboratory using transmission electron microscopy (18) showed that by 48 h, cells infected by *R. rickettsii* begin to display the characteristic dilatation of the membranes of the endoplasmic reticulum. Because of the coincident time relationship, it is tempting to speculate that the increased levels of peroxides and the intracellular morphological changes relate to one another as cause and effect.

Because the hydroxyl radical is highly reactive with most macromolecules of cells and tissues, including nucleic acids, proteins, and lipids (5), we sought to determine whether it might play a role in injury to cells infected by *R. rickettsii*. The iron chelator desferrioxamine can be used to prevent the formation of the hydroxyl radical from superoxide anion and hydrogen peroxide (11). Accordingly, we incorporated this compound into our culture system at several concentrations to determine its effect on intracellular peroxide levels. To assure that any observed changes were not due to antirickettsial activity of the drug, we also examined the effect of desferrioxamine on the growth of *R. rickettsii* over a 72-h period. Our results showed that desferrioxamine was indeed effective in reducing the levels of peroxides in endothelial cells infected by this organism and, at the concentrations used, had no detectable effect on the growth of the organism. Although it is attractive to speculate that the increased peroxides in *R. rickettsii*-infected cells may be lipid hydroperoxides, which would imply an increased level of peroxidation of membrane lipids, further experiments are necessary before this can be confirmed. Moreover, even if the increased peroxides eventually are shown to be lipid hydroperoxides, it will be important to determine whether lipid peroxidation is an actual cause of cell injury (12) in these cells or merely a consequence triggered by another mechanism important to the pathogenesis of the organism.

Unfortunately, preliminary studies using desferrioxamine at the concentrations effective in reducing intracellular peroxide levels were not effective in preventing the morphological changes seen by electron microscopy in infected cells. This may indicate that the drug is not blocking the formation of the hydroxyl radical to the extent necessary to protect the cells or, possibly, that other radicals, such as the protonated superoxide radical $HO_2\cdot$, which is also capable of directly attacking fatty acids and causing peroxidation (7), are responsible for generating increased peroxide levels. It is not clear at this time what the specific source of the oxidant(s) is in our system, since generation of free radicals and, in particular, the superoxide radical is a common byproduct of a variety of spontaneous and enzymatic oxidative reactions that occur within cells. Future studies in our laboratory will attempt to address these questions.

It should be pointed out that even if peroxidation of membrane lipids proves to be an important mechanism of cellular injury caused by *R. rickettsii*, other mechanisms may also play a role, as suggested by the work of Walker et al. (26, 29). In one study (29), they found that synthetic protease inhibitors of the amidine type prevent plaque formation by *R. rickettsii*, suggesting that a trypsinlike protease might be involved in the pathogenesis of disease caused by this organism. This could occur either by indirect proteolytic activation of phospholipase (during entry or exit of the organism from the cell) or by direct proteolytic attack on the host cell membrane. In another study (26), it was shown that the phospholipase A inhibitor phentermine hydrochloride significantly reduces the capacity of *R. rickettsii* to form plaques, presumably by interfering with the mechanism of entry of the organism into the host cell. These observations, together with those presented above, indicate the possible complexity of cell injury caused by *R. rickettsii* and emphasize the need for further exploration.

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