INFECTION AND IMMUNITY, June 1994, p. 2619–2621
0019-9567/94/$04.00+0

Vol. 62, No. 6

Infection of Human Endothelial Cells by *Rickettsia rickettsii* Causes a Significant Reduction in the Levels of Key Enzymes Involved in Protection against Oxidative Injury

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Received 24 January 1994/Returned for modification 18 February 1994/Accepted 30 March 1994

The activities of glucose-6-phosphate dehydrogenase, catalase, and glutathione peroxidase were significantly decreased in human endothelial cells infected with the obligate intracellular bacterium *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever. This observation lends additional support to our hypothesis implicating oxidative damage in endothelial cell injury caused by this microorganism.

Previous studies from our laboratory have suggested that oxidative mechanisms play a role in injury to human endothelial cells infected by the obligate intracellular bacterium *Rickettsia rickettsii* (10, 12–14). Initial observations from transmission electron microscopic studies consistently showed extensive dilatation of the rough-surface endoplasmic reticulum–outer nuclear envelope complex following infection with *R. rickettsii* (12). In a subsequent study we demonstrated increased levels of intracellular peroxide in endothelial cells during infection by this microorganism (13). Concomitantly, we observed an early and sustained increase in the level of the enzyme superoxide dismutase, a scavenger of superoxide radicals (10), as well as a reduction in the levels of intracellular glutathione (14). In a later study (10), we found that when a certain ratio of *R. rickettsii* to endothelial cells was used for infection, significant quantities of superoxide radical were released into the culture medium.

In the present study, we have selected three specific enzymes considered to be important in normal cellular defenses against active oxygen species to determine how infection of endothelial cells by *R. rickettsii* affects their endogenous levels. Glucose-6-phosphate dehydrogenase (G-6-PD) was selected since it plays a key role in the generation of reduced glutathione through the hexose monophosphate shunt and since our previous observations had shown a significant reduction in the reduced form of this antioxidant in infected endothelial cells (14). G-6-PD is coupled with another enzyme of the glutathione redox cycle, glutathione peroxidase, which catalyzes the oxidation of reduced glutathione and, simultaneously, the conversion of toxic peroxides to innocuous by-products.

A third enzyme, catalase, was selected for study for two reasons: (i) it is capable of specifically detoxifying hydrogen peroxide, a potential mediator of oxidation, by converting it to water, and (ii) it was previously reported to be present only in negligible amounts in human umbilical vein endothelial cells (11). We sought not only to confirm this latter finding in uninfected cells but also to determine the levels of catalase in endothelial cells infected by *R. rickettsii*.

We found that the activity of all three enzymes was significantly reduced in endothelial cells infected by *R. rickettsii*, suggesting that these cells are compromised in their ability to defend against toxic oxygen species and therefore are vulnerable to oxygen-mediated injury. These results lend additional support to our hypothesis that oxygen radicals contribute to endothelial cell injury sustained following infection by *R. rickettsii*.

Endothelial cells were isolated from freshly acquired human umbilical veins by a modified method of Gimbrone (3) as described previously (13). Confluent endothelial cell monolayers in 35-mm-diameter Nunclon dishes were infected with an average of 0.5 rickettsia (*R. rickettsii* Sheila Smith) per cell and incubated at 37°C. Uninfected cells served as controls. The degree of infection was monitored by staining the cells by the method of Gimenez (4).

At the end of the experimental period, the medium was aspirated and 0.5 ml of 1% Triton X-100 in distilled H_2O was added directly to the cell monolayer. The cells were scraped and immediately placed into 0.5 ml of cold 10 mM phosphate buffer, pH 7.0. The cells were then lysed by sonication for 30 s, and these sonicates (endothelial cell extracts) were used for each enzyme assay.

G-6-PD activity was determined according to the method described in the *Worthington Manual* (17). The reaction mixture was placed in a 4-ml cuvette and consisted of 2.7 ml of 55 mM Tris-HCl buffer with 33 mM $MgCl_2$ (pH 7.8), 0.1 ml of 6 mM $NADP^+$, and 0.1 ml of 0.1 M glucose 6-phosphate (Sigma). The reaction was initiated by the addition of 0.1 ml of endothelial cell extract. The increase in optical density at 340 nm due to the reduction of $NADP^+$ was measured spectrophotometrically for 4 min at 1-min intervals. Enzyme activity was linear over this time period and was calculated by using the millimolar extinction coefficient of NADPH (6.22). The results are expressed as milliunits of enzyme per milligram of cell protein. One milliunit of G-6-PD is defined as that amount which is capable of reducing 1 nmol of $NADP^+$ per min.

The catalase assay is based upon the spectrophotometric measurement, at 240 nm, of the decomposition of H_2O_2 (1). Briefly, 1 ml of a 10 mM solution of H_2O_2 in 50 mM phosphate buffer, pH 7.0, was placed into a cuvette, and the reaction was started by the addition of 0.1 ml of the endothelial cell extract. The decrease in optical density was monitored for 5 min at 1-min intervals. Enzyme activity was linear over this period and was calculated by using the molar extinction coefficient of H_2O_2 (43.6 M^{-1} cm^{-1}). The results are expressed as milliunits of enzyme per milligram of cell protein. One milliunit of

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TABLE 1. Levels of catalase, glutathione peroxidase, and G-6-PD in human endothelial cells

Time (h)	Level (mU/mg of protein)[a] of:					
	Catalase in:		Glutathione peroxidase in:		G-6-PD in:	
	Uninfected cells	Infected cells	Uninfected cells	Infected cells	Uninfected cells	Infected cells
24	2,139 ± 337	1,820 ± 282	102.34 ± 7.5	58.40 ± 4.8	22.34 ± 0.83	16.84 ± 0.92
48	2,720 ± 250	1,180 ± 160	113.37 ± 7.8	38.87 ± 4.2	25.22 ± 0.77	4.30 ± 0.46

[a] Each value represents the mean ± standard error of the mean from three to five individual experiments. Infected samples had significantly lower values than did their respective controls, at a P value of <0.0001, except in the case of catalase at 24 h, for which the difference is not significant.

catalase is that amount of enzyme that decomposes 1 nmol of H_2O_2 per min.

Glutathione peroxidase was measured according to the method of Gunzler et al. (5). The reaction mixture in each cuvette initially contained 0.9 ml of 50 mM phosphate buffer with 0.5 mM DETAPAC (diethylenetriaminepentaacetic acid) (pH 7.0), 100 μl of either buffer (blank) or cell extract, 20 μl of glutathione (0.1 M), and 20 μl of glutathione reductase (50 U/ml) (Sigma). These were allowed to equilibrate for 10 min before addition of 20 μl of NADPH (4 mM) and 20 μl of *t*-butyl hydroperoxide (3 mM) to start the reaction. The decrease in A_{340} was recorded for 6 min at 1-min intervals. Enzyme activity was linear over this time period and was calculated by using the millimolar extinction coefficient of NADPH (6.22). The results are expressed as milliunits of enzyme per milligram of cell protein. One milliunit of glutathione peroxidase oxidizes 1 nmol of glutathione per min. The protein content of the endothelial cell extracts for all of the above experiments was determined by the method of Smith et al. (15).

The activities of G-6-PD, glutathione peroxidase, and catalase, three key enzymes in cellular defense against oxygen-mediated injury, were all significantly decreased in human endothelial cells following infection with *R. rickettsii* (Table 1). In uninfected endothelial cells, the activity of G-6-PD was about 22 and 25 mU/mg of protein at 24 and 48 h, respectively. Following infection with *R. rickettsii*, there was a modest drop of 28% in activity (to about 17 mU) at 24 h and a very dramatic drop of more than 80% (to about 4 mU) at 48 h postinfection.

The activity of glutathione peroxidase decreased more rapidly during the first 24 h following infection with *R. rickettsii*. The enzyme levels in uninfected endothelial cell extracts at 24 and 48 h were 102 and 113 mU/mg, respectively. By 24 h postinfection, glutathione peroxidase levels decreased by 43% to 58 mU; by 48 h they decreased by 65% (to 39 mU). In contrast to glutathione peroxidase, the activity of catalase remained fairly stable during the first 24 h after infection. By 48 h, however, the activity of this enzyme also decreased significantly, dropping to 57% of the values in corresponding uninfected cells.

Reduction in the activity of all three enzymes between 24 and 48 h postinfection was accompanied by the usual increase in the number of intracellular rickettsiae. Typically, at 24 h after infection, there were 5 to 7 rickettsiae per infected endothelial cell, with about 50% of the cells infected, and at 48 h, there were 10 to 15 rickettsiae per infected cell, with 100% of the cells infected. There was no significant difference in the protein content between infected and uninfected cells at 24 h; however, at 48 h the protein content of infected cells decreased by no more than ≈20%.

The primary objective of this study was to examine the status of selected enzymes involved in protection against oxygen-mediated injury in human endothelial cells infected with *R. rickettsii*. The decrease in activity of G-6-PD in infected cells probably caused the decrease in glutathione levels reported by us previously (14). We speculate that this decrease in G-6-PD levels may be due to an inhibition in its synthesis or to some oxidative modification in enzyme structure, such as carbonyl formation, which has been shown to cause inhibition of G-6-PD activity as well as that of several other enzymes (8). The epidemiological studies of Walker et al. (16) revealed an association between G-6-PD deficiency and Rocky Mountain spotted fever. They have shown that African-American males, 12% of whom are deficient in G-6-PD, experienced a more severe form of the disease than did white males with Rocky Mountain spotted fever. The results of our study with this enzyme appear to support their finding and confirm the importance of G-6-PD in the pathogenesis of rickettsial infection.

Catalase and glutathione peroxidase also protect cells from oxidant-mediated injury. We speculate that the decreased levels of these two enzymes in infected endothelial cells could account for the elevated intracellular peroxide levels (13). In the previous literature, there have been conflicting reports as to what constitutes normal cellular levels of catalase in endothelial cells. Shingu et al. (11) reported that human umbilical vein endothelial cells possess little or no catalase activity, while Jornot and Junod (6) reported significant activity of this enzyme in similar cells. We have found catalase levels in uninfected umbilical vein endothelial cells that are comparable to those of Jornot and Junod (6) (≈3,000 mU/mg of protein) and also similar to those found in bovine aortic endothelial cells (9). At present, we have no explanation for the decrease in catalase activity in infected endothelial cells; however, the possibility of oxidative inhibition by superoxide radical cannot be ruled out (7). The decreased activity of glutathione peroxidase may also be due to inhibition by superoxide radicals (2) or to inhibition of its synthesis.

Whatever the mechanism may be, the decrease of these key antioxidant enzymes could account for the depletion of reduced glutathione and increased intracellular peroxides observed in our earlier studies (13, 14). Further studies involving the measurement of hexose monophosphate shunt activity, the enzymes of glutathione synthesis, and glutathione reductase may further clarify the role of oxidative damage and antioxidant defenses in the pathogenesis of endothelial cell injury by *R. rickettsii*.

Furthermore, if this hypothesis can be verified in a relevant animal model system (a goal we intend to pursue), then the use of adjunct antioxidant therapy may become warranted in the treatment of Rocky Mountain spotted fever.

This work was supported by Public Health Service grant AI-17416 from the National Institute of Allergy and Infectious Diseases (to D.J.S.).

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