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Heparin Protects Human Endothelial Cells Infected by *Rickettsia rickettsii*

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Routine culture of endothelial cells currently includes the use of heparin, which significantly reduces cell doubling time and increases cell population size. Heparin protects cultured arterial endothelial cells from damage by toxic oxygen metabolites produced by the action of xanthine and xanthine oxidase. Because of our hypothesis implicating free radicals in cell injury caused by *Rickettsia rickettsii*, we have carried out a series of experiments to examine the effects of heparin on injury to endothelial cells infected by this microorganism. These studies showed that heparin does not inhibit replication of *R. rickettsii* in the cytoplasm of endothelial cells. Furthermore, heparin appears to exhibit a protective effect on the infected host cell as measured by (i) reduced plaque size, (ii) increased longevity of the cell monolayer, (iii) reduction in the amount of lactic dehydrogenase released from infected cells, and (iv) reduction in the levels of intracellular peroxides formed in infected cells. Electron microscopic studies also show a significant reduction in dilatation of the rough-surfaced endoplasmic reticulum of the infected cells in the presence of heparin. These observations appear to lend additional support to involvement of an oxidative mechanism in human endothelial cell injury caused by *R. rickettsii*.

Rickettsia rickettsii is an obligate intracellular bacterial parasite that infects humans, thereby causing Rocky Mountain spotted fever. A prominent feature of Rocky Mountain spotted fever disease pathogenesis is an endovasculitis that commonly leads to widespread hemodynamic disturbances. Because the endothelial cell is the putative target cell in human infections, our studies over the past several years have focused on the interaction of *R. rickettsii* with endothelial cells. The kinetics of infection of endothelial cells in culture (10) parallel that shown by Wisseman et al. (20) for chicken embryo fibroblasts and mouse L cells. Typically, following internalization by uptake and subsequent escape from the phagosome into the cytoplasm (17, 18), the microorganisms replicate by binary fission and rapidly spread to adjacent host cells in the culture. In a small percentage of infected host cells, the bacteria also enter the nucleus, a characteristic common to members of the spotted fever group rickettsiae (20).

In our earlier studies, which compared host cells infected by the typhus and spotted fever rickettsiae by electron microscopy, we reported striking differences in the cytopathic effects induced by these microorganisms (13, 14). Host cells infected by the Sheila Smith strain of *R. rickettsii* had severe dilatation of the rough-surfaced endoplasmic reticulum (ER) by 48 to 72 h postinfection, while there were no significant changes apparent in host cells infected by the Breinl strain of *Rickettsia prowazekii*. These observations, together with the determination that host cells infected by *R. rickettsii* had increased levels of intracellular peroxides, led us to hypothesize that host cell injury caused by this microorganism is mediated by free-radical-induced lipid peroxidation (11). Indeed, in host cells infected by *R. rickettsii*, the concentrations of cellular thiols, which, among other molecules, serve to detoxify peroxides, were dramatically reduced. This change has the potential to render the host cells vulnerable to oxidative injury (12).

In a recent report, heparin was shown to be protective to aortic endothelial cells exposed to xanthine and xanthine oxidase, a system that generates high levels of the superoxide radical capable of causing endothelial cell injury (6). In light of our observations which support a role for oxidative stress in *R. rickettsii*-induced host cell injury, we have examined the effectiveness of heparin in the protection of human endothelial cells infected by *R. rickettsii*. We report that heparin (i) increases the longevity of infected host cells as indicated by viability and injury measurements, (ii) reduces the size of plaques formed in endothelial cells, (iii) diminishes the distention of the ER membranes as seen by electron microscopy, and (iv) reduces intracellular peroxide levels of infected host cells.

MATERIALS AND METHODS

Bacteria. A plaque-purified isolate of the Sheila Smith strain of *R. rickettsii* was used in all experiments. The bacteria were propagated in Vero cells, strain C1008 (American Type Culture Collection, Rockville, Md.). A standardized plaque assay technique was used to determine the infectious bacteria in the seed stocks (19).

Culture of human endothelial cells and Vero cells. Endothelial cells were isolated from freshly acquired human umbilical veins by the method of Gimbrone (3) with a 0.1% solution of collagenase, type A (Boehringer Mannheim Corp., Indianapolis, Ind.), prepared in phosphate-buffered saline (PBS). These cells were cultured directly in 60-mm Nunc (Interlab, Newbury Park, Calif.) plastic tissue culture dishes (or, for the purpose of monitoring the infection of host cells, on coverslips placed in the culture dishes), with McCoy's 5A medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% heat-inactivated fetal bovine serum (Upstate Biotechnology, Inc., Saranac Lake, N.Y.) and 30 μg of H-Neurext endothelial cell growth supplement

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(UBI, Inc.) per ml. Where appropriate, sodium heparin (Sigma Chemical Co., St. Louis, Mo.) was added to the cultures. Endothelial cells were used after either the first or the second passage with trypsin-EDTA. Vero cells were used to propagate the *R. rickettsii* and to determine viable rickettsial titers by plaque assay. Infected Vero cells were cultured in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum. All host cells were grown at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Infection of endothelial cells. We used an average of 0.5 bacteria per cell as the infecting dose for endothelial cells. The inoculum, in McCoy's 5A medium, was added to the cell monolayers, incubated for 2 h at 37°C, and removed by aspiration. Fresh growth medium, with and without heparin, was added to the cultures. Progression and spread of the infection of endothelial cells by *R. rickettsii* was monitored by removing coverslips from parallel cultures, staining the cells by the method of Gimenez (4), and determining the percentage of host cells infected and the average number of bacteria per infected cell.

Endothelial cell plaque assay. Confluent monolayers of endothelial cells were exposed for 2 h to three dilutions of the *R. rickettsii* seed stock (10^{-5}, 10^{-6}, and 10^{-7}) in the presence of 0, 50, and 100 μg of sodium heparin per ml. The medium was removed and replaced with fresh medium containing the appropriate concentrations of heparin prepared in an agarose overlay. After 6 days of incubation, the cells were stained with neutral red, the plaques were counted, and their sizes were measured with a Bausch and Lomb micrometer.

LDH activity and cell viability. The assay for lactic dehydrogenase (LDH) activity was performed on culture medium samples from infected and uninfected host cells at 24, 48, and 72 h in the presence and absence of heparin (Sigma diagnostic kit 228UV). Briefly, 50 μl of the cell supernatant fluids was added to 1 ml of prewarmed (30°C) reagent in disposable cuvettes and mixed by inversion. The initial A_{340} was recorded with a Beckman model DU 64 spectrophotometer (Beckman Instruments, Fullerton, Calif.), and additional readings were taken at 30, 60, 90, and 120 s. LDH activity in units per milliliter was determined by the following formula: LDH activity (U/ml) = (A × TV)/(6.22 × SV × LP), where A represents the change in A_{340} per min, TV represents the total reaction mixture volume (1.05 ml), SV represents sample volume (50 μl), 6.22 is millimolar absorptivity of NADH, and LP represents lightpath (1 cm). The LDH released was calculated and expressed in units per milligram of protein. Three LDH standards (Beckman) were used to generate a standard curve. As a control, Renografin-purified suspensions of *R. rickettsii* were tested for LDH activity. Cell viability was determined by scraping cells from the surfaces of culture dishes into the medium and mixing 100 μl of this suspension with 20 μl of 0.4% trypan blue dye. After 5 min, percent viability of the samples was determined by counting the cells in a hemacytometer. trypan blue staining was also carried out on cells attached to the plastic tissue culture dishes and on the corresponding culture supernatants. All attached cells were found to be viable by direct staining, and all floating cells were nonviable.

Transmission electron microscopy. Infected endothelial cells grown in the presence and absence of heparin were examined at 48 and 72 h postinfection. At the appropriate times, the culture medium was removed, and the cells were washed 1× in Hank's balanced salt solution and fixed in 2% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer. After postfixation in 1% osmium tetroxide, the cells were stained with 2% uranyl acetate, dehydrated in an ascending series of ethanol, and embedded in PolyBed 812 (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were cut on a Sorvall MT-1 ultramicrotome, picked up on collodion-carbon-coated copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL-JEM 1200 CX electron microscope (JEOL, Ltd., Peabody, Mass.) operating at 60 kV.

Measurement of intracellular peroxide levels. Peroxide levels in infected and uninfected endothelial cells that were either treated or not treated with heparin were determined at 48 h as previously described (11) by using the probe 5 (and 6)-carboxy-2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, Oreg.). Briefly, the cells were exposed to the probe for 10 min at room temperature and washed three times in PBS, and 1.2 ml of distilled water was added to each dish. Ten microliters of an aqueous solution of 10% Nonidet P-40 (Sigma) was added to lyse the cells. The cells were scraped with a rubber policeman, and the contents of each dish were transferred to disposable Ultravu cuvettes (American Scientific Products, Inc., McGaw Park, Ill.). 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. Protein concentrations of each sample were determined by the method of Lowry et al. (8), and peroxide levels were expressed as fluorescence units per milligram of protein.

RESULTS

Growth of *R. rickettsii* in endothelial cells in the presence of heparin. To assess the effect of heparin on the growth of *R. rickettsii* in endothelial cells, a study was carried out to determine the average number of bacteria per infected cell and the percentage of cells infected over a 72-h period in the absence of heparin and in the presence of 50 and 100 μg of heparin per ml. Counting a minimum of 400 Gimenez-stained cells per treatment (Fig. 1), we found no significant differences between cultures treated with 50 μg of heparin per ml and cultures that were not treated. At 100 μg/ml, however, there was a reduction at 72 h in the average number of bacteria per infected cell, but no difference in the percentage of cells infected. These results show that heparin, at a concentration of 50 μg/ml, did not impair bacterial replication or the microorganism's ability to spread from cell to cell.

Effect of heparin on viability and injury of endothelial cells infected by *R. rickettsii*. The viability of endothelial cells infected by *R. rickettsii* was tested in the presence and absence of heparin (Fig. 2) by using trypan blue dye exclusion. As early as 24 h postinfection, there was evidence that heparin exerted a protective effect on infected cells. By 72 h, the number of viable infected cells in the presence of heparin was at least twice that of the untreated cells. This result was also supported by direct visual observation of the cultures with an inverted tissue culture microscope; the number of detached cells in the non-heparin-treated cells was clearly greater than in those treated with heparin.

The release of LDH by endothelial cells was used as an indicator of cell injury caused by *R. rickettsii*, and the effects of heparin on LDH release were determined (Fig. 3). The results show that heparin is effective in reducing the amount

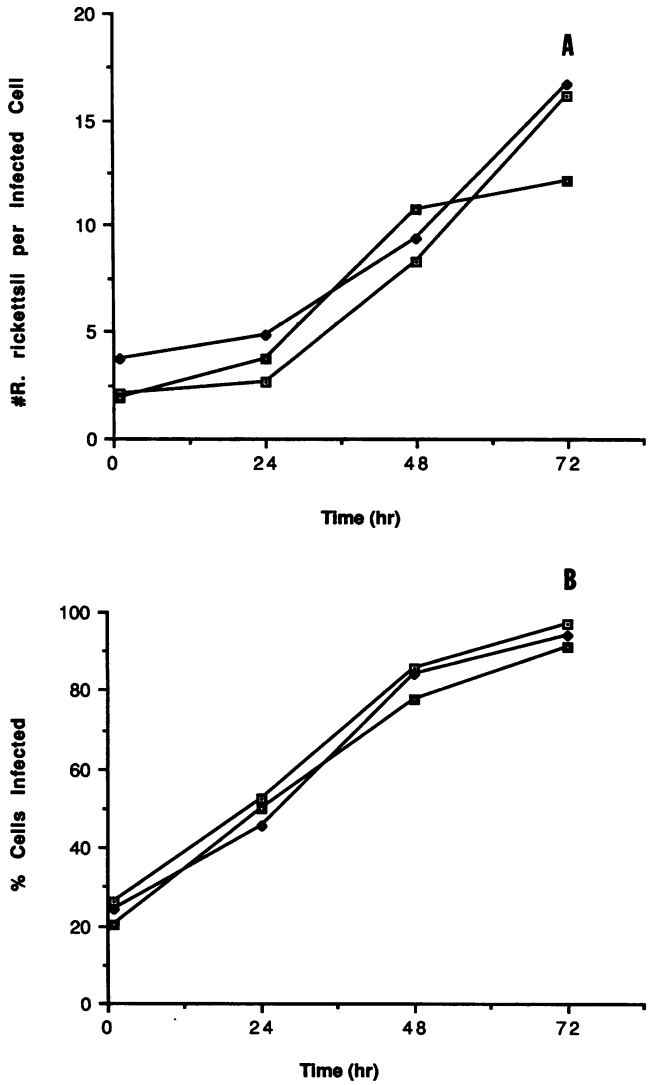

FIG. 1. Growth of *R. rickettsii* in endothelial cells with and without heparin as determined by Gimenez staining. (A) Average number of *R. rickettsii* per infected cell; (B) percentage of cells infected. ⊡, 0 μg of heparin per ml; ◆, 50 μg of heparin per ml; ■, 100 μg of heparin per ml.

of LDH released from infected cells. This observation was first made at 48 h postinfection, when the LDH activity in the medium of non-heparin-treated infected cultures was approximately threefold higher than in those cultures that were treated with heparin. A similar difference also was seen at 72 h postinfection. Purified suspensions of *R. rickettsii*, when tested for LDH under the same conditions, showed no activity.

Effects of heparin on plaque size in endothelial cells infected by *R. rickettsii*. The effect of heparin on the formation of plaques in endothelial cells was examined. No difference in the number of plaques formed was seen in heparin-treated cultures. However, there was a marked reduction (>40%) in the size of the plaques formed in cultures treated with 50 μg of heparin per ml. Average plaque size in untreated cultures was 1.02 mm, and in cultures treated with 50 μg of heparin per ml it was 0.58 mm. Doubling the concentration of

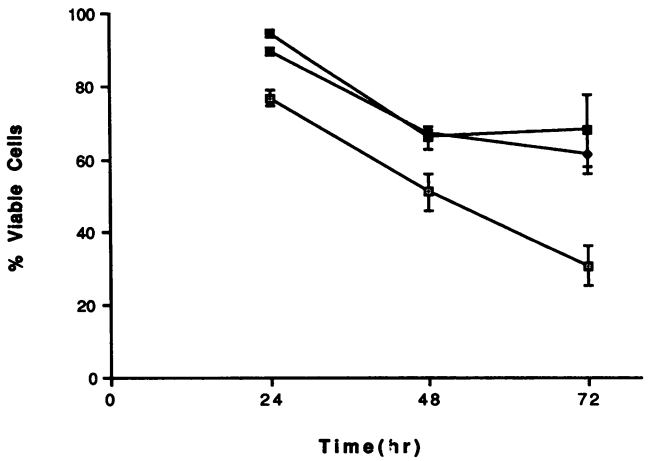

FIG. 2. Effects of heparin on the viability of endothelial cells infected by *R. rickettsii* as determined by trypan blue dye exclusion. ⊡, 0 μg of heparin per ml; ◆, 50 μg of heparin per ml; ■, 100 μg of heparin per ml. Data represent the mean values plus or minus the standard error of the mean.

heparin did not significantly reduce plaque size beyond that observed with 50 μg/ml.

Transmission electron microscopy of heparin-treated and untreated endothelial cells infected by *R. rickettsii*. Endothelial cells infected by *R. rickettsii* and grown in the presence and absence of heparin were examined by transmission electron microscopy at 48 and 72 h postinfection. Figure 4A shows cell injury typical of that seen in cells infected by *R. rickettsii* in the absence of heparin at 72 h after infection. The most prominent characteristic feature of this infected cell is

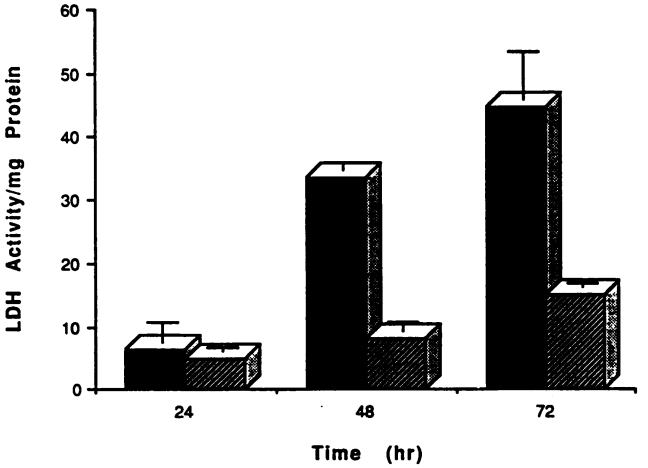

FIG. 3. Effects of heparin on the release of LDH from endothelial cells infected by *R. rickettsii*. Heparin, where used, was first introduced into the cell culture medium at the time of primary isolation of the cells and was again added to the cultures every other day (when the culture medium was changed) and at the time of infection by *R. rickettsii*. Solid bars, LDH activity in infected cultures not treated with heparin; hatched bars, LDH activity in infected cultures treated with 50 μg of heparin per ml. LDH activity from uninfected control cultures in the presence and absence of heparin was subtracted from the activity in the corresponding infected cultures. Data represent the mean values plus or minus the standard error of the mean.

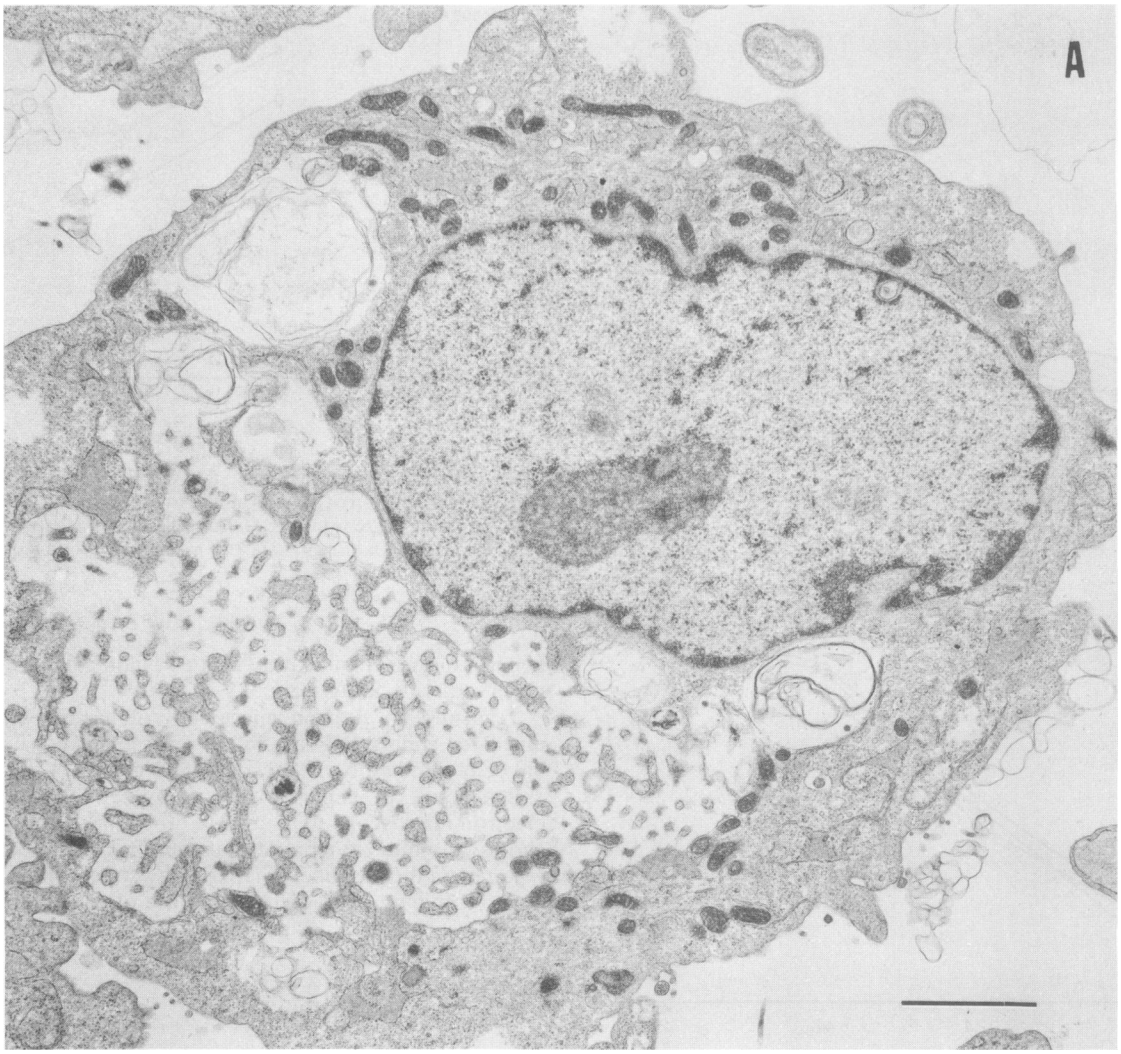

FIG. 4. Transmission electron photomicrographs of human endothelial cells 72 h after infection by *R. rickettsii*. (A) Cells grown in the absence of heparin. Note distention of endoplasmic reticulum. Bar, 2 μm. (B) Cells grown in the presence of 50 μg of sodium heparin per ml. Distention of the endoplasmic reticulum is noticeably reduced. Bar, 0.5 μm.

the significant distention of the ER-outer nuclear envelope complex. Cultures treated with 50 μg of heparin per ml, on the other hand (Fig. 4B), generally showed much less distention of these membranes.

Measurement of intracellular peroxides in infected and uninfected endothelial cells in the presence and absence of heparin. We used the molecular probe 5 (and 6)-carboxy-2′,7′-dichlorofluorescin diacetate to determine the levels of intracellular peroxide (measured as either hydrogen peroxide or lipid peroxide) in infected and uninfected endothelial cells in the presence and absence of heparin. This compound becomes deacetylated when it passes through the plasma membrane of cells (2), and upon reaction with either hydrogen peroxide or lipid peroxides, it is oxidized to the fluorescent compound 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (2). Figure 5 shows that heparin has the ability to significantly reduce intracellular peroxide levels in endothelial cells at 48 h postinfection with *R. rickettsii*. There was not a significant difference in peroxide levels between heparin-treated and non-heparin-treated uninfected endothelial cells.

DISCUSSION

The ultrastructural changes that occur in chicken embryo cells infected by the Breinl strain of *R. prowazekii* and the Sheila Smith strain of *R. rickettsii* (13, 14) demonstrate striking differences between the strains with respect to host cell cytopathology. Virtually no evidence of injury could be detected in cells infected by *R. prowazekii* even after 72 h when large numbers of microorganisms were apparent in thin sections through infected cells. The conclusion was made that these cells die eventually because of their large bacterial burden. On the other hand, *R. rickettsii* does not accumulate in cells to the extent that *R. prowazekii* does, yet the microorganism has the capacity to cause noticeable cell injury within 48 to 72 h postinfection. This injury appears to be unique to *R. rickettsii* and is manifested by progressive swelling of the rough-surfaced ER-outer nuclear membrane complex. This, in turn, leads to the formation of large intracellular cisternae and apparent envelopment of the bacteria by internal host membranes. Similar observations

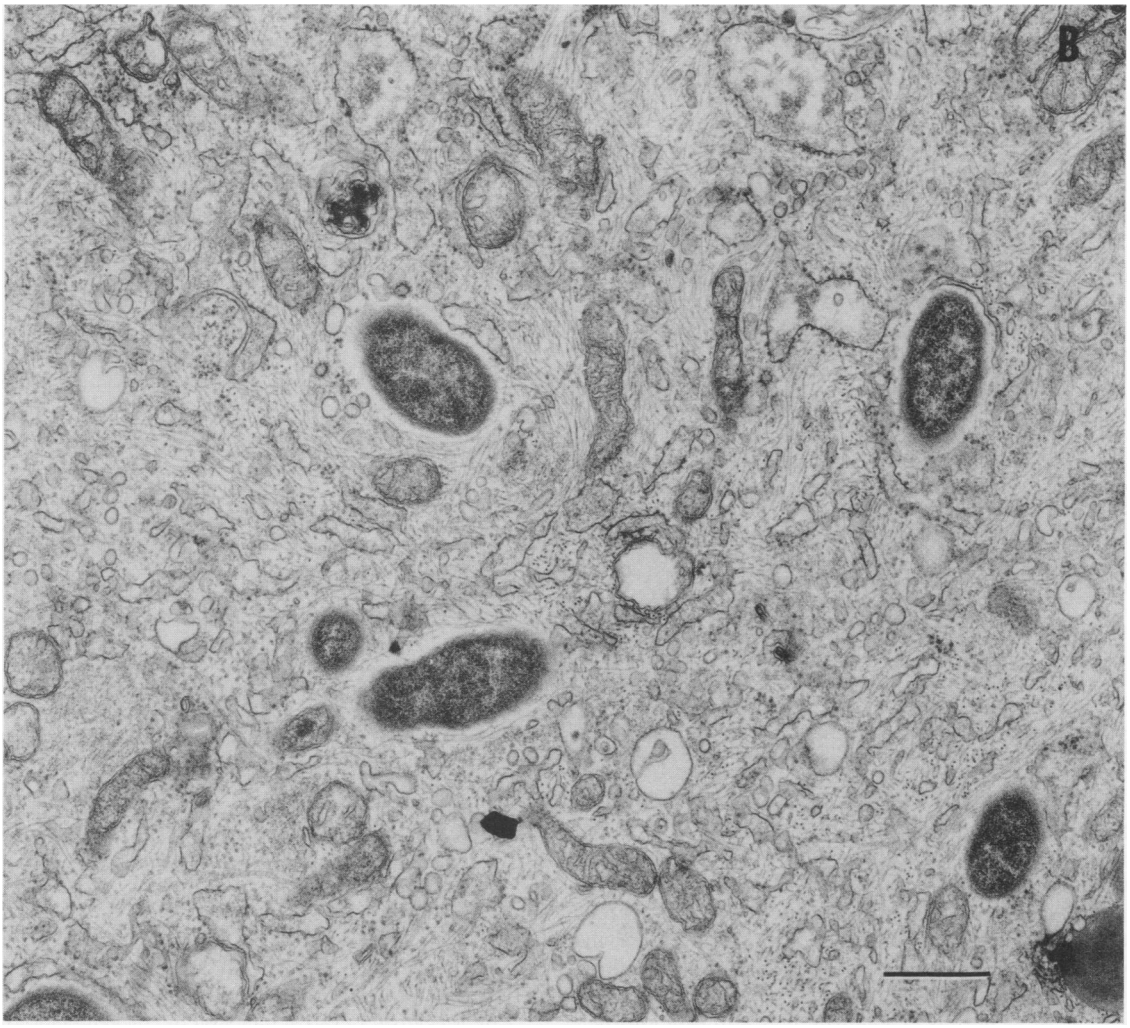

FIG. 4—*Continued.*

were made in human endothelial cells infected by this microorganism (9, 16). In other studies, we showed that *R. rickettsii*-infected cells have increased levels of peroxides (11).

Collectively, these observations have led us to test the hypothesis that injury of host cells by this microorganism is caused by oxidative stress. Endothelial cells infected by this microorganism also have reduced glutathione levels (12), which may compromise the cell's ability to detoxify intracellular peroxides. Restoration of glutathione levels in infected cells by the addition of its dipeptide precursor, gamma glutamylcysteine, resulted in a significant reduction in the size of plaques formed in human endothelial cells without apparent inhibitory activity toward the growth of the bacteria (12).

The glycosaminoglycan heparin has antioxidant activity and the capacity to protect aortic endothelial cells exposed to the free-radical-generating system xanthine-xanthine oxidase (6). Heparin is commonly used in the culture of endothelial cells because it reportedly reduces cell doubling time and increases cell population size (15). Until recently, we did not use heparin for culture of human umbilical vein endothelial cells. When we first introduced heparin into our isolation and culture system, it was necessary to evaluate the effect on growth of *R. rickettsii*. Heparin did not inhibit growth of the microorganism at a concentration of 50 μg/ml but did show some inhibition at 100 μg/ml. Furthermore, upon examination of the treated and untreated cultures with an inverted tissue culture microscope, it was apparent that the cells in the infected population that was treated with heparin (50 and 100 μg/ml) seemed to survive two and sometimes three days longer than cells that were not treated with heparin.

The viability studies and LDH determinations that we carried out supported our observation that heparin prolonged the life of *R. rickettsii*-infected endothelial cell cultures. Therefore, we decided to examine other parameters that could strengthen this observation. The formation of plaques in monolayer cultures is a measure of a microorganism's ability to infect and lyse cells and is routinely used to determine the titers of *R. rickettsii* seed stocks. We tested the effect of heparin on plaque formation by *R. rickettsii* in endothelial cells. Heparin significantly reduced the size, but not the number, of plaques formed. Since heparin, at a concentration of 50 μg/ml, inhibits neither *R. rickettsii* growth nor its capacity to spread from cell to cell, the

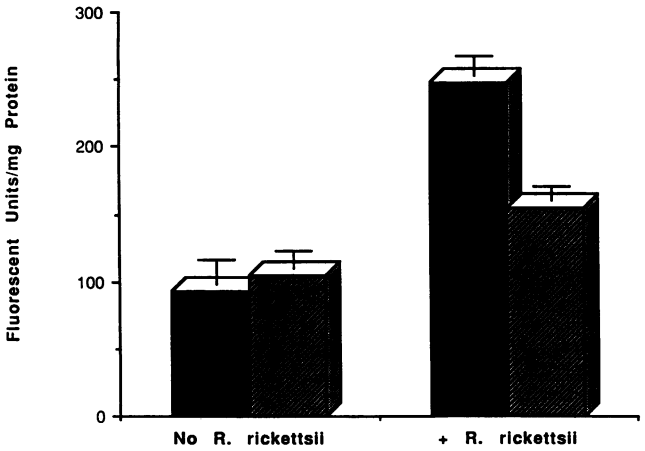

FIG. 5. Intracellular peroxide levels in human endothelial cells infected for 48 h by *R. rickettsii* in the presence and absence of heparin. Solid bars, endothelial cells grown in the absence of heparin; hatched bars, endothelial cells grown in the presence of 50 μg of heparin per ml. Data represent the mean values plus or minus the standard error of at least four determinations.

reduced plaque size apparently could not be attributed to an inability of the microorganism to spread to and injure adjacent cells. Electron microscopic observations provided the initial impetus for investigation of oxidative injury in *R. rickettsii*-infected endothelial cells. These studies showed that heparin is capable of modifying the characteristic injury seen in infected cells as indicated by reduction in the degree of distention of the ER. Finally, heparin is effective in reducing the elevated levels of peroxides that are formed in *R. rickettsii*-infected cells (11).

The mechanism of action of heparin as a putative antioxidant is unknown. However, heparin binds to endothelial cells and is internalized by endocytosis (1, 5, 7). The purported antioxidant activity of heparin could reduce cell injury by exerting action through one of several mechanisms: (i) it may directly detoxify substances, such as peroxides; (ii) it may act as a free-radical scavenger in cells to neutralize superoxide or hydroxyl radical, or (iii) it may prevent the formation of lipid peroxides by inhibiting an intermediary step in their formation. Further study of the activity of heparin is needed before such speculations can be verified.

Rocky Mountain spotted fever is effectively treated with either the tetracyclines or chloramphenicol when diagnosed early. However, the disease can be rapidly progressing in untreated cases or in misdiagnosed cases in which therapy is instituted too late. Our results suggest that oxidative injury is, in part, responsible for damage to the endothelium after infection by *R. rickettsii*. The use of an antioxidant in humans might be desirable adjunctive therapy which can be substantiated in an appropriate animal model.

ACKNOWLEDGMENT

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