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Effects of ascorbic acid on biologically obtained diaziquone free radicals

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The antitumor activity of quinone-containing agents is believed to be influenced by their ability to undergo biological activation via a free radical mechanism involving their quinone moieties [1–4]. In the case of diaziquone (AZQ), it is possible that bioreductive alkylation is part of the mechanism of action of this drug [5, 6]. AZQ has been shown to be easily reduced to its free radical species by rat liver microsomes [4], by NADPH cytochrome *c* reductase [4] and by cells in culture [7]. The reduced AZQ thus obtained produces a five line electron spin resonance (ESR) spectrum which we have characterized by electrochemical reduction and ESR [8]. We report here the influence of ascorbic acid on the AZQ free radical generated by human erythrocytes and by L1210 murine leukemic cells.

Methods

Diaziquone (AZQ), 2,5-diaziridinyl-3,6-bis(carboethyoxyamino)-1,4-benzoquinone (see Fig. 1), was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A., and shown to be 99% pure by high pressure liquid chromatography [9]. L1210 murine leukemia cells were maintained *in vitro* by serial culture in RPMI 1640 medium, as previously described [7]. L1210 cells were harvested by centrifugation, washed twice with 0.15 M NaCl, and resuspended at a concentration of 10^7 cells/ml in calcium, magnesium free Hanks' balanced salt solution (HBSS), pH 7.5. Blood was obtained from human volunteers and anticoagulated with heparin (50 units/ml). Erythrocytes were separated by centrifugation at 900 *g*. The plasma and "buffy coat" were removed, and the cells were resuspended in 1 ml HBSS, treated with 1 mM AZQ, and subjected to electron spin resonance analysis. In some cases the "buffy coat" was not removed. Oxygen uptake was determined with a Clark-type electrode in a Biological Oxygen Monitor (model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Oxygen consumption rates were calculated as previously described [4, 7]. The 1-ml reaction mixtures contained AZQ (1 mM), sodium borohydride ($NaBH_4$) as a reducing agent or ascorbic acid (AH_2). ESR spectra were obtained with an X-band (9.3 GHz) Varian E-109 Century Series spectrometer equipped with 100 kHz field modulation. A dual rectangular cavity was used which contained strong pitch (g = 2.0028) in one section and the sample in an ESR flat cell in the other. The concentration of the AZQ free radical was calculated by double integration with a Nicolet 1180 computer (Madison, WI) using 2,2-diphenyl-1-picrylhydrazyl as a standard [7]. AZQ was reduced to $AZQH_2$ with $NaBH_4$ at a 3 to 1 ratio of $NaBH_4$ to AZQ. AZQ solutions (1 mM) in phosphate-buffered saline (PBS), pH 7.5, became colorless after the addition of sodium borohydride (3 mM). Fifty microliters of this colorless solution was injected into a 1-ml PBS solution, pH 7.5, and the oxygen consumption from this solution was measured in the oxygen monitor described above. The final concentrations for the oxygen consumption measurements were 50 μM for AZQ and 150 μM for sodium borohydride respectively. Oxygen consumption evaluation was based on a 100% oxygen concentration of 200 μM, calculated for our conditions as in Ref. 4.

Results and discussion

Diaziquone free radicals were observed when the drug (1 mM final concentration) was added to a suspension of red blood cells (Fig. 1). These free radicals gave the same five line ESR spectra previously reported for the reduction of AZQ by microsomes, purified NADPH cytochrome *c* reductase [4] and L1210 murine leukemic cells [7]. If the "buffy coat" was only partially removed or not removed, a rapid decay of the AZQ free radical was observed with the appearance of a 1.8 G doublet (Fig. 1). This doublet was strongly suggestive of the ascorbyl radical, the presence of which was confirmed by the appearance of the same doublet when ascorbic acid, either as a solid or in solution (*ca* 2 mM), was added to a mixture of AZQ (1 mM) and L1210 cells which had reduced AZQ to its free radical anion (Fig. 2B). In preparations where the "buffy coat" was mixed in with the red blood cells, the ascorbyl radical appeared at various degrees of intensity and at various times after the addition of AZQ depending on the blood donor. The 16-min time in Fig. 1 is one of the fastest times observed. The "buffy coat" of a red blood cell preparation is rich in leukocytes which are second only to the adrenals in containing the most ascorbic acid in humans (250–350 mg/kg net wt) [10].

The most likely equations involving endogenous or exogenous monodehydro ascorbate (AH_2, ascorbic acid)* that can explain the observations above are equations 1–3. Ascorbic acid is oxidized to the ascorbyl radical ($\dot{A}H$) while the AZQ free radical ($AZ\dot{Q}H$) is fully reduced to the hydroquinone ($AZQH_2$) (Eq. 1). This is an electron transfer reaction which has been observed by Schuler for *p*-semiquinones [11] and by Borg *et al.* for a variety of free radicals including 6-hydroxy dopamine and dialuric acid [12]. In the latter case, the ascorbyl radical was always observed [12].

* We have chosen to write all chemical reactions in the protonated form for simplicity.

	$AZ\dot{Q}H + AH_2$	$\rightarrow$	$\dot{A}H + AZQH_2$	(1)
	$AZQ + AH_2$	$\rightarrow$	$\dot{A}H + AZ\dot{Q}H$	(2)
	$2\dot{A}H$	$\rightleftarrows$	$AH_2 + A$	(3)
1 + 2 + 3	$AZQ + AH_2$	$\rightarrow$	$AZQH_2 + A$	(4)
	$AZ\dot{Q}H + O_2$	$\rightarrow$	$AZQ + O_2^{\cdot} + H^+$	(4a)
	$AZQH_2 + O_2$	$\rightarrow$	$AZ\dot{Q}H + O_2^{\cdot} + H^+$	(5)
	$AZQH_2 + O_2^{\cdot} + H^+$	$\rightarrow$	$AZ\dot{Q}H + H_2O_2$	(6)
	$2AZ\dot{Q}H$	$\rightleftarrows$	$AZQH_2 + AZQ$	(7)
5 + 6 + 7	$AZQH_2 + O_2$	$\rightarrow$	$AZQ + H_2O_2$	(8)
Sum of 4 and 8	$AH_2 + O_2$	$\rightarrow$	$A + H_2O_2$	(9)

In the case of AZQ, the sum of equations 1 through 3 leads to fully reduced AZQ and fully oxidized ascorbic acid (Eq. 4). Equation 1 is known to be relatively fast for *p*-benzoquinone free radicals (*k ca* $5 \times 10^6\ M^{-1}\ sec^{-1}$) [11] when compared to the relatively slow reactivity of AH_2 with quinones, Eq. 2 [12]. Equation 3, the disproportionation of the ascorbyl radical, is relatively fast and pH dependent, with *k ca* $3 \times 10^5\ M^{-1}\ sec^{-1}$ at pH 8.7 [13] and $10^8\ M^{-1}\ sec^{-1}$ at pH 4.8 [14]. In our case, pH 7.5, Eq. 3 would be expected to have an intermediate rate constant value close to that of Eq. 1. With this in mind, equations 1 through 3 represent a cycle that accounts for the disappearance of $AZ\dot{Q}H$ and the appearance of $\dot{A}H$ in Fig. 1, as well as the absence of $AZ\dot{Q}H$ (or quantities too small to detect) and the presence of $\dot{A}H$ when AH_2 is added to a solution containing AZQ (Fig. 2C).

The possibility that $AZQH_2$ autoxidizes to its free radical anion (Eq. 5) and eventually to the parent compound or other quinoid products (e.g. AZQ with open aziridine rings) (Eq. 8) was investigated by measuring oxygen consumption. Equations 5–7 are an important part of the cycle described above (Eq. 1–3) because of the additional source of AZQ free radicals. The reduction of AZQ with excess sodium borohydride ($NaBH_4$) to AZQ hydroquinone (see Methods) was verified by the lack of ESR spectra (Fig. 2F) and by the fact that the solution became colorless within 30 sec after the addition of sodium borohydride. Shaking the test tube (equivalent of oxygenating) temporarily turned the solution yellow, the color of the AZQ free radical as confirmed by ESR. With time, or by passing O_2 through the solution, the reddish-brown color of the parent compound and other quinoid products returned (Eq. 8). The concentration of free radicals obtained by this mode of oxygenation was 0.3 to 0.4 mM in a PBS solution containing 1 mM AZQ.

The colorless $AZQH_2$ compound consumed oxygen (total O_2 consumed 44 μM, rate 4.4 μM O_2/min) from an

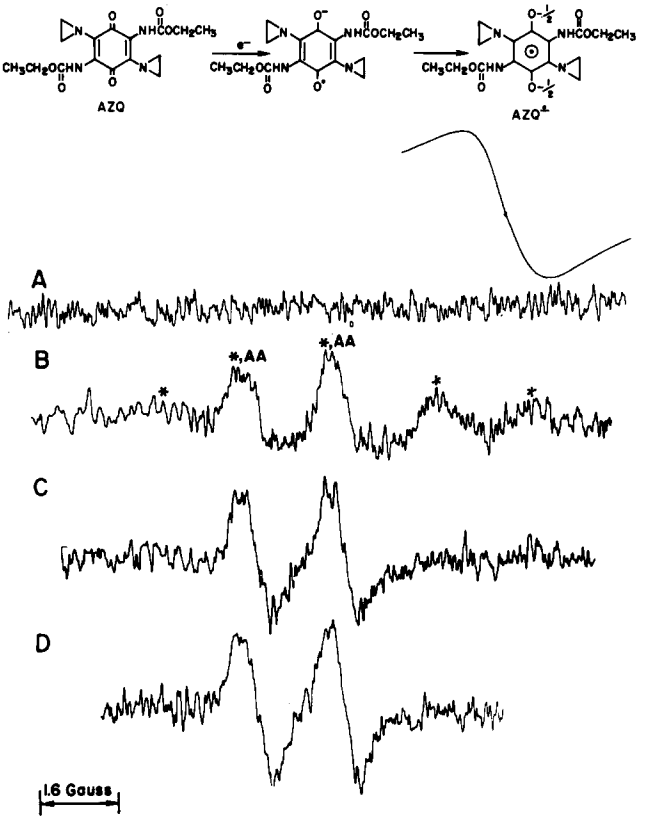

Fig. 1. (A) ESR spectrum of red blood cells. (B) ESR spectrum of AZQ (1 mM) added to red blood cells showing the decrease of the AZQ free radical (*) and the increase of the ascorbyl free radical (AA). (C) Same as (B) 16 min after the addition of AZQ. (D) Same as (B) 32 min after the addition of AZQ. All solutions were prepared in HBSS, pH 7.5. The ESR conditions at room temperature were: 9.3 GHz, 10 mW incident microwave power, and 1 G modulation amplitude.

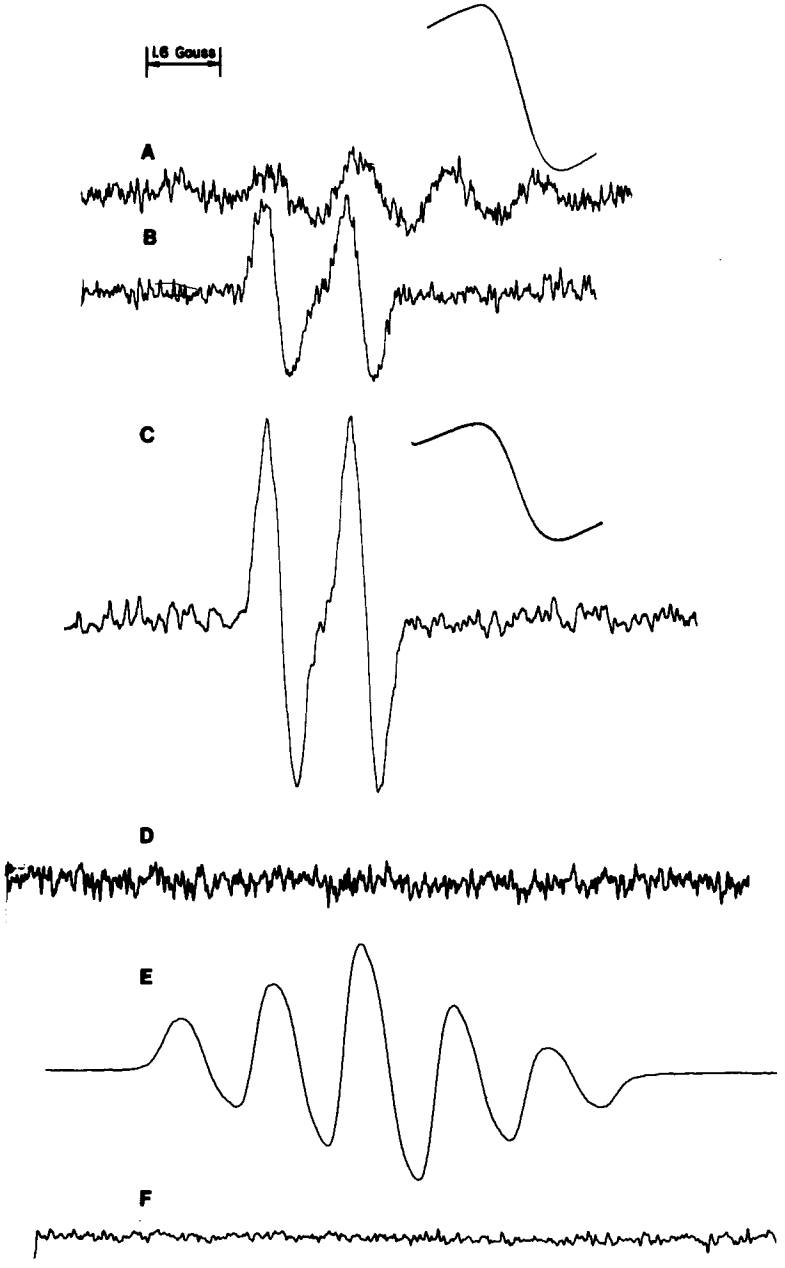

Fig. 2. (A) ESR spectrum of the AZQ free radical produced by 10^7 L1210 cells after the addition of 1 mM AZQ. (B) Same as (A) after the addition of ascorbic acid crystals. (C) ESR spectrum of the ascorbyl free radical in a solution of 1 mM AZQ and 2 mM ascorbic acid in HBSS, pH 7.5. (D) ESR spectrum of ascorbic acid (2 mM) in HBSS, pH 7.5 (control for C). (E) ESR spectrum of the AZQ free radical obtained by the oxidation of reduced AZQ ($AZQH_2$) by superoxide anion. Potassium superoxide (1 mM) was added to the colorless, diagmagnetic solution of $AZQH_2$ whose ESR spectrum is shown in (F). The experimental conditions were the same as in Fig. 1, except that the sensitivity (gain) in (F) was ten times that of (E).

aerated solution of phosphate-buffered saline, pH 7.5 (Fig. 3A). These data indicate that $AZQH_2$ can autoxidize to produce superoxide anion and AZQ free radical anion (Eq. 5). The presence of $AZ\dot{Q}H$ was confirmed by ESR (data not shown). The AZQ free radical also consumed oxygen (Fig. 3, B and E). In Fig. 3B, the AZQ free radical was obtained by aerating a 1 mM AZQ solution previously reduced with $NaBH_4$ (3 mM). In Fig. 3E, the free radical was immediately obtained as AZQ was reduced by $NaBH_4$. The rate of O_2 consumption was 16 μM O_2/min in Fig. 3E as opposed to 4.7 μM O_2/min in Fig. 3B. The total O_2 consumed was 48 μM for Fig. 3E and 24 μM for Fig. 3B.

Ascorbic acid augments the oxygen uptake in all these reactions, e.g. Fig. 3C and Eqs. 1, 2, 4, 5 and 6. The total O_2 consumed was 80 μM, at a rate of 5.2 μM O_2/min for both Fig. 3C and 3D. Equation 6 was verified by reacting colorless, diamagnetic $AZQH_2$ (Fig. 2F) with 1–5 mM potassium superoxide in PBS, pH 7.5, and observing the

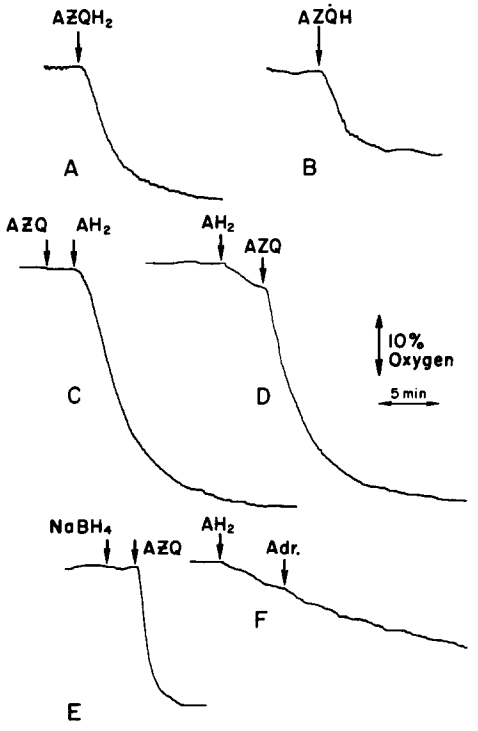

Fig. 3. (A) Oxygen consumption of sodium borohydride reduced AZQ (50 μM). (B) Oxygen consumption of AZQ free radical (15 μM) obtained by aerating the colorless solution in (A) until it became yellow (see text). (C) Oxygen consumption of 50 μM AZQ followed by 100 μM ascorbic acid injection. (D) Endogenous oxygen consumption of ascorbic acid (100 μM) augmented by AZQ (50 μM). (E) Oxygen consumption of AZQ free radical (AZQH) produced by adding 50 μM AZQ to a solution of PBS, pH 7.5, containing 150 μM sodium borohydride. (F) Endogenous oxygen consumption of ascorbic acid (150 μM) to which Adriamycin (50 μM) was added. All concentrations are final concentrations in PBS, pH 7.5.

five line ESR signal of AZQH (Fig. 2E). The pH of this buffered solution did not change appreciably after the addition of KO_2 (i.e. from pH 7.50 to pH 7.65 after the addition of KO_2). The comproportionation–disproportionation reaction (Eq. 7) was studied by reducing 1 mM AZQ with 0.03 mM $NaBH_4$ under anaerboic conditions in PBS, pH 7.5. Under these conditions, the AZQ free radical was stable for up to 32 hr.

In general, the oxygen consumption measurements indicate that $AZQH_2$ can autoxidize and that AZQ acts as a catalyst to augment the autoxidation of ascorbic acid (Fig. 3C and D). This catalytic effect can be seen theoretically by adding Eqs. 4 and 8 to yield Eq. 9. In contrast, Adriamycin does not augment the autoxidation of ascorbic acid (Fig. 3F).

If AZQH is involved as an obligatory intermediate in the activity of AZQ, then our observations of the effects of ascorbic acid imply two things. Ascorbic acid can act as a detoxifying agent if AZQH itself is involved in the activity of the drug as the DNA experiments of King *et al.* imply [5]. On the other hand, if oxygen free radicals and the generation of hydrogen peroxide (Eq. 9) are an important aspect of toxicity, ascorbic acid will enhance AZQ activity because hydrogen peroxide can be reduced by semiquinones to yield the highly toxic hydroxyl radicals even in the absence of metal ions [15].

The ease with which AZQ autoxidizes may explain why AZQ free radicals can be seen in biological systems under aerobic conditions. In contrast, Adriamycin free radicals cannot be observed under the same conditions despite the fact that fully reduced Adriamycin autoxidizes. The reason for this is that perhaps the equivalent of Eq. 5 for Adriamycin is slow and/or because the fully reduced Adriamycin metabolite is an aglycone, an insoluble compound different from the parent compound and the Adriamycin semiquinone.

In general, our data show that ascorbic acid at biological concentrations can reduce biologically generated AZQ free radicals while oxidizing itself to the ascorbyl radical. AZQ acts as a catalyst for the autoxidation of ascorbic acid and this vitamin can be involved in modifying the activity of AZQ. The fact that Adriamycin does not catalyze the autoxidation of ascorbic acid points to different mechanisms of action in these two drugs. Our results indicate that the redox reactions observed for Adriamycin [2, 3] also occur for AZQ (Eq. 4a) and that the autoxidation of AZQ (Eq. 5) is important and may be responsible for observing AZQ free radicals under aerobiosis [4].

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