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GENERATION OF DAUNOMYCIN RADICALS ON THE OUTER SIDE OF THE ERYTHROCYTE MEMBRANE

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The generation of the daunomycin semiquinone was studied in intact red blood cells under CO atmosphere by ESR spectroscopy. The undialyzed hemolysates and the spin broadening agent chromium oxalate quenched the ESR signal, suggesting external location of the ESR-detectable radicals and their slow diffusion inside. A constant outward flow of O_2^- was detected by monitoring the approach to the steady state of the ESR signal of Cu,Zn superoxide dismutase externally added to red blood cells plus daunomycin in air. This suggests a reductase on the outer side of the erythrocyte membrane as the source of daunomycin radicals.

The antitumor activity and the undesired side effects of anthracycline antibiotics (daunomycin and adriamycin) have been related to their involvement in enzyme-catalyzed redox cycling with the formation of free semiquinone radicals and noxious oxygen species (1). By means of ESR spectroscopy, all these radicals have been shown to be formed by various cytosolic and membrane reducing enzymes or intact cells (1-2). Moreover, results with agarose-coupled adriamycin point to the cell surface as a site of anthracycline activation (3), although they were obtained from cytotoxicity experiments lacking any direct spectroscopic evidence. Recent ESR studies, although clearly demonstrating, by the use of spin trapping

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agents, that adriamycin-induced O_2^- and OH· radicals were produced outside tumor cells (4-6), gave no indication on whether the site of anthracycline semiquinone production was intra- or extracellular, mainly because the spin adducts formed are relatively unstable species and freely diffusible through membranes. Studies on complex cells are rendered complicated by the presence of internal membrane systems and by the likely occurrence of different sites of radical formation; it can therefore be an advantage to use a system like the red blood cell, which lacks internal membranes and where the contribution of hemoglobin as intracellular redox activator for quinonoid drugs can be ruled out by its convertion into carbomonoxy hemoglobin (7). By proper use of CO flushing, the ESR spectrum of the daunomycin radical could for the first time be detected in intact erythrocytes and isolated red blood cell membranes (8). In the present report we show that the erythrocyte is a convenient model system to investigate, by ESR spectroscopy, the vectorial aspects of the process as well, and that the ESR-detectable radicals are not only located but also generated on the outside of the cells.

MATERIALS AND METHODS

Daunomycin was a kind gift of Drs. V. Malatesta and A. Suarato, Farmitalia, Milano.

Samples of human red blood cells at 50% hematocrit in 140 mM NaCl, 20 mM phosphate buffer, pH 7.4, containing 5 mM glucose and 1 mM daunomycin, were prepared as previously described (8). Hemolysis was estimated as in (8), after a 5 fold dilution of the samples. Cell hemolysates were obtained by freeze/thaw cycles. Low molecular weight components were removed from hemolysates by exhaustive dialysis against 1,000 volumes of the suspension buffer.

X-band room temperature ESR spectra were recorded with a Bruker ESP 300 instrument using flat ESR cells. Anaerobiosis was achieved by flushing the samples with CO directly in the ESR flat cell, via the capillary opening at its bottom. The radical concentration was calculated from comparison with a Tempamine spin label standard.

The rate of O_2^--formation by red blood cells in the presence of daunomycin was measured in aerobic conditions by monitoring the reduction of the ESR signal of externally added oxidized bovine Cu,Zn superoxide dismutase. In the presence of a constant flow of O_2^-, the steady state, where the enzyme-

bound copper is 50 % in the reduced state, is reached according to a first order process, from which the rate of O_2^- generation can be calculated (9). Other sources than O_2^- for reduction of Cu,Zn superoxide dismutase could be ruled out, because no reduction was observed in the absence of O_2 or daunomycin, and catalase in catalytic amounts (0.2 μM) was added to the system to avoid slow steady-state attainment due to H_2O_2 (10).

In all experiments the measurements were made within the first 2 h of incubation, to avoid artifacts from O_2 diffusion or hemolysis.

RESULTS AND DISCUSSION

Location of the ESR-detectable daunomycin radicals

When daunomycin was incubated with whole blood or intact human erythrocytes under CO atmosphere (8), its semiquinone radical was detected by the appearance of its typical broad ESR signal (11). The formation of the radical was a slow process (Fig. 1): after a variable lag phase, which might depend on the presence of residual oxygen, the radical accumulated slowly for 1 h at the rate of $2\text{-}4\times10^{-11}$ Ms^{-1}, reaching a maximum steady-state level of 0.1-0.2 μM. Introduction of air caused the immediate loss of the ESR signal with no observable recovery within one hour, a further indication for slow formation of the radical.

It should be noticed that the time course of Fig. 1, does not actually refer to a single homogenous species. In fact, Fig. 2 shows that initial spectra are more anisotropic than the final ones. The anisotropic spectra very likely reflect some immobilization of the radicals due to binding to the membranes, as has been observed for other membrane systems (11). The changes in time can either mean that only a limited number of binding sites is available, or that at later times the radical is slowly diffusing out of the membrane.

No radicals were found in plasma or hemolyzed cell suspension; the semiquinone ESR signal only appeared after dialysis of the hemolysates, with kinetics that are similar to the curves seen for intact cells. This indicates

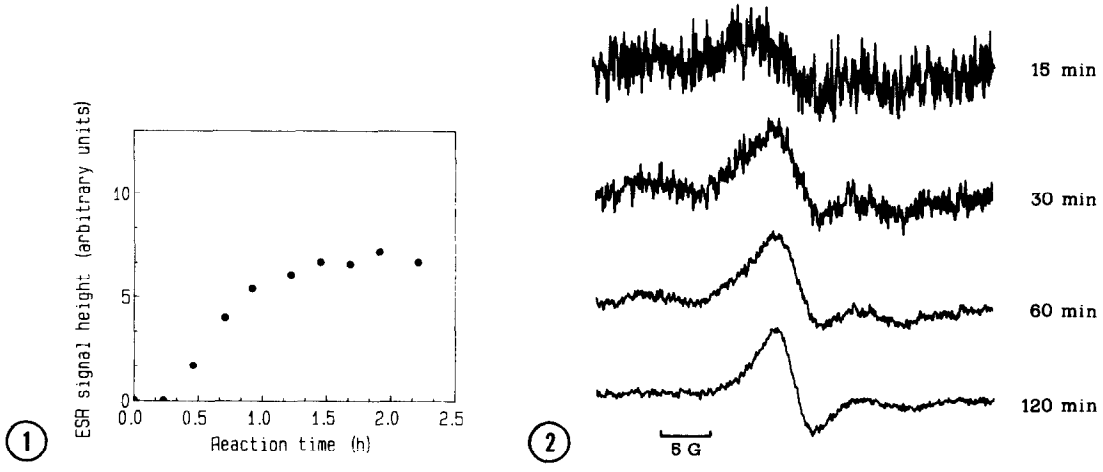


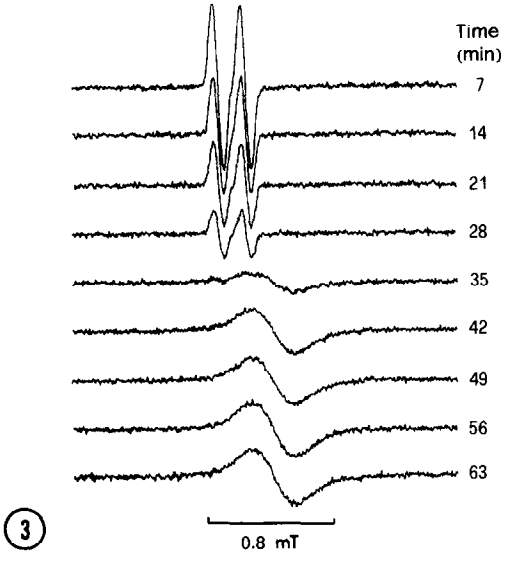
Figure 1. Time course of the formation of daunomycin radicals by red blood cells. ESR spectra of the radical generated by red blood cells in the presence of 1 mM daunomycin were recorded using 1.0 G modulation, 40 G scan width, 25 mW power, 84 s scan time and a time constant of 82 ms. 10 scans were accumulated, the amount of radical was measured arbitrarily as the peak-to-peak height of the signal.

Figure 2. ESR spectra of daunomycin radicals formed in samples of red blood cell containing 1mM daunomycin. Measurements were made as in Fig. 1 but with continuous accumulation of scans; the time-dependent increase in the signal size is caused both by the larger number of scans accumulated and by the increasing semiquinone concentration. The spectra are drawn using different scales to emphasize the changes in spectral line shape.

that low molecular weight radical scavengers, like glutathione or ascorbate, can prevent the accumulation of the radicals inside the cells. To test this hypothesis the reaction between daunomycin semiquinone and ascorbate was examined by ESR. The daunomycin semiquinone was formed directly by anaerobic reduction of the drug via the xanthine-xanthine oxidase systems (12). Fig. 3 shows that in presence of ascorbate the semiquinone signal was detected only after disappearance of the ascorbyl radical. Since identical results were obtained irrespective of the ascorbate excess added, a reasonable explanation is the reaction of the semiquinone with the ascorbyl radical rather than with ascorbate itself.

The above-mentioned experiments suggest that part of the formed radicals, if in contact with the intracellular milieu, may escape ESR

detection; however, they also show that the radicals observed by ESR in intact cell samples cannot be located inside the cells. In line with this conclusion, addition of the membrane-impermeant spin-broadening agent chromium oxalate (13) to samples of red blood cells broadened the radical signal to invisibility. The fact that the accumulation of the radicals observed by ESR in intact human erythrocytes proceeds for hours (Fig. 1) and that they are located outside the cells means that the diffusion of these radicals across the plasma membranes must be slow, otherwise they should



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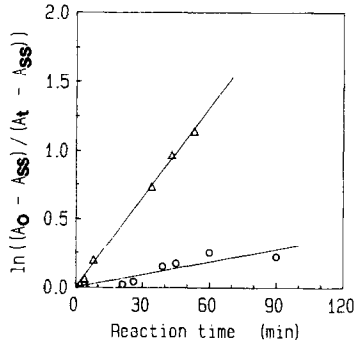

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Figure 3. Time-dependence of the formation of daunomycin radicals by xanthine oxidase in the presence of ascorbate. The ESR spectra of an anaerobic sample containing 12.5×10^{-3} unit/ml xanthine oxidase, 0.5 mM xanthine, 0.5 mM daunomycin, 1 mM ascorbate in the phosphate buffer, were recorded by 5 scans accumulations as in Fig. 1. Similar signal intensities and time-dependence were obtained with 10 mM ascorbate. In control experiments without ascorbate the daunomycin semiquinone signal was already stable after the first 7 min.

Figure 4. Generation of O_2^- by intact red blood cells upon incubation with daunomycin. The decrease of the room temperature ESR signal of 50 μM bovine Cu,Zn-superoxide dismutase added to a suspension of intact red blood cells was recorded using the following instrumental settings: 25 mW microwave power, 1,000 G scan range, 10 G modulation at 1.56 kHz frequency, 42 s scan time using a time constant of 41 ms. 4 single scans were accumulated and the exact signal height was determined after Fourier transform noise reduction. Data from a single experiment are reported for 1 mM (○) or 5 mM (△) daunomycin, and plotted as a first order reaction after subtraction of the steady state signal level (9).

enter the cells and become reduced. This slow diffusion of the semiquinone radical of daunomycin through the erythrocyte cell membrane is in line with the slow diffusion of adriamycin out of resealed red blood cells (14), but is in contrast with the recent suggestion that the adriamycin semiquinone readily crosses the membranes of tumor cells (6).

Site of formation of the ESR-detectable daunomycin radicals

The anaerobic experiments are not able to establish the site of radical generation. In fact the demonstration that the ESR spectra of Figs. 1 and 2 arise from externally-located daunomycin radicals does not rule out the possibility that they can be generated inside and give rise, by disproportionation or reduction by intracellular compounds, to the hydroquinone form, which in turn may diffuse out slowly and produce the radical outside by comproportionation with the quinone (1). In the presence of physiological oxygen concentrations the reaction with oxygen to give O_2^- is faster than any other reaction of the anthracycline radical (2,15,16), and can be used, under proper conditions, as a probe for the sidedness of the process. In this context, the use of a freely diffusible spin trap (6) is not appropriate, also because it gives no direct kinetic informations. Therefore, daunomycin was aerobically incubated with red blood cells in the presence of externally added Cu,Zn-superoxide dismutase which was used as a specific, erythrocyte-impermeant (17), O_2^--detector system (9). The approach of the enzyme to its steady state was a first-order process at both daunomycin concentrations used (Fig. 4), indicating that a constant flow of O_2^- was established from the beginning of the incubations and was maintained throughout. Any contribution from intracellular O_2^- sources could be ruled out by the high level of intracellular enzyme activity, the rate of O_2^- diffusion through the membrane being low compared to the high rate of its enzymatic

dismutation (18); moreover, insignificant hemolysis occurred within the relatively short time of these experiments. Any intracellular source of semiquinones could be ruled out as well, since in the presence of oxygen they would have been quenched (2,15,16) before having time to diffuse outside. It can therefore be safely concluded that the data of Fig. 4 are only accounted for by a source of O_2^- on the cell surface.

An obvious conclusion of this work is that anthracyclines intravenously injected during antitumor therapy may generate O_2^- in the plasma, where antioxidant enzyme activities are much weaker than in the blood cells. A further point regards the mechanism responsible for the formation of the daunomycin semiquinones on the cell membrane. Mild heat-denaturation of erythrocyte membranes abolished completely the formation of the radicals, (data not shown) indicating that a membrane redox enzyme is involved, probably an enzyme activated by intracellular electron sources, given that only glucose is available as electron donor in the whole cell experiments. A candidate may be the NADH ferricyanide reductase located on the outside of the red blood cell (19). Actually, similar reductases have been found on all other cell plasma membranes examined so far, including tumor cells (19); thus anthracycline activation may occur on the surface of the plasma membrane also in other cell types, where the more direct and quantitative experimental approach used here with the red blood cells cannot be applied.

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REFERENCES

1. Gianni, L., Corden, B. J. and Myers C. E. (1983) in: Reviews in Biochemical Toxicology (Hodgson, E., Bend, J. & Philpot, R. Eds.) pp. 1-82, Elsevier/North Holland, New York.

2. Powis, G. (1989) Free Rad. Biol. Med. 6, 63-101.
3. Tritton, T. R. and Yee, G. (1982) Science 217, 248-250.
4. Sinha, B.K., Katki, G.A., Batist, G., Cowan, K.H. and Myers C. (1987) Biochemistry 26, 3776-3781.
5. Sinha, B. K., Dusre, L. Collins, C. and Myers, C. E. (1989) Biochim. Biophys. Acta 1010, 304-310.
6. Alegria, A. E., Samuni, A., Mitchell, J. B., Riesz, P. and Russo, A. (1989) Biochemistry 28, 8653-8658.
7. Mavelli, I., Ciriolo, M.R. and Rotilio, G. (1985) Biochim. Biophys. Acta 847, 280-284.
8. Pedersen, J.Z., Marcocci, L., Rossi, L., Mavelli, I. and Rotilio, G. (1988) Ann. N. Y. Acad. Sci. 551, 121-127.
9. Rigo, A., Ugo, P., Viglino, P. and Rotilio, G. (1981) FEBS Lett. 132, 78-80.
10. Viglino, P., Scarpa, M., Rotilio, G. and Rigo, A. (1988) Biochim. Biophys. Acta 952, 77-82.
11. Kalyanaraman, B., Perez-Reyes, E. and Mason, R. P. (1980) Biochim. Biophys. Acta 630, 119-130.
12. Schreiber, J., Mottley, C., Sinha, B. K., Kalyanaraman, B. and Mason, R. P. (1987) J. Am. Chem. Soc. 109, 348-351.
13. Berg, S. P. and Nesbitt, D. M. (1979) Biochim. Biophys. Acta 548, 608-615.
14. De Flora, A., Benatti, U., Guida, L. and Zocchi, E. (1986) Proc. Natl. Acad. Sci.83, 7029-7033.
15. Butler, J., Hoey, B.M. and Swallow, A.J. (1985) FEBS Lett. 182, 95-98.
16. Houée-Levin, C., Gardès-Albert, M., Ferradini, C., Faraggi, M. and Klapper, M. (1985) FEBS Lett. 179, 46-50.
17. Michelson, A.M. and Puget, K. (1980) Acta Physiol. Scand. 492, 67-80.
18. Lynch, R. E. and Fridovich I. (1978) J. Biol. Chem. 253, 4697-4699.
19. Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C. and Löw, H. (1985) Biochim. Biophys. Acta 811, 233-264.