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Interaction of the 2,6-dimethoxysemiquinone and ascorbyl free radicals with Ehrlich ascites cells: A probe of cell-surface charge

(redox potential/electron-transfer kinetics/cytotoxicity/sulfhydryl groups)

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ABSTRACT **The rate of quenching by Ehrlich ascites cells of anionic 2,6-dimethoxy-*p*-semiquinone and ascorbyl free radicals is investigated as a function of cell concentration, the blocking of cell-surface sulfhydryl groups by *N*-ethylmaleimide, and the reduction of cell-surface charge by neuraminidase. The rate of quenching is found to be proportional to cell viability and to the number of free cell-surface sulfhydryl groups. The enzymatic action of neuraminidase results in an increase of the free radical quenching rate, indicating that this rate can be used as a probe of cell-surface charge. Measurements as a function of the ionic strength of the suspending electrolyte gave a value of $-1.22\ \mu C \cdot cm^{-2}$ for the charge density at the ascites cell surface. This is equivalent to a surface membrane potential of −14 mV for a 150 mM NaCl electrolyte and is a value in good agreement with published electrophoresis data.**

Previous studies (1) have shown that a direct correlation exists between the cytotoxic action of quinone/ascorbate mixtures against Ehrlich ascites tumor cells and the ability of such mixtures to produce stable populations of semiquinone and ascorbyl free radicals. These findings were considered to support the proposal by Bachur *et al.* (2) that the cytotoxic action of quinone anticancer drugs such as adriamycin is mediated through the production of semiquinone free radical metabolites. The main direction of our work follows from earlier considerations of the oxidation–reduction and cytotoxic properties of naturally occurring methoxyquinones (3, 4).

We report here investigations of the nature and kinetics of the interaction of semiquinone and ascorbyl radicals with Ehrlich ascites cells in physiological media. Evidence has been obtained to show that the rate at which the free radicals are quenched by the ascites cells is a function of cell viability and is controlled by the cell-surface electrical potential. The charge transfer interactions responsible for the free radical quenching appear to be dominated by the presence of cell-membrane sulfhydryl groups. Attention has been given to the 2,6-dimethoxyquinone (2,6-DMQ)/ascorbate mixture as this combination was observed (1) to produce the largest dynamic population of free radicals and the most effective treatment of ascites tumors in mice.

MATERIALS AND METHODS

For all the studies reported here, 20 mM sodium ascorbate (Sigma)/superoxide dismutase (2800 units·ml^{-1}) (Sigma S-8254) was mixed with a suspension of Ehrlich ascites cells. These cells were obtained from a cell line maintained in our laboratory by transplantation into female CD_1 mice (Charles River Breeding Laboratories). A previous determination had been made of cell concentration, and trypan blue and neutral red staining (5) had been used to determine cell viability. Then, 2 mM 2,6-dimethoxy-*p*-quinone (2,6-DMQ, supplied by G. Fodor, West Virginia University) was mixed with the ascorbate/ascites suspension and 1 ml of this total combination was immediately syringed into an aqueous flat cell already situated in a Varian E-109 (ESR) spectrometer. The final concentrations in the samples undergoing ESR investigation were 5 mM sodium ascorbate superoxide dismutase at 700 units·ml^{-1}/0.5 mM 2,6-DMQ, and the ascites cell concentrations varied in different experiments between 0 and 5 × 10^7 cells·ml^{-1}. The sodium ascorbate was sufficient to buffer the suspensions at pH 7.2. To maximize sensitivity, the modulation amplitude of the ESR spectrometer was set to 0.4 mT so as to broaden the ascorbyl and semiquinone radical spectra into a single line, onto which the spectrometer was tuned using the Varian E-272 B field/frequency lock. The intensity of this spin-resonance line was recorded as a function of time, and a spin-density calibration was obtained using a 1 mM aqueous solution of $MnCl_2$. To prevent the sample from absorbing oxygen, a nitrogen atmosphere was maintained above the aqueous flat cell, and problems associated with air bubbles or the gravitational settling of cells were minimized by using a reciprocating syringe pump to keep the sample in constant motion.

Various concentrations of up to 2.0 mM *N*-ethylmaleimide (Aldrich) were incubated for 15 min at 22°C with 5 × 10^7 Ehrlich ascites cells per ml, and these treated cells then reacted with the 2,6-DMQ and ascorbic acid mixture in the ESR aqueous cell. The extent of blocking of the cell-surface sulfhydryl groups by *N*-ethylmaleimide was determined by the method of Ellman (6). For the investigations of cell-surface charge effects, the cells were twice washed in 0.9% saline, either before suspension in NaCl/sucrose mixtures or prior to treatment with neuraminidase. For the enzymatic reduction of cell-surface charge by neuraminidase, the method described by Weiss (7) was adopted, with the following modifications: 3 × 10^6 cells were suspended in 6 ml of Hanks' saline containing 600 units of neuraminidase (Calbiochem and Sigma), incubated for 125 min at 37°C, and washed with phosphate-buffered saline (pH 7.4).

RESULTS AND DISCUSSION

Our previous work (1) indicated that the ability of a member of the family of methoxy-substituted quinones, when mixed with ascorbic acid, to produce a long-lived dynamic population of semiquinone and ascorbyl free radicals was directly related to the difference between the standard midpoint potentials of the quinone and ascorbate. Ascorbic acid was used as a nontoxic electron donor to the quinones, through a coupling of the ascorbate–dehydroascorbate and quinone—hydroquinone redox couples. It was found that the production of long-lived free radical populations was a maximum when the difference between the quinone and ascorbate midpoint potentials was a minimum. An understanding of this observation can be approached through semiconductor and

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Abbreviation: 2,6-DMQ, 2,6-dimethoxy-*p*-benzoquinone.

electrochemical concepts such as those used to describe *p–n* junction and corrosion phenomena. The analogy can be made that the rate (dn/dt) of electron transfer between ascorbic acid (A) and a quinone (Q), having standard midpoint potentials E_A and E_Q, is given by (8)

$$\left(\frac{dn}{dt}\right) \propto \exp\left(\frac{F(E_Q - E_A)}{4RT}\right), \quad [1]$$

where F, R, and T are the Faraday constant, the gas constant, and absolute temperature, respectively. Redox reactions are normally envisaged in terms of two-electron transfer processes, but they can also be envisaged as two consecutive single-electron transfer events. Eq. 1 indicates that for $E_A \cong E_Q$ the driving force for electron transfer is small. Consequently, the closer E_A and E_Q become, the slower will be the rate of one-electron transfer steps and the higher will be the dynamic population of free radical intermediates as the system moves toward final thermodynamic equilibrium. For example, for the mixture of 2,3-dimethoxyquinone (E_Q at pH 7.4 = 183 mV) with ascorbate (E_A at pH 7.4 = 47 mV) the observed (1) ascorbyl and semiquinone radical lifetimes were more than an order of magnitude lower than those observed for the 2,6-DMQ (E_Q at pH 7.4 = 78 mV) and ascorbate mixture. For the case of 2,6-DMQ and ascorbate, the free radicals produced are of the form shown in Fig. 1, in which it can be seen that at pH 7.2 both the semiquinone and ascorbyl radicals carry a net negative charge. The object of the studies reported here was to investigate the way in which the ascites cells quench these free radical species.

Measurements made of the individual ascorbyl and semiquinone free radicals, using the spectral features described (1), showed that the rate of quenching of the semiquinone radicals by the ascites cells was oxygen dependent. When superoxide dismutase was included in the ascorbate plus cell mixture before the addition of 2,6-DMQ, quenching rates in the presence of oxygen were obtained that were equal to those observed in nitrogen-saturated samples. This indicated that superoxide radicals were able to mediate the rapid quenching of the semiquinone radicals by the ascites cells. With the addition of superoxide dismutase, 100 sec after the initial mixing of the 2,6-DMQ with the cells plus ascorbate mixture, the quenching rates of the semiquinone and ascorbyl radicals were found to be identical.

The typical decay observed for the combined semiquinone and ascorbyl radicals in the presence of ascites cells is shown in Fig. 2. After 100 sec, the free radical decay obeys a relationship of the form

$$N = N_0 \exp(-kt), \quad [2]$$

where k is the indicated first-order decay constant, and t is the time after the addition of 2,6-DMQ to the ascorbate/ascites mixture. It may be seen from Figs. 2 and 3 that the rate of free radical decay increases in direct proportion to the number of viable ascites cells in the test sample. Measurements have been made for mixtures of viable and nonviable cells, and the decay kinetics have consistently reflected the number of viable cells. For example, for a total cell population of 5×10^7 cells per ml if all the cells were nonviable (or were fragmented), the observed free radical decay was the same as that observed for the suspending medium containing no cells. This indicated that the electron-transfer process responsible for the free radical decay was intimately related to cellular metabolic activity. Furthermore, progressive blocking of cell-surface sulfhydryl groups with *N*-ethylmaleimide resulted in a correspondingly progressive decrease in the rate of free radical decay, which indicated that the reaction of the ascorbate and/or semiquinone radical was directly influenced by, or directly involved with, cell-surface sulfhydryl groups.

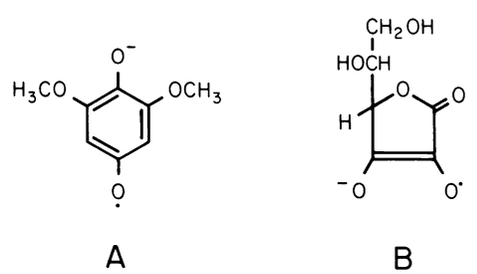

FIG. 1. (*A*) The 2,6-dimethoxy semiquinone free radical and (*B*) the ascorbyl free radical. The pK values are such that at neutral pH each radical carries a net negative charge.

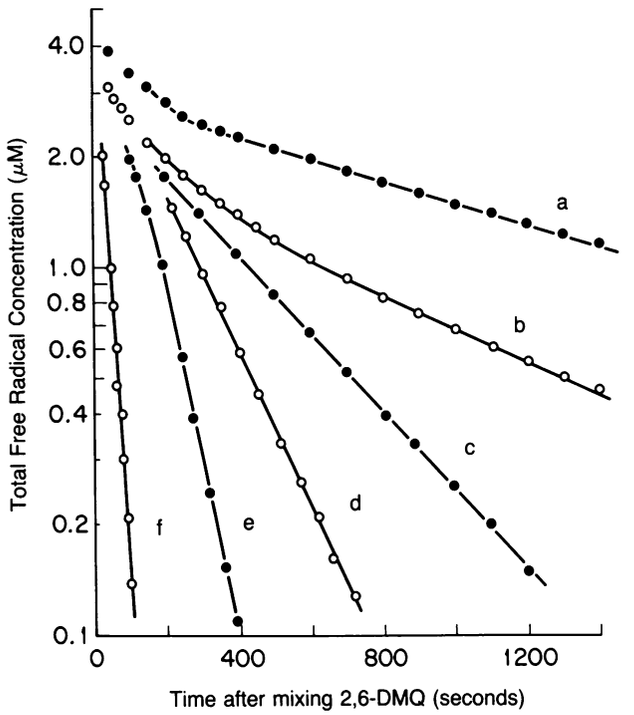

FIG. 2. Decay of the semiquinone plus ascorbyl free radicals for differing ascites cell concentrations at 28°C. The suspending medium was the supernatant obtained by centrifuging the fluid from the peritoneal cavity of ascites-bearing mice. The viable cell concentrations ($\times 10^6$ cells per ml) were as follows: curve a, 0.09; curve b, 2.25; curve c, 4.5; curve d, 11.3; curve e, 26; curve f, 45.

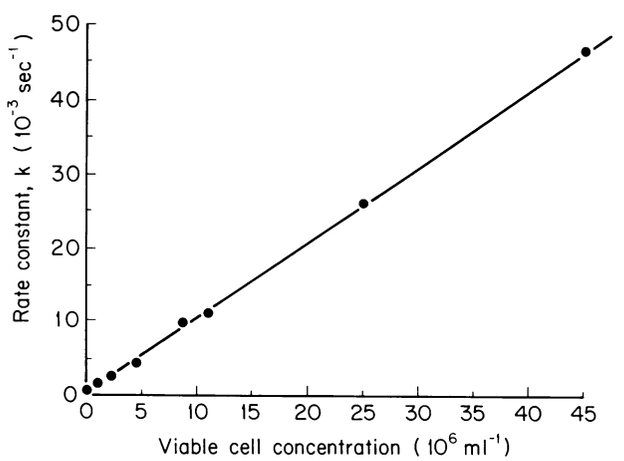

FIG. 3. Variation of the reaction rate constant k of Eq. 2 as a function of the viable ascites cell concentration.

In common with other cells, Ehrlich ascites cells carry a net negative charge at neutral pH. This will tend to decrease the rate at which the negatively charged semiquinone and ascorbate radicals (see Fig. 1) approach the cells, and the radical concentration n_s at the membrane surface with respect to that (n_b) in the bulk suspending medium will be governed by the Boltzmann distribution

$$n_s = n_b \exp(-zF\psi_s/RT). \quad [3]$$

Here, ψ_s is the surface electrical potential and the valency, z, of the charged radicals is unity.

If the activity coefficient f does not vary with distance from the cell surface, then everywhere the activity (a) of a species whose concentration is C is given by

$$a = Cf.$$

Eq. **3** can then be written in the form

$$a_s = a_b \exp(-zF\psi_s/RT), \quad [4]$$

where the subscripts s and b refer to the surface and bulk, respectively. If a charge transfer reaction of the form

$$A^- + B \overset{k_o}{\rightleftharpoons} A + B^-,$$

involving a reagent A in the suspending electrolyte and a membrane-bound component B takes place at the membrane surface, then the observed rate of oxidation of A^- will depend on its activity at the membrane surface. This activity in turn will depend on the cell-surface potential as given by Eq. **4**. In our experiments, the apparent rate (k) of oxidation of A corresponds to the observed rate of quenching (dn/dt) of the semiquinone and ascorbate radicals and will be given by

$$\frac{dn}{dt} = -ka_{rs}[S_q], \quad [5]$$

where a_{rs} is the activity of the radical species at the cell surface and $[S_q]$ is the effective concentration of the membrane-bound quenching reagent. It will be assumed that the activity of the quenching reagent remains constant during the course of its reaction with the ascorbate and semiquinone radicals. From Eqs. **4** and **5**, it can be deduced that the apparent rate constant k will be related to the surface potential ψ_s of the cell membrane and to the true rate constant, k_o (which would be observed for an electrically neutral membrane surface) by the expression

$$\ln(k) = \ln(k_o) - zF\psi_s/RT. \quad [6]$$

With the usual assumptions concerning the Gouy–Chapman theory of electrical double layers, as developed by Grahame (9), then the membrane surface charge σ can be related to the ionic concentration C_b of a bulk z-z electrolyte by the expression

$$\sigma^2 = 2RT\varepsilon_o\varepsilon_r \cdot C_b [\exp(-zF\psi_s/RT) - 1] \quad [7]$$

where ε_o is the permittivity of free space and ε_r is the relative permittivity of the electrolyte. For low values of ψ_s (<40/z mV), with T = 294 K, ε_r = 80 and a uni-univalent electrolyte, numerical substitution into Eq. **7** gives

$$\sigma = 11.77 C_b^{1/2} F\psi_s/2RT, \quad [8]$$

with C_b having units of mol·liter^{-1} and σ having units of μC·cm^{-2}. Substitution of Eq. **8** into Eq. **6** gives (for $z = -1$)

$$\ln(k) = \ln(k_o) + 0.170\sigma C^{-1/2}. \quad [9]$$

A plot of $\ln(k)$ versus $C^{-1/2}$ should then provide a straight line with a slope proportional to the effective surface-charge density near to the site of the membrane-bound quenching reagent.

As shown in Fig. 4, increasing the NaCl molarity of an isotonic suspending electrolyte resulted in an increase of the observed free radical quenching rate. The osmosity of the various NaCl solutions was maintained equivalent to that of 150 mM NaCl using sucrose (10). The variation of the observed quenching rate (corrected for cell concentration and viability) is shown in Fig. 5, and from the slope (−0.207 $M^{-1/2}$) of the straight-line plot, a value of −1.22 μC·cm^{-2} was obtained for the cell-surface charge density. From Eq. **8**, this charge density corresponds to the surface potential value of −13.8 mV at a NaCl concentration of 150 mM. Weiss (7) has determined an electrophoretic mobility of -1.2×10^{-8} m^2·V^{-1}·sec^{-1} for Ehrlich ascites cells at 28.5°C in half-strength Hanks' saline (approximately equivalent to a 71 mM uni-univalent electrolyte). The Helmholtz–Smoluchowski equation

$$\zeta = \frac{\eta\mu}{\varepsilon_o\varepsilon_r} \quad [10]$$

relates the electrophoretic mobility μ to the zeta potential ζ at the hydrodynamic shear plane, where η and ε_r are taken to be the viscosity and relative permittivity of the bulk electrolyte. For water at 28.5°C, $\eta = 8.22 \times 10^{-4}$ N·sec·m^{-2} (see ref. 10, p. F-51) and ε_r = 77.1 (see ref. 10, p. E-61), so that from Eq. **10** the value for ζ of −14.6 ± 0.9 mV can be derived from the electrophoretic mobility measured by Weiss (7). From Eq. **8** with C_b = 71 mM and T = 302 K, then ψ_s = −20.3 mV for our deduced value of the cell-surface charge. Since ψ_s is always more negative than ζ by an amount corresponding to the potential drop across the region between the membrane surface and the hydrodynamic shear plane, these

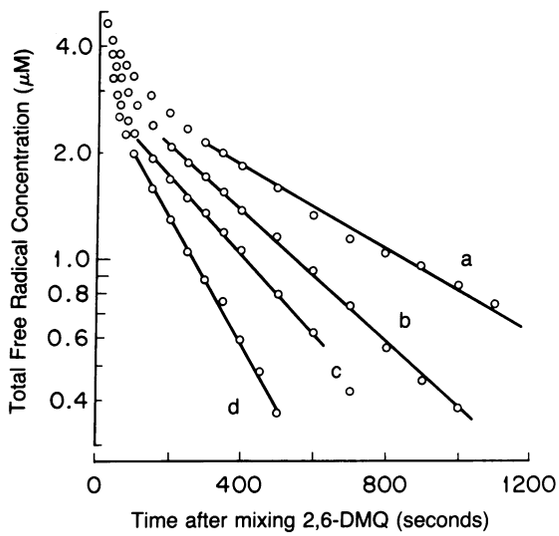

FIG. 4. Variation of the decay of the semiquinone plus ascorbyl free radicals at 22°C for ≈25 × 10^6 ascites cell per ml as a function of NaCl concentration in the isotonic suspending fluid. The concentrations of NaCl and sucrose, respectively, were as follows: curve a, 37.5 mM, 196 mM; curve b, 50 mM, 179 mM; curve c, 100 mM, 89 mM; curve d, 150 mM, no sucrose.

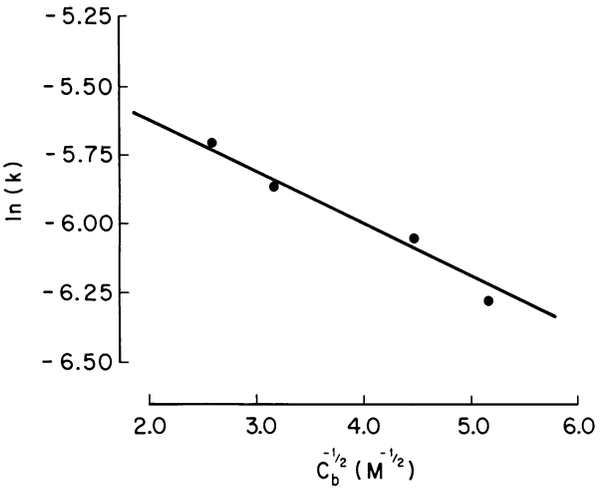

FIG. 5. Variation of the quenching rate constants obtained from Fig. 4 as a function of the reciprocal square root of the NaCl concentration. Small corrections were made for cell count and cell viability.

results can be considered to be in very good agreement. Preliminary tests using divalent counter ions ($MgSO_4$, $CaCl_2$, and $MgCl_2$) have given the increased rates of radical quenching predicted by Eq. **6**.

Two cell samples, of viable cell concentrations 2.5 and 2.7 $\times 10^5$ cells·ml^{-1}, that had been incubated with neuraminidase, exhibited larger free radical quenching rates as compared with control cells incubated in the absence of neuraminidase. When analyzed in terms of Eq. **9**, the limited data available from these two measurements indicated that the observed increases in the quenching rates resulted from a reduction of the surface-charge density as well as from an increase in the rate constant k_o at the charge-transfer reaction site. The best estimate was consistent with neuraminidase having reduced the cell-surface charge by some 25–50% and having increased k_o by a factor of 4.0–4.5. This result does confirm, however, that the rate of quenching of the semiquinone and ascorbyl radicals is directly related to the effective surface-charge density and electrical potential at the charge-transfer reaction site on the cell surface.

Finally, tests have been made of the viability of the Ehrlich ascites cells after they have interacted with the 2,6-DMQ/ ascorbate mixtures for up to 30 min. The viability of the cells was found to be largely unaffected; however CD_1 mice inoculated with such treated cells did not develop ascites tumors. This suggests that as a result of their quenching the semiquinone and ascorbate radicals the ascites cells lost their ability to replicate.

CONCLUSIONS

The results presented here indicate that the cytotoxicity against Ehrlich ascites tumor cells reported (1) for 2,6-DMQ/ascorbate mixtures is related to the quenching of semiquinone and ascorbyl radicals by sites on the membrane surfaces of the ascites cells. Treatment of the ascites cells with *N*-ethylmaleimide resulted in a reduction of the free radical quenching sites, and this provided strong evidence that cell-surface sulfhydryl groups were either directly involved in or directly influenced the charge-transfer interactions responsible for the quenching of the semiquinone and ascorbyl radicals. Furthermore, the observation that the presence of nonviable cells, or cellular debris, did not influence the rate of free radical quenching indicates that the cell-surface charge-transfer reactions involved are associated with a cellular metabolic process. The cytotoxicity appears to be associated with an inability of the treated cells to replicate, rather than in their being destroyed directly.

Further studies of the specific charge-transfer processes involved at the cell surface may lead to suggested modifications of the chemical structure of either the quinone or ascorbate molecule (e.g., to give greater lipophilicity) and hence an improvement of the observed cytotoxicity. As indicated in our previous work (1), such structural modifications need to take account of the correct balancing of electrochemical potentials required for the efficient production of a stable population of free radicals.

An interesting consequence of the semiquinone and ascorbyl radicals being negatively charged at neutral pH is that their rate of quenching by cells is directly dependent on the magnitude of the cell-surface charge, a conclusion confirmed by the effect of neuraminidase. Altering this effective charge by varying the concentration of counter ions leads to data such as those presented in Fig. 5. These provide a value for the surface charge density of -1.22 μC·cm^{-2} at the specific site of the quenching interaction, which corresponds to a surface potential of -14 mV for a 150 mM NaCl electrolyte. The surface-potential value of -20.3 mV (71 mM NaCl) obtained for ascites cells corresponds very favorably with the zeta potential of -14.6 mV derived from the electrophoresis results of Weiss (7) for a near equivalent saline concentration. Unlike the electrophoretic technique, which provides the potential at an ill-defined hydrodynamic plane of shear beyond the membrane surface, the free radical quenching technique described here provides the surface-potential value for a specific site on the membrane surface itself. The value of 5.7 mV deduced here for the difference between the potentials at the cell surface and the hydrodynamic plane of shear is of interest, since at present no satisfactory theory exists that predicts the magnitude of this potential drop. This new technique for probing cell-surface charge may provide a useful tool for the investigation of cell-membrane potentials and surface charge.

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