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Superoxide Dismutase-Dependent, Catalase-Sensitive Peroxides in Human Endothelial Cells Infected by *Rickettsia rickettsii*

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The generation and intracellular accumulation of reactive oxygen species have been shown to be associated with the infection of human umbilical vein endothelial cells (HUVEC) by *Rickettsia rickettsii*. In response to the oxidant superoxide, the activity of the enzyme superoxide dismutase (SOD) increases following infection by this obligate intracellular bacterium. Other oxidants which are capable of oxidizing the fluorescent probe 2′,7′-dichlorofluorescin (DCFH) also accumulate intracellularly within infected cells. In the study reported here, we show that (i) an inhibitor of SOD, diethyldithiocarbamic acid, reduces the observed rise in SOD activity in infected cells by 40 to 60% and at the same time reduces the degree of intracellular oxidation of DCFH; (ii) catalase-sensitive peroxides can be detected in supernatants of *R. rickettsii*-infected cells shortly after rickettsial exposure; and (iii) fluorescence-activated cell sorter analysis demonstrates significant intracellular oxidant activity in infected cells within 5 h after exposure to *R. rickettsii*. The results of these experiments indicate that hydrogen peroxide is a major oxidant associated with infection of HUVEC by *R. rickettsii* and that intracellular oxidant activity sensitive to SOD inhibition is detectable early and prior to significant rickettsial multiplication and much earlier than the ultrastructural manifestations of cell injury seen by electron microscopy.

Rickettsia rickettsii is an obligate intracellular bacterium and the causative agent of Rocky Mountain spotted fever in humans. The clinical manifestations and pathology of this disease have been well documented (1, 6–8, 11, 18–20). However, the structural and metabolic properties of *R. rickettsii* which contribute to its pathogenesis remain unclear. The putative target cells in naturally acquired human infection are endothelial cells. For the past several years, our laboratory has examined the interaction of this rickettsia with human umbilical vein-derived endothelial cells (HUVEC), which in culture retain properties of endothelium in vivo. As a result of these collective studies, we believe that there is strong evidence to suggest that reactive oxygen species play a role in *R. rickettsii*-induced cellular injury (13–16). Among the studies which seem to corroborate this theory are the detection of superoxide in endothelial cell supernatants during internalization of *R. rickettsii* and elevation in the levels of intracellular superoxide dismutase (SOD) following infection (12).

Hydrogen peroxide represents a major oxidant that may contribute to cell injury. Peroxides increase in endothelial cells infected by *R. rickettsii* as a function of time postinfection (15). In previous studies, peroxides were first detected at 24 h postinfection of HUVEC. Earlier time points were not tested because of the lack of sensitivity of the assay used. In the present study, however, utilizing the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and fluorescence-activated cell sorter analysis (FACS), we can now show significant intracellular oxidant activity within hours after rickettsial contact with HUVEC. This observation suggests for the first time that relatively small numbers of rickettsiae (and perhaps the lack of significant rickettsial multiplication) are sufficient for oxidant induction to occur.

Initial studies indicating the presence of peroxides within infected cells were relatively nonspecific in that they could not distinguish between hydrogen peroxide and lipid peroxides (15). DCFH is capable of being oxidized not only by the former but also by the latter (3). By incorporating the SOD inhibitor diethyldithiocarbamic acid (DDC) into the current studies, we now have determined that a significant proportion of the peroxides present in *R. rickettsii*-infected HUVEC is hydrogen peroxide. Also, in a previous study (12) we reported the release of superoxide to culture supernatants following exposure of endothelial cells to *R. rickettsii*. We now report the detection of catalase-sensitive extracellular peroxide (hydrogen peroxide) within several hours after exposure of endothelial cells to *R. rickettsii*. The data presented in this study extend previous observations that reactive oxygen species, and especially peroxides, are associated with *R. rickettsii* infection of endothelial cells. The data resulting from use of the SOD inhibitor DDC and the detection of catalase-sensitive peroxides in infected cell supernatant fluids confirm that hydrogen peroxide is a major oxidant produced during infection of HUVEC by *R. rickettsii.*

MATERIALS AND METHODS

Isolation and culture of endothelial cells. Endothelial cells were isolated from freshly acquired human umbilical veins by a modification of the method of Gimbrone as previously described (4). Cells were removed from the umbilical vein by using a 0.1% type I collagenase solution (Gibco BRL, Grand Island, N.Y.) prepared in phosphate-buffered saline. They were then cultured on the surface of 60-mm-diameter Nunclon tissue culture dishes (Nunc, Kamstrup, Denmark) in McCoy's 5A medium (Gibco BRL) supplemented with 20% heat-inactivated fetal bovine serum (Upstate Biotechnology Inc., Lake Placid, N.Y.), 30 μg of H-Neurext endothelial cell growth supplement (Upstate Biotechnology Inc.) per ml, and 50 μg of sodium heparin (Upstate Biotechnology Inc.) per ml. Cultures were grown to confluent monolayers in a humidified atmosphere at 5% CO_2–95% air at 37°C. Only first- or second-passage cells were used in experiments.

Rickettsiae. The rickettsial seed used in these studies was a plaque-purified isolate of the Sheila Smith strain of *R. rickettsii*, which was propagated in C1008 Vero cells (American Type Culture Collection, Rockville, Md.). After harvest, the infected Vero cell preparation was passed through a sterile 25-gauge needle (Becton Dickinson & Co., Franklin Lakes, N.J.) in order to break up clumps of cells and rickettsiae and to release additional rickettsiae from fragile cells and

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cell debris. This suspension was centrifuged at low speed (800 rpm for 2 min), which removes most of the host cell debris, leaving a semipurified rickettsial preparation in the supernatant fraction. Enumeration of these rickettsial seeds was carried out by plaque assay using a modification of the method of Wike and Burgdorfer (22). Aliquots of the rickettsiae were used to infect HUVEC at a dose of 0.5 rickettsia per cell unless otherwise noted. The rickettsiae were added directly to the culture medium and incubated for 1 h at 37°C. After 1 h, the inoculum was removed and fresh culture medium was added to the cells. In studies described below with the SOD inhibitor DDC, HUVEC were preincubated for 1 h in McCoy's 5A medium with the inhibitor, where appropriate, at the specified concentrations. Rickettsiae were added and removed after 1 h of incubation, and the medium was replaced with fresh McCoy's which contained the specified concentrations of DDC. The culture medium containing the inhibitor was replaced with fresh medium without inhibitor after an additional incubation period of 4 h.

Measurement of SOD activity in HUVEC. SOD was assayed by the method of Oberley and Spitz (10), using the reduction of nitroblue tetrazolium. The reaction solution contained final concentrations of 10^{-4} M xanthine (freshly prepared every week), 5.6×10^{-5} M nitroblue tetrazolium (stored in brown bottle), 1 mM diethylenetriaminepentaacetic acid (DETAPAC), and 1 U of catalase per ml, all prepared in 0.05 M potassium phosphate buffer (pH 7.8). From this reaction buffer aliquots of 0.8 ml were placed in four-sided clear cuvettes (Fisher Scientific, Pittsburgh, Pa.). Blanks (without SOD) were set up by adding 100 μl of the 0.05 M phosphate buffer to each of at least five cuvettes. At least five dilutions of xanthine oxidase (1.3 U/mg of protein) were prepared in phosphate buffer with 1.33 mM DETAPAC at the time of the assay and added to the blanks in 100-μl amounts. The rate of change in absorbance at 560 nm was then recorded for 10 min at 1-min intervals on a Beckman DU 650 spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). That dilution of xanthine oxidase which effected an absorbance rate between 0.015 and 0.025 optical density unit/min was used for all subsequent standards and lysate samples in the experiment. To establish a standard curve of SOD activity, various amounts (1 to 1,000 ng) of pure SOD from bovine erythrocytes at 4,000 U/mg of solid were added in 100 μl aliquots (in place of the phosphate buffer in the blanks) to the reaction mixture. To prepare HUVEC samples for SOD determination, cells were first trypsinized from the culture dishes, washed once by centrifugation to remove trypsin, and resuspended in 0.5 ml of phosphate buffer. The cells were sonicated for 1 min, using four 15-s bursts in a model 450 Sonifier (no. 10 output) at a constant pulse (Branson Instruments, Danbury, Conn.), yielding the cell extract used for SOD analysis. Previous studies in our laboratory have shown that the SOD activity detected in the sonicates originated from host cells and not from disrupted intracellular rickettsiae (12). All chemicals and enzymes used in the reaction mixture were obtained from Sigma Chemical Co., St. Louis, Mo.

Studies with the SOD inhibitor DDC. HUVEC were treated with the sodium salt of DDC (Sigma), a copper-chelating agent, to inactivate SOD. Initially, acceptable concentrations of DDC in which the uptake and spread of *R. rickettsii* in endothelial cells were not affected had to be determined. We monitored the effects of various concentrations of DDC on infected and uninfected HUVEC by pretreating the cells for 1 h with the appropriate test dilutions of DDC in the tissue culture medium and maintaining exposure to the inhibitor through the duration of the experiment. Cells were stained by the method of Gimenez (5) after 0, 24, and 48 h to assess the progression of the infection. A minimum of 200 endothelial cells per time point and condition were examined by light microscopy to determine the average number of rickettsiae per infected cell as well as the percentage of cells infected. DDC at concentrations of 10^{-4} M or higher was shown to be toxic to rickettsiae. A similar conclusion was reached from studies on plaque formation in Vero cells, in which no plaques could be observed in wells treated at those concentrations. In addition, we found that the Vero cell monolayers in the plaque assay became increasingly disrupted as the dose of DDC was increased. DDC at concentrations of 10^{-5} and 10^{-6} M had no effect on either rickettsial growth, as determined by Gimenez-stained preparations, or plaque-forming ability. We thus chose 10^{-5} and 10^{-6} M as concentrations for use in our studies.

Analysis of intracellular oxidant levels in HUVEC. Intracellular oxidant levels in uninfected and infected endothelial cells were determined by using the dye incorporation studies of Carter et al. (2), employing the carboxylated form (C-400) of DCFH-DA (Molecular Probes Inc., Eugene, Oreg.). DCFH-DA freely diffuses across cell membranes, is diacetylated, and incorporates into hydrophobic lipid regions of the cell (2). After the appropriate amount of time following rickettsial infection, endothelial cells were exposed to the dye at a concentration of 1 μM. Two milliliters of this DCFH-DA–phosphate-buffered saline solution was sufficient to cover the cell monolayer in a 60-mm-diameter culture dish. Endothelial cells were incubated with the dye at 37°C for 15 min. After incubation, the dye was aspirated and the cells were trypsinized and washed once by centrifugation at 1,000 rpm for 5 min to remove trypsin and extracellular DCFH-DA. HUVEC from each 60-mm-diameter culture dish were resuspended in 0.5 ml of 1% paraformaldehyde, kept cold, and protected from light until ready for analysis on a FACScan flow cytometer (Becton Dickinson & Co.) set at 488-nm excitation. Emission filters were 530/30-nm bandpass. For each tube containing a total cell volume of 0.5 ml from a single culture dish, approximately 20,000 cells were collected and analyzed. To exclude extraneous cell debris and dead cells, a gate was created around the main live cell population based on the forward versus side scatter distribution. In this way, only viable cells were evaluated for fluorescence and applied to fluorescence histograms.

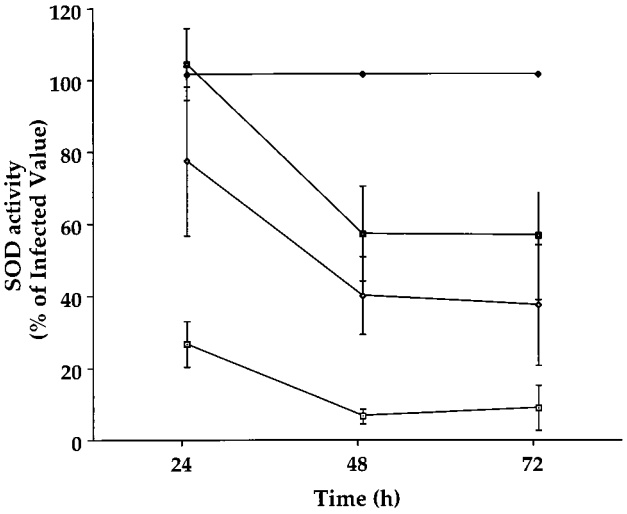

FIG. 1. Relative percentage SOD activity in control (⊡) (uninfected, untreated) and infected, DDC-treated (■, ◇) endothelial cells in comparison to untreated (◆) cells infected with *R. rickettsii*. SOD concentrations found in untreated, infected cells were set at 100%. The data presented represent mean values ± the standard errors of at least three separate experiments at each time point.

Extracellular oxidants and the effects of catalase and glutathione peroxidase. Extracellular oxidants produced or released into the culture medium following exposure of HUVEC to *R. rickettsii* were measured by using DCFH, the reduced, nondiacetylated form of the probe. Confluent monolayers of HUVEC were grown as previously described. Prior to infection with *R. rickettsii* (inoculum of approximately 6 rickettsiae per HUVEC), the culture medium was removed and replaced with Hanks' balanced salts solution (HBSS) without phenol red and containing horseradish peroxidase (Sigma) at a concentration of 2 U/ml. Those samples to which catalase was added received 100 μl of a stock catalase solution containing 6.09×10^4 U/ml prepared in HBSS. In other conditions, either 100 μl of a 5×10^2-U/ml stock solution of glutathione peroxidase (Sigma) was added in lieu of the catalase or 100 μl of catalase and 100 μl of glutathione peroxidase were added to the samples. The infections were carried out at 37°C for 1 h. After this time, the supernatants were aspirated from the dishes and either (i) fresh HBSS with or without catalase or (ii) glutathione peroxidase was added. A 2 mM stock solution of DCFH was prepared in absolute ethanol. This stock solution was further diluted to 10 nM in HBSS, and 10 μl was added to each dish (preparation of the preceding solutions was carried out in relative darkness, using a red, no. 1A photographic filter due to DCFH's sensitivity to light). The cells were returned to the incubator for 4 h. Following this incubation, the supernatants from each dish were transferred to microcentrifuge tubes and centrifuged for 2 min at 13,000 rpm in a Beckman Microfuge II to remove cell debris and extracellular rickettsiae. The supernatants were decanted into disposable Ultravu cuvettes (Fisher Scientific), and the fluorescence of the samples was measured in an MPF-66 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) operating at an emission wavelength of 535 nm and an excitation wavelength of 505 nm. Peroxide levels were expressed as fluorescence units per 10^6 cells.

RESULTS

Inhibition of SOD by DDC in infected endothelial cells. Following infection by *R. rickettsii*, endothelial cells show significant increases in the levels of SOD activity. These increases specifically can be prevented by pretreating cells prior to infection with DDC, an inhibitor of SOD. The effectiveness of DDC is dose dependent, but the compound has its limitations in our system since at higher doses, it is toxic to *R. rickettsii*. Figure 1 shows the kinetics of inhibition of SOD by DDC. The effectiveness of the DDC treatment regimen is expressed as a percentage of those values achieved from infected non-DDC-treated samples. Uninfected endothelial cells were used as

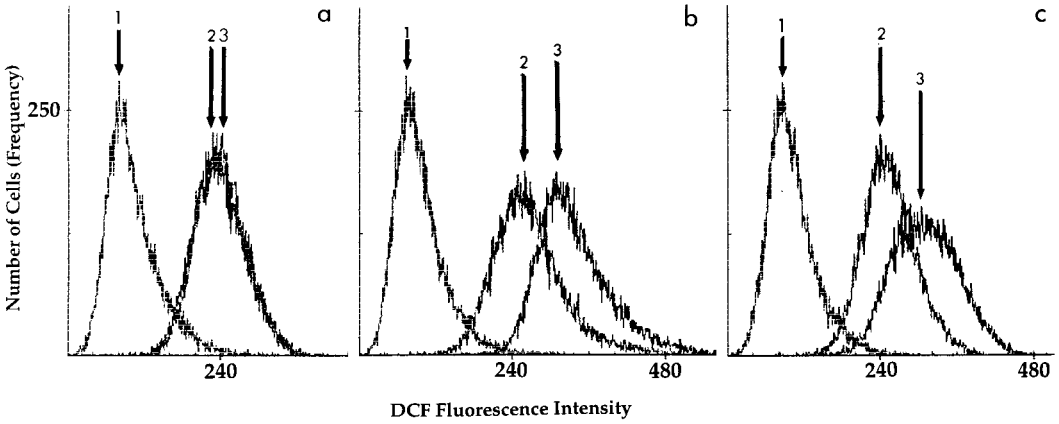

FIG. 2. Representative FACScan fluorescence histograms constructed from HUVEC loaded with DCFH-DA and analyzed at 1 (a), 5 (b) and 24 (c) h after infection with *R. rickettsii*. Cells were incubated with 1 μM DCFH-DA at 37°C for 15 min and fixed in 1% paraformaldehyde before being analyzed on a FACScan flow cytometer. Excitation was 488 nm and emission was collected at 530/30 nm. Peak 1, unstained, uninfected endothelial cells; peak 2, uninfected endothelial cells stained with DCFH-DA; peak 3, *R. rickettsii*-infected endothelial cells stained with DCFH-DA. Each panel is a composite of separate runs, and the histogram represents the DCFH fluorescence of approximately 20,000 endothelial cells.

control cell populations. At 24 h postinfection, DDC concentrations of 10^{-5} and 10^{-6} had no statistically significant effect on SOD activity in infected cells. At 48 h postinfection, however, both concentrations of DDC reduced SOD levels below that of infected untreated samples, 10^{-6} M by about 40% and 10^{-5} M by about 60%. Inhibitory levels similar to those achieved at 48 h were maintained at 72 h postinfection. Baseline levels of SOD activity in uninfected untreated endothelial cells throughout the duration of the experiments ranged from 9 to 27% of those in the infected untreated samples. DDC treatment at the levels used did not affect the kinetics of rickettsial infection. On average, the number of rickettsiae per infected cell and the percentage of cells infected (value in parentheses) as determined by Gimenez staining were, at 24, 48, and 72 h, respectively, 3 (65), 10 (100), and 15 (100) in both treated and untreated populations.

Demonstration of intracellular oxidants in *R. rickettsii*-infected endothelial cells by FACS analysis. Oxidation of DCFH by hydrogen peroxide and lipid peroxides can be measured by FACS analysis as well as by spectrofluorometric methods. An approximate relationship exists between the intensity of fluorescence of a cell population and the amount of hydrogen peroxide or other hydroperoxides produced intracellularly. The SOD inhibitor DDC, also can be used to determine the contribution of newly formed hydrogen peroxide to this fluorescence. Figure 2 shows representative histograms comparing the fluorescence of oxidized DCFH in uninfected endothelial cells with the fluorescence in cells infected by *R. rickettsii* at 1, 5, and 24 h postinfection. Figure 2a is a histogram comparing the fluorescence of infected and uninfected cells 1 h postinfection. The mean fluorescences of the uninfected and infected cell populations at this time interval are 241 and 237, respectively, and the peaks overlap nearly perfectly, indicating little or no difference between the two cell populations. At 5 h postinfection (Fig. 2b), there was a significant difference in the mean fluorescences observed between uninfected and infected cells. The uninfected population had a mean fluorescence value of 266, while that of the infected cell population was 337. Aside from the difference in mean fluorescence between uninfected and infected cells, the two peaks were uniform in size and shape, indicating that the two populations were fairly similar in fluorescence distribution. At 24 h after infection by *R. rickettsii*, the difference in mean fluorescence between uninfected and infected populations was about the same as at 5 h (253 and 310, respectively), but the shapes of the peaks, relative to one another, changed, with the infected cell population peak becoming shorter and more broad.

Effect of DDC on oxidant-mediated fluorescence generated in *R. rickettsii*-infected endothelial cells. The inhibitory effect of DDC on SOD translated into reduced fluorescence as measured by FACS analysis (Fig. 3). At 1 h postinfection, the difference in fluorescence between infected and uninfected endothelial cells, as well as DDC-treated cells, was not significant. At 5 h postinfection, both concentrations of DDC noticeably reduced the fluorescence intensity in the infected cell populations: 10^{-6} M DDC by about 4% ($P < 0.025$) and 10^{-5} M DDC by about 12% ($P < 0.003$). Uninfected cell samples at this time period had about 10% less fluorescence than the corresponding infected cell populations. At 24 h postinfection,

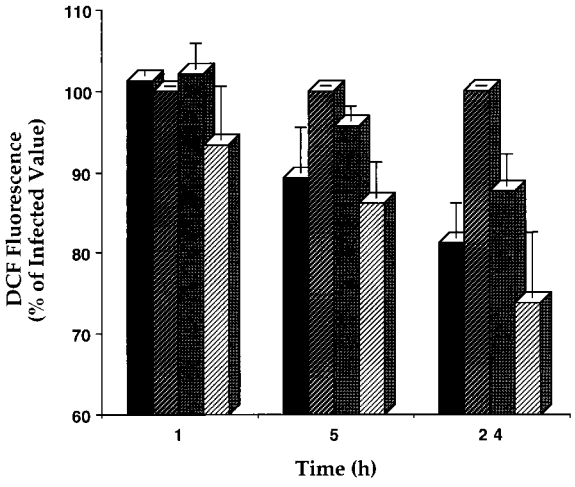

FIG. 3. Relative percentage of DCFH fluorescence in *R. rickettsii*-infected endothelial cells not treated with DDC (column 2), infected endothelial cells treated with 10^{-6} and 10^{-5} M DDC (columns 3 and 4, respectively), and uninfected, untreated endothelial cells (column 1) at 1, 5, and 24 h. DCFH fluorescence found in untreated, infected cells (column 2) was set at 100%. The data presented represent mean values ± standard errors of at least four separate experiments at each time point.

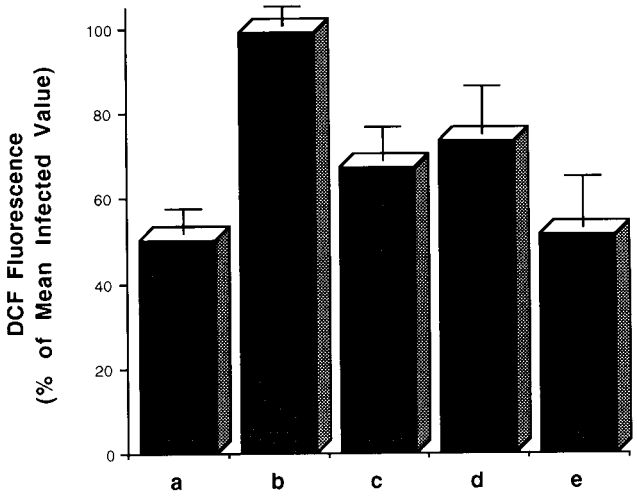

FIG. 4. Relative percentage of DCFH fluorescence in supernatant fluids of uninfected, untreated control (column a) and *R. rickettsii*-infected (column b) endothelial cells in comparison to catalase-treated infected cells (column c), glutathione peroxidase-treated infected cells (column d), and infected cells treated with both catalase and glutathione peroxidase (column e). DCFH fluorescence was normalized against the untreated infected cell mean, set at 100%. The data represent mean values ± standard error of the means of two separate experiments with three determinations at each condition. Statistically significant values compared with (column b), the untreated infected cell population: (columns a, c, and e, $P < 0.01$; column d, $P < 0.05$.

the differences in fluorescence became progressively greater. At a concentration of 10^{-6} M DDC, cell fluorescence was 12% less than that found in the untreated infected cell population, while at 10^{-5} M DDC, there was a 25% reduction in fluorescence. The difference in fluorescence between infected and uninfected cell populations at this time point was about 17%.

Effects of catalase and glutathione peroxidase on oxidant-mediated fluorescence observed in culture supernatants of *R. rickettsii*-infected endothelial cells. To determine whether hydrogen peroxide and/or lipid peroxides were responsible for the increased oxidant-mediated fluorescence observed in culture supernatants of cells infected by *R. rickettsii* (Fig. 4), catalase or glutathione peroxidase was added to the cells prior to and during exposure of the cells to the rickettsiae. After a 4-h incubation in the presence of catalase, fluorescence of the supernatant fluids was reduced to about 35% of that of the infected cell population (and to approximately 15% above the background fluorescence of uninfected cell supernatants), suggesting that hydrogen peroxide was largely responsible for oxidation of DCFH. Glutathione peroxidase by itself in infected supernatants reduced fluorescence to about the same levels as that of catalase and, when added in combination with catalase, although further reducing fluorescence slightly, caused no statistically significant change compared with either enzyme alone.

DISCUSSION

Previous studies have shown that superoxide radical is released to the culture medium following incubation of *R. rickettsii* with human endothelial cells (12). Because this phenomenon occurs soon after exposure of the cells to the rickettsiae, we believe that its generation may represent part of the rickettsial internalization process. This oxidative burst may, in some respects, parallel that seen with professional phagocytes, one major difference being that the rickettsiae may be only transiently enclosed within an intact phagocytic vesicle (17). In that light, the release of superoxide to the extracellular space may reflect an abortive attempt by the endothelial cell to phagocytize the rickettsiae.

Shortly after exposure and internalization of *R. rickettsii* into endothelial cells, there is a rapid increase in the level of SOD (12), a metalloenzyme which scavenges the superoxide radical and converts it to hydrogen peroxide. Cellular increases in SOD typically occur in response to increased levels of intracellular superoxide. Although our previous experiments (because of technique limitations) have detected only extracellular superoxide, this radical could conceivably find its way into the cytoplasmic compartment by at least one of two mechanisms. It could be released directly into the cell cytoplasm during internalization of the rickettsiae, that is, follow the rickettsiae in, or it could be generated intracellularly as a by-product of rickettsial or host cell metabolism.

It has previously been demonstrated that HUVEC infected by *R. rickettsii* show elevated levels of intracellular peroxides (15). It was postulated, although not proven, that these peroxides were the result of increased cytoplasmic superoxide. In the present study, we have used the SOD inhibitor DDC to determine both its capacity to reduce the activity of SOD in *R. rickettsii*-infected HUVEC and to examine its effects on the levels of intracellular peroxides. As expected, the levels of SOD in infected HUVEC postinfection were 4- to 10-fold higher than in uninfected control cells over a 72-h time period. The typical in vitro experimental dose of DDC used to inhibit SOD is 10^{-2} M. However, in our hands, concentrations greater than 10^{-5} M DDC (for example, 10^{-4} M) were toxic to *R. rickettsii*. and could not be used. Concentrations of 10^{-5} and 10^{-6} M, however, were not toxic and despite the potential limitations because of the lower usable concentrations were used in the experiments. DDC was clearly effective in reducing the activity levels of SOD in infected HUVEC by as much as 60%. However, total inhibition to uninfected cell baseline levels was not achieved, possibly because the concentrations of the inhibitor that were used were not high enough to completely block enzyme activity. It should be pointed out that DDC, although clearly an efficacious SOD inhibitor, is also a modestly good reducing agent as well as a free radical scavenger (9). Nevertheless, its ability to directly inhibit SOD activity in the experiments reported here provides justification for its use.

Our previous study reporting the accumulation of intracellular peroxides in *R. rickettsii*-infected HUVEC was carried out by using spectrofluorometric analyses (15). The earliest sample examined in this study was taken at 24 h postinfection and the last at 72 h. In the present study, we have used a more sensitive technique (FACS analysis) to determine whether oxidant activity could be detected at earlier time points. The results of our experiments showed that there was no increase in oxidant activity in infected HUVEC 1 h after rickettsial exposure with the inoculum size that was used (0.5 rickettsia/HUVEC) as measured by DCFH fluorescence. However, within 5 h postexposure, there was a distinct shift in mean fluorescence between infected and uninfected cell populations, indicative of oxidant activity. The shift to the right in mean fluorescence was also observed at 24 h postinfection. The characteristic nature of this peak suggests that the infected cell population has a greater percentage of cells with a higher degree of fluorescence. The broadness of the peak indicates an increased heterogeneity in fluorescence among the cell population compared with the infected cell population at 5 h.

The effect of DDC on the mean fluorescence produced in these infected HUVEC populations also was examined by

FACS analysis. At a concentration of 10^{-6} M, DDC was not effective in reducing the mean fluorescence. This correlated well with the inability of DDC at this concentration to inhibit SOD activity at 24 h postinfection as described above. The inhibitor at a concentration of 10^{-5} M was marginally effective at reducing DCFH fluorescence at 5 h and was more effective by 24 h. It is possible that the limited ability of DDC to inhibit SOD and to reduce intracellular fluorescence due to oxidant activity again is the result of the relatively low concentrations of DDC that can be used in these experiments because of toxicity for *R. rickettsii*. It may equally suggest that lipid peroxides, which are not sensitive to SOD inhibition but also are capable of oxidizing DCFH, contribute to oxidant-mediated fluorescence and potentially to oxidant-mediated cell injury. Nonetheless, these studies do indicate that elevated levels of hydrogen peroxide are present soon after infection of endothelial cells by *R. rickettsii.*

Because of the potential for diffusion of peroxides from the intracellular milieu to the cell surface, it was of interest to determine whether peroxides also could be found in culture supernatant fractions after exposure of endothelial cells to *R. rickettsii*. Since lipid peroxides as well as hydrogen peroxide are capable of oxidizing DCFH, catalase, and glutathione peroxidase were used to help monitor the specificity of oxidation of DCFH. The addition of catalase to endothelial cell cultures prior to rickettsial exposure reduced fluorescence by 35% compared with untreated infected samples and to within 15% of the baseline levels of fluorescence of uninfected cells, suggesting that hydrogen peroxide was the major oxidant present in the extracellular milieu. The inability of catalase to neutralize fluorescence totally to baseline levels (the additional 15%), however, also suggested that lipid peroxides may be present since these peroxides also are capable of oxidizing DCFH (3). The addition of glutathione peroxidase which detoxifies hydrogen peroxide as well as lipid peroxides had no effect in reducing fluorescence beyond that observed with catalase alone, nor when it was used in conjunction with catalase was fluorescence reduced significantly beyond that achieved with either enzyme alone. Thus, the identity of the oxidant responsible for the remaining small percentage of nonneutralizable fluorescence could not be determined through use of these enzymes. Even though superoxide is apparently generated at the time of rickettsial internalization, in these experiments, the inoculum was removed prior to the addition of DCFH and 4 h prior to fluorescence analysis, a time at which superoxide is not detected at other than baseline levels in the supernatant (unpublished observations). Superoxide also reportedly does not oxidize DCFH (23) and therefore would not contribute to fluorescence of the samples.

Nitric oxide (formation of peroxynitrite from superoxide and nitric oxide) should be mentioned as a possible contributor to oxidant-mediated injury caused by *R. rickettsii*. Previous studies from our laboratory (unpublished data), however, have shown no increase in nitrite levels in infected HUVEC, suggesting that nitric oxide does not play a major role in the in vitro cell injury caused by *R. rickettsii*. Walker et al. (21) have shown that nitric oxide is produced in interferon gamma and tumor necrosis factor alpha-stimulated murine endothelial cells infected by *R. conorii* (a member of the spotted fever group of rickettsiae) and is rickettsiacidal to these organisms, while in a human endothelial cell line, hydrogen peroxide, and not nitric oxide, is involved in the killing of these rickettsiae. How these studies may relate to ours is not yet clear, although it can be said with some confidence that reactive oxygen species appear to play an important role in the rickettsia-host cell interactions.

In summary, the experimental results reported here indicate that cells infected by *R. rickettsii* exhibit early intracellular oxidant activity and that hydrogen peroxide is present in infected cell populations at significantly high levels. The presence of high levels of intracellular hydrogen peroxide, and presumably superoxide, because of elevated levels of SOD is highly suggestive of the potential for oxidant-mediated cell injury. While the specific role that these reactive oxygen species play in contributing to endothelial cell injury remains speculative, their very presence at abnormally high levels, together with other supporting data (12, 14–16), reenforces the likelihood that they contribute to cell injury. In support of this statement, studies currently in progress in our laboratory demonstrate that restoration of antioxidant levels in infected HUVEC (including glutathione peroxidase and reduced glutathione [thiols]) with the natural antioxidant α-lipoic acid has the effect of decreasing the levels of intracellular peroxides and increasing the longevity of infected cell populations (3a).

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