Doxorubicin Enhances Complement Susceptibility of Human Melanoma Cells by Extracellular Oxygen Radical Formation*

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In two recent publications we showed that rapid inactivation of cell-bound C3b is a protective mechanism of human melanoma cells against killing by the R24 monoclonal antibody and human complement (Panneerselvam, M., Welt, S., Old, L. J., and Vogel, C.-W. (1986) J. Immunol. 136, 2534-2541) and that this protective mechanism can be inhibited by both the free and immobilized anthracycline glycoside doxorubicin (adriamycin) resulting in an enhanced complement susceptibility (Panneerselvam, M., Bredehorst, R., and Vogel, C.-W. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9144-9148). In this paper we show that the complement enhancing effect of both free and immobilized doxorubicin is caused by the generation of reactive oxygen species including superoxide anion radical, hydrogen peroxide, and hydroxyl radical. The complement-enhancing effect of the anthracyclines can be completely inhibited by the reactive oxygen scavengers superoxide dismutase, catalase, and dimethyl sulfoxide. Consistent with this observation, 5-iminodaunorubicin, an anthracycline glycoside with an imine-substituted quinone moiety and, therefore, with a significantly reduced ability to form oxygen radicals, did not cause an enhanced-complement susceptibility. The complement-enhancing effect of the anthracyclines could also be inhibited by bivalent metal chelators but was unaffected by sulfhydryl-blocking reagents or glutathione. Our results suggest that the anthracycline glycosides generate in a metal- (most probably iron) dependent reaction superoxide anion radicals with subsequent formation of hydrogen peroxide and hydroxyl radicals. These reactive oxygen species then cause alterations in the melanoma cells resulting in the enhanced complement susceptibility. While the target molecule(s) of the reactive oxygen species responsible for the enhanced complement susceptibility is not known, the data obtained with immobilized doxorubicin suggest that the target molecule(s) is located in the cell membrane.

The anthracycline glycoside doxorubicin (former generic name, adriamycin) is a widely used anticancer agent. In addition to its well-known direct cytotoxic activity doxorubicin has another activity, the enhancement of complement susceptibility as we have recently demonstrated for human melanoma cells (2). The melanoma cell line SK-MEL-170 is resistant to complement-mediated killing using the complement-activating R24 murine IgG3 monoclonal antibody to the GD3 ganglioside melanoma antigen for sensitization and human serum as the source of complement (3). Rapid inactivation of C3b was identified as a protective mechanism of the SK-MEL-170 cells to resist complement attack (3). Pretreatment of the cells with the doxorubicin inhibited the degradation of C3b and converted the cells into susceptible ones (2). Doxorubicin exerted this effect also after immobilization onto glycerol-coated glass beads by which cellular uptake of the anthracycline glycoside was prevented (2). Drug release as an explanation for the observed effect could be excluded, since immobilized doxorubicin caused a significant increase in complement-mediated cell killing even at concentrations where free doxorubicin was no longer effective (2). While these data demonstrate that doxorubicin can induce biochemical alterations without entering the cell leading to a marked increase in complement susceptibility, the molecular mechanism of this anthracycline effect remains to be investigated.

With regard to free doxorubicin considerable evidence from in vitro and in vivo studies has accumulated which strongly suggests that activation of the drug to the semiquinone-free radical is an important step in the cascade of doxorubicin-induced events (for reviews see Refs. 4 and 5). In the presence of molecular oxygen, doxorubicin semiquinone radicals are rapidly reoxidized in a process which generates superoxide anion radicals (6-8) and other partially reduced species of oxygen, including hydrogen peroxide (9, 10) and hydroxyl radicals (11, 12). Such activated oxygen species can damage a variety of cellular macromolecules including enzymes (13-15) and unsaturated membrane lipids (16-18). Therefore, oxygen radical formation could also be responsible for alterations resulting in an enhanced complement susceptibility.

In the present paper we describe the effect of free and immobilized anthracycline glycosides on the enhancement of complement-mediated cell killing in the presence of different reactive oxygen scavengers. The data presented here document that the enhancement of complement susceptibility by both free and immobilized doxorubicin is caused by the generation of superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. In addition, oxygen radicals generated extracellularly appear to be mainly responsible for this effect since anthracycline glycosides have a significantly higher complement-enhancing activity when immobilized.

EXPERIMENTAL PROCEDURES

Materials—Daunorubicin and 5-iminodaunorubicin were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institutes of Health, Bethesda, MD. Dox-
orubicin hydrochloride (adriamycin), superoxide dismutase (EC 1.15.1.11) (from bovine erythrocytes; 3,000 units/mg), catalase (EC 1.11.1.6) (from bovine liver; 11,000 units/mg), reduced glutathione (GSH),\(^1\) bathophenanthroline sulfonate, diethylenetriaminepentaacetic acid, and \(\beta\)-hydroxymercuribenzoate were purchased from Sigma. Carboxyldimidazole-activated glycerol-coated controlled pore glass beads (CDI-CPG beads; 74-125-\(\mu\)m diameter; 130 \(\mu\)m\(^2\)/g surface area) and N-\((\beta\)-iodoethyl\)trifluoroacetamide were from Pierce. N-ethylmaleimide, thiourea, MeSO, mannotil, EDTA, \(\beta\)-mercaptoethanol, and iodoacetamide were obtained from Aldrich. The human melanoma cell line SK-MEL-170 was kept in continuous culture (using minimal essential medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% antibody (19, 20).\(^2\)

**Immunobilization of Anthracycline Glycosides—Immobilization of doxorubicin, daunorubicin, and 5-iminodaunorubicin onto CDI-CPG beads was performed as described in a recent publication (2). Briefly, 10 mg of each anthracycline glycoside in 10 ml of 0.1 M sodium borate, pH 8.0, was incubated with 1.5 mg of dry CDI-CPG beads for 72 h at 4°C in the dark until use. After incubation, the beads were washed, treated with 0.1 M \(\mathrm{NH}_2\mathrm{OH}, \text{pH 8.0} (1 	ext{ h at } 4^\circ \text{C}) \) to block unreacted imidazolyl groups, and then washed excessively with borate buffer, acetonitrile, and methanol for 4-5 days at 4°C until free anthracycline glycosides could no longer be detected. By this procedure the anthracycline glycosides were coupled via the amino group in the sugar moiety resulting in an N-alkyl carbamate linkage (2). As determined by acid-catalyzed release of the aglycone moiety (2), the degree of substitution was approximately 30 \(\mu\)g of anthracycline glycoside/100 \(\mu\)l of packed wet beads. The immobilized anthracycline glycosides were stored at 4°C in the dark until use.

**Preparation of Doxorubicin-Ferric Iron Complexes—Using free doxorubicin, equal volumes of 2 mM doxorubicin in 0.1 M glycine buffer, pH 3.0, and 1 mM FeC\(_4\) in the same buffer were mixed and titrated to pH 7.4 with 0.05 M NaOH (21). Using immobilized doxorubicin, 1 ml of a suspension of doxorubicin beads (300 \(\mu\)g of immobilized doxorubicin) in 0.1 M glycine buffer, pH 3.0, was mixed with 1 ml of 0.28 mM FeC\(_4\) in the same buffer, adjusted to pH 7.4 with 0.05 M NaOH and then washed twice with phosphate-buffered saline at pH 7.4.

**Treatment of Cells with Free and Immobilized Anthracycline Glycosides in the Absence and Presence of Reactive Oxygen Scavengers or Other Agents—SK-MEL-170 cells were harvested with trypsin (3). Subsequently, 5 \(\times\) \(10^6\) cells were incubated at 37°C for 15 h in a total volume of 1 ml of complete culture medium (if not otherwise stated) containing either 40 \(\mu\)g free (schebled ferric ion) or 15 \(\mu\)g (50 \(\mu\)l) of immobilized anthracycline glycoside (doxorubicin, daunorubicin, or 5-iminodaunorubicin) (schebled ferric iron). The incubations were performed in the absence and presence of different reactive oxygen scavengers (superoxide dismutase, catalase, thiourea, MeSO, mannotil), metal ion chelators (EDTA, diethylenetriaminepentaacetic acid, bathophenanthrolione sulfonate, GSH, \(\beta\)-mercaptoethanol, and sulfhydryl-blocking agents (\(\beta\)-hydroxymercurobenzoate, N-ethylmaleimide, iodoacetamide, N-\((\beta\)-iodoethyl\)trifluoroacetamide) at concentrations as indicated. Controls were run with heat-inactivated superoxide dismutase and catalase (heat-inactivated by autoclaving for 60 min) and without anthracycline glycosides. Prior to cytotoxicity testing, cells were separated from anthracycline glycoside beads by differential sedimentation (after vortexing), while free anthracycline glycosides and other soluble agents were removed by washing. All experiments were performed in duplicate.

**Cytotoxicity Assay—** Determination of complement-mediated cytotoxicity was performed as described recently (3). Briefly, SK-MEL-170 cells (5 \(\times\) \(10^6\)) were sensitized with 200 \(\mu\)l of the R24 antibody (1:5 dilution of R24 ascites). After 15 min at 37°C the cells were washed once and incubated with 500 \(\mu\)l of normal human serum (stored at -70°C until use; 1:2 dilution) for 60 min at 37°C. Cytotoxicity was determined by dye exclusion with 0.4% trypan blue in a hemocytometer. Unsensitized SK-MEL-170 cells were used as control.

\(^1\) The abbreviations used are: GSH, reduced glutathione; CDI-CPG beads, carboxyldimidazole-activated glycerol-coated controlled pore glass beads; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

**RESULTS**

**Effect of Doxorubicin, Daunorubicin, and 5-Iminodaunorubicin on Complement Susceptibility—** Fig. 1 (upper panel) shows the effect of different concentrations of the anthracycline glycosides doxorubicin, daunorubicin, and 5-iminodaunorubicin on the R24 antibody and human complement-mediated killing of human SK-MEL-170 melanoma cells. Pretreatment of the cells with the quinone-containing anthracyclines doxorubicin and daunorubicin caused a dose-dependent enhancement of complement-mediated cytotoxicity from approximately 30 to \(\geq\) 70%. In contrast, no enhancement of complement susceptibility was observed with 5-iminodaunorubicin even when the drug was present at a 4-fold higher concentration than the minimal concentration of doxorubicin and daunorubicin necessary to exert their maximal effect. 5-Iminodaunorubicin has the identical structure as daunorubicin except for the quinone moiety. In the 5-iminodaunorubicin molecule, one oxygen of the quinone ring is replaced by an imino group. Therefore, these data indicate an important role of the quinone moiety for the conversion of complement-resistant cells into susceptible ones.

Similar results were obtained with these anthracyclines after their covalent coupling onto glycerol-coated glass beads (diameter 75-125 \(\mu\)m) to prevent their cellular uptake. As shown in Fig. 1 (lower panel), both immobilized doxorubicin and immobilized daunorubicin enhanced the complement susceptibility of SK-MEL-170 cells reaching the same degree of cytotoxicity (\(\geq 70\%\)) as it had been observed with the free anthracycline glycosides, whereas immobilized 5-iminodaunorubicin had no effect. However, doxorubicin and daunorubicin are more effective after immobilization, causing maximal increase at a 3-fold lower concentration than the free anthracyclines. We have previously shown that the effect of immo-

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**FIG. 1.** Effect of different anthracycline glycosides on complement susceptibility of human SK-MEL-170 melanoma cells. Cells were treated with increasing concentrations of free (upper panel) or immobilized (lower panel) doxorubicin (closed circles), daunorubicin (open circles), and 5-iminodaunorubicin (closed triangles). Complement-mediated cytotoxicity was determined as described under "Experimental Procedures."
Doxorubicin Enhances Complement Susceptibility

Bilized doxorubicin is not due to released drug: when immobilized doxorubicin is present at a concentration that is sufficient for maximal enhancement, the amount of doxorubicin removed from doxorubicin-coated glass beads by SK-MEL-170 cells does not effect any increase in complement-mediated cell killing if supplied as free drug (2). It is evident from these data that quinone-containing anthracycline glycosides can, without entering the cell, induce biochemical alterations resulting in an enhanced complement susceptibility.

Inhibition of Anthracycline-induced Enhancement of Complement Susceptibility by Reactive Oxygen Scavengers—The requirement of quinone-containing anthracycline glycosides for the conversion of complement-resistant cells into susceptible ones suggests reactive oxygen radicals as likely participants in the sequence of the biochemical events. To test this hypothesis we incubated SK-MEL-170 cells with free and immobilized doxorubicin in the presence of different reactive oxygen scavengers. Both free and immobilized doxorubicin were present at concentrations which produced maximal enhancement of complement-mediated cell killing. As shown in Fig. 2 (left panel), the addition of superoxide dismutase inhibits the effect of both free and immobilized doxorubicin by up to 50%, indicating an important role of superoxide anion radicals for this anthracycline effect. Identical amounts of heat-inactivated superoxide dismutase had no inhibitory effect on the complement-enhancing activity of free and immobilized doxorubicin. Other reactive oxygen species including hydrogen peroxide and hydroxyl radical are also involved.

First, the enzyme catalase diminishes the effect of free and immobilized doxorubicin by up to 50% (Fig. 2, right panel). No inhibition of the complement-enhancing activity of free and immobilized doxorubicin was observed when controls were run with identical amounts of heat-inactivated catalase. Second, thiourea, Me₂SO, and mannitol, all of which are potent hydroxyl radical scavengers, caused a 65–70% inhibition (Fig. 3). These data suggest that hydroxyl radicals are involved and represent the predominant attacking oxygen species.

Table I lists the inhibitory effect of different scavenger combinations. All scavengers were applied at concentrations where they effected maximal inhibition. As is evident from Table I, superoxide dismutase and catalase together are only slightly more inhibitory than each of the enzymes alone. The combination of superoxide dismutase, catalase, and Me₂SO inhibits the effect of both free and immobilized doxorubicin completely implicating that anthracycline-induced enhancement of complement susceptibility is initiated by a cascade of reactive oxygen species including superoxide anion radical, hydrogen peroxide, and the hydroxyl radical. The complete inhibition of the doxorubicin effect by the scavenger combination employed suggests that singlet oxygen does not play a significant role. Furthermore, the experiments with immobilized doxorubicin demonstrate that the sequence of events can be initiated by extracellular generation of these reactive oxygen species.

Role of Iron in Anthracycline-induced Oxygen Radical For-

**Fig. 2.** Effect of superoxide dismutase and catalase on doxorubicin-induced complement susceptibility. SK-MEL-170 cells were incubated with free doxorubicin (40 μg/ml) (closed circles) or immobilized doxorubicin (15 μg/ml) (open triangles) in the presence of increasing concentrations of superoxide dismutase and catalase. Controls were performed in the absence of doxorubicin (open circles).

**Fig. 3.** Effect of hydroxyl radical scavengers on doxorubicin-induced complement susceptibility. SK-MEL-170 cells were incubated with free doxorubicin (40 μg/ml) (closed circles) or immobilized doxorubicin (15 μg/ml) (open triangles) in the presence of increasing concentrations of thiourea, Me₂SO, and mannitol. Controls were performed in the absence of doxorubicin (open circles).
Doxorubicin Enhances Complement Susceptibility

Doxorubicin chelates iron and these complexes undergo redox cycling to reduce molecular oxygen (21-23). We prepared doxorubicin-Fe³⁺ complexes and tested their effectiveness in enhancing complement susceptibility of SK-MEL-170 cells. As is evident from Fig. 4 (left panel), doxorubicin is 2-fold more effective when complexed with Fe³⁺. The complement-enhancing effect of doxorubicin-Fe³⁺ complexes could be inhibited with the same reactive oxygen scavengers in a comparable fashion as demonstrated for non-iron-complexed doxorubicin (data not shown) indicating that the doxorubicin-Fe³⁺ complexes exert their effect by the same reactive oxygen species. Thus, iron chelation by free doxorubicin seems to enhance but not to alter the mechanism of radical formation. In contrast, no significant change in complement-enhancing activity was seen when immobilized doxorubicin was subjected to the same procedure as free doxorubicin to prepare Fe³⁺ chelates (Fig. 4, right panel).

It is known that superoxide anion radicals can act as a reductant for Fe³⁺, and, in turn, Fe³⁺ can reduce hydrogen peroxide in the Fenton reaction to generate hydroxyl radicals (for review see Ref. 24). The overall reaction has been referred to as an iron-catalyzed Haber-Weiss reaction (25-27). We incubated SK-MEL-170 cells with doxorubicin in the presence of different metal ion chelators to elucidate the role of this mechanism for the enhancement of complement susceptibility. As shown in Table II, the effect of free doxorubicin is completely blocked by the metal ion chelating agents EDTA, diethylenetriaminepentaacetic acid, and bathophenanthroline sulfonate. Identical results were obtained with doxorubicin-Fe³⁺ chelates and immobilized doxorubicin. These data demonstrate that a transition metal, most probably iron, is required for the anthracycline-induced enhancement of complement-mediated cell killing, which implies an important role for the iron-catalyzed Haber-Weiss reaction in the sequence of biochemical events.

Role of Sulfhydryl Groups—Membrane protein sulfhydryl groups have been reported to play an essential role in lipid peroxidation of erythrocyte ghost membranes by doxorubicin-Fe³⁺ complexes (28). We incubated SK-MEL-170 cells with doxorubicin, doxorubicin-Fe³⁺ chelates, and immobilized doxorubicin in the presence of four different sulfhydryl-blocking agents: p-hydroxymercuribenzoate, N-ethylmaleimide, iodoacetamide, N-(β-iodoethyl)trifluoroacetamide. As shown in Table III, none of these agents affected the anthracycline-induced enhancement of complement susceptibility to any significant extent. Identical results were obtained, when the cells were preincubated with p-hydroxymercuribenzoate (1 mM; 2 h at 37 °C) and then exposed to doxorubicin.

To evaluate the effect of soluble sulfhydryl groups, SK-MEL-170 cells were incubated with doxorubicin, doxorubicin-Fe³⁺ complexes, and immobilized doxorubicin in the presence of reduced GSH which has been shown to enhance doxorubicin-Fe³⁺-mediated destruction of erythrocyte ghost membranes more than 3-fold (21). In order to detect enhancing effects of GSH, the anthracyclines were present at concentrations which cause only partial enhancement of complement susceptibility. However, the data in Table IV demonstrate...
that even high concentrations of GSH have no significant effect. Comparable results were obtained with β-mercaptoethanol (data not shown).

**Cellular Location of the Target**—The inhibition of C3b degradation and enhancement of complement susceptibility by immobilized doxorubicin demonstrates that this anthracycline effect can be initiated by extracellular formation of oxygen radicals. However, the cellular location of the target for the reactive oxygen species and in particular of hydroxyl radicals remains uncertain. Hydrogen peroxide, derived from spontaneous dismutation of superoxide anion radical, crosses all membranes easily (24) and could generate hydroxyl radicals in different parts of the cell whenever meeting a transition metal ion. In order to distinguish whether reactions of the generated oxygen radicals with intracellular target molecules or with cell membrane components are more important for the enhancement of complement susceptibility, we added constant quantities of free and immobilized doxorubicin to different concentrations of SK-MEL-170 cells. Free doxorubicin is known to accumulate in the cells by more than two orders of magnitude as compared to the extracellular concentrations (29–31) and to localize primarily in the nucleus (32, 33). Therefore, the effectiveness of free doxorubicin should be more reduced by a higher cell concentration due to an increased cellular uptake than that of immobilized doxorubicin, if cell membrane components are the main target for the reactive oxygen species.

As shown in Fig. 5, the enhancement of complement susceptibility by free doxorubicin is significantly decreased when the cell concentration is increased from 10^6 cells/ml to 10^7 cells/ml, while the effect of immobilized doxorubicin proved to be rather independent of the cell concentration within this range. It should be noted that both free and immobilized doxorubicin were added at a concentration just sufficient to achieve maximal enhancement of complement susceptibility for 5×10^6 cells/ml.

**DISCUSSION**

The present study demonstrates that reactive oxygen species initiate the sequence of biochemical events leading to the anthracycline-mediated inhibition of C3b degradation and enhancement of complement susceptibility of complement-resistant human melanoma cells. By a combination of different reactive oxygen scavengers, the enhancement can be completely inhibited. Consistent with the proposed role of reactive oxygen species is our finding that the anthracycline glycoside 5-iminodaunorubicin which has the identical structure as daunorubicin except for the imine-substituted quinone moiety and, therefore, does not undergo significant redox cycling with concomitant production of reactive oxygen species (34–37), has no effect on complement-mediated cell killing. The data obtained from the experiments with the immobilized anthracycline glycosides, doxorubicin and daunorubicin, document that the enhancing effect can be induced by extracellular oxygen radical formation, most probably by anthracycline semiquinone-mediated reduction of molecular oxygen to the superoxide anion radical (6–8).

The data obtained with different reactive oxygen scavengers strongly implicate that superoxide anion radical, hydrogen peroxide, and hydroxyl radical are the oxygen species responsible for the alterations leading to the enhancement of complement susceptibility. The hydroxyl radical, a powerful oxidant (38, 39), appears to be the predominant attacking species, since the enhancing effect of both free and immobilized doxorubicin can be inhibited by up to 70% when hydroxyl radical scavengers are present. Superoxide anion radicals and hydrogen peroxide also seem to represent attacking oxygen species, since the additional presence of catalase and superoxide dismutase is required to achieve complete inhibition of the

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**TABLE III**

| Sulfhydryl-blocking agent | % Complement-mediated cytoxicity |
|---------------------------|---------------------------------|
|                           | No Dox | Free Dox | Dox-Fe⁺ chelate | Immobilized Dox |
|                           | 40 µg/ml | 20 µg/ml | 15 µg/ml | 30 µg/ml |
| None                      | 33 ± 2    | 72 ± 3    | 73 ± 1    | 71 ± 4    |
| p-Hydroxymercuribenzoate (1 mM) | 35 ± 2    | 68 ± 3    | 69 ± 2    | 70 ± 2    |
| N-Ethylmaleimide (1 mM) | 31 ± 3    | 68 ± 4    | 64 ± 3    | 68 ± 3    |
| Iodoacetamide (10 mM) | 34 ± 1    | 70 ± 2    | 70 ± 1    | 64 ± 3    |
| N-(β-Iodoethyl)trifluoroacetamide (10 mM) | 32 ± 2    | 67 ± 4    | 67 ± 4    | 66 ± 2    |

**TABLE IV**

| Doxorubicin | % Complement-mediated cytoxicity |
|------------|---------------------------------|
|             | No GSH | 15 mM GSH | 30 mM GSH |
| None        | 33 ± 2  | 32 ± 4    | 33 ± 3    |
| Free Dox    | 54 ± 4  | 51 ± 1    | 47 ± 2    |
| Dox-Fe⁺ chelate (10 µg/ml) | 52 ± 4    | 54 ± 2    | 48 ± 4    |
| Immobilized Dox (7.5 µg/ml) | 54 ± 4    | 51 ± 3    | 49 ± 4    |

**FIG. 5. Effect of cell concentration on complement-enhancing activity of doxorubicin.** Different concentrations of SK-MEL-170 cells were incubated with 40 µg/ml (closed circles) free doxorubicin, or 16 µg/ml immobilized doxorubicin (open circles) and analyzed for complement susceptibility as described under "Experimental Procedures." Control incubations were performed in absence of doxorubicin (closed triangles).
doxorubicin effect. Consistent with this conclusion is a recent report describing toxic effects of superoxide anion radicals without formation of hydroxyl radicals (40).

The complete inhibition of the anthracycline-induced enhancement of complement susceptibility by different agents which chelate transition metal ions strongly suggests that a transition metal, most probably iron, is intrinsically involved in the generation of the activated oxygen species. The simplest explanation is that ferric iron participates in a secondary oxidation-reduction cycle, the iron-catalyzed Haber-Weiss reaction (25–27). Superoxide anion radical (derived from doxorubicin semiquinone oxidation) reduces Fe3+ to Fe2+, which, in turn, reduces hydrogen peroxide by the Fenton reaction to the hydroxyl radical (24). For this cycling process only trace (catalytic) amounts of iron are required. This mechanism would explain why superoxide anion radical, hydrogen peroxide, and hydroxyl radical are involved in the enhancement of complement susceptibility. The completely inhibitory effect by metal ion chelators indicates furthermore that metal (iron)-independent mechanisms for hydroxyl radical formation such as direct reaction of the semiquinone radical with hydrogen peroxide (41–43) are not important for this anthracycline effect. It should be mentioned that the inhibitory effect of EDTA on the complement-enhancing activity of free and immobilized doxorubicin is in contrast to other studies in which EDTA has been shown to be a potent catalyst of the Haber-Weiss reaction (26, 27). On the other hand, doxorubicin-stimulated mouse heart and liver microsomal lipid peroxidation has been reported to be completely inhibited in the presence of 10^-4 M EDTA (44) and the destruction of erythrocyte ghosts by doxorubicin-iron complexes could be totally blocked by the addition of 10^-3 M EDTA (21). Whether the inhibitory effect of EDTA is caused by removing iron from the doxorubicin complex as suggested (21) or by other mechanisms remains to be investigated.

The completely inhibitory effect of metal ion chelators implies that the hydroxyl radical should be the only attacking oxygen species, since the role of iron in the Haber-Weiss reaction cycle is the reduction of hydrogen peroxide to the hydroxyl radical. But even in the presence of high concentrations of the effective hydroxyl radical scavengers Me2S0 and thiourea (45), the doxorubicin-induced enhancement of complement susceptibility is inhibited by not more than 70%. Superoxide dismutase and catalase have also to be present to effect complete inhibition. These data suggest that superoxide anion radical and/or hydrogen peroxide are also generated by a metal-dependent mechanism. One possible explanation is that doxorubicin-iron complexes are involved. It is known that doxorubicin can form metal complexes with both ferrous and ferric ion (21). The affinity of doxorubicin for ferric ion is extremely high, with step association constants of 10^18 M^-3, 10^17 M^-2, and 10^14 M^-1, respectively, for the association of the first, second, and third doxorubicin molecule with Fe^3+ (46). It has been shown that doxorubicin-iron complexes undergo redox cycling in the presence of air resulting in electron transfer to molecular oxygen (23, 47). O2 consumption by doxorubicin-iron complexes has been blocked by the high affinity Fe^2+ chelator bathophenanthroline (47), indicating that molecular oxygen is not reduced by doxorubicin when cycling of bound iron is inhibited. According to this mechanism, metal ion chelators would be expected to be completely inhibitory as observed in our studies, while the inhibitory effect of hydroxyl radical scavengers may be limited depending on the presence of other toxic oxygen species, e.g., superoxide anion radicals.

It is likely that both mechanisms, the iron-catalyzed Haber-Weiss reaction and the doxorubicin-iron complex-mediated generation of oxygen radicals, are involved in the enhancement of complement susceptibility. The extremely high affinity of doxorubicin for iron suggests that doxorubicin-iron complexes are formed even at very low iron concentrations. In our experiments fetal calf serum added to the incubation medium and, most likely, iron present as a contamination in buffer solutions (up to 20 μM (48)), served as iron source. However, the quantity of iron in the incubation medium does not seem to be sufficient to form iron complexes with all doxorubicin molecules. Doxorubicin:Fe^2+ complexes prepared from equal volumes of a 2 mM doxorubicin solution and a 1 mM ferro iron solution at pH 3 proved to be 2- to 3-fold more effective in enhancing complement susceptibility than doxorubicin without prior iron complexation. In contrast, the effectiveness of immobilized doxorubicin could not be increased by prior treatment with ferric iron solutions at pH 3. Whether this is due to the limitation of the drug/iron ratio (maximal 1 doxorubicin molecule/iron molecule), to the lack of formation of an unidentified intermediate upon interaction with the phospholipid bilayer (49), or to other unknown mechanisms remains to be investigated.

Regardless of the mechanism by which molecular oxygen is reduced by doxorubicin-iron complexes, the anthracycline molecule has to be activated to the semiquinone free radical. Intracellularly, doxorubicin can be activated enzymatically, e.g., by NADPH cytochrome P-450 reductase (NADPH:ferrocytochrome oxidoreductase, EC 1.6.2.4) (6, 9, 10). However, the mechanism by which doxorubicin is activated extracellularly, when immobilized on beads, is not known. Recently, doxorubicin has been reported to cause peroxidation of erythrocyte ghost membranes in the presence of Fe^2+, O2, and membrane protein sulphydryl groups without enzymatic activation to the semiquinone free radical (28). From our experiments there is no evidence for the requirement of membrane protein sulphydryl groups since blockade of such groups with four different agents had no significant effect. Soluble sulphydryl compounds such as reduced glutathione or β-mercaptopoethanol up to concentrations of 30 mM had also no significant effect on the complement-enhancing activity of free and immobilized doxorubicin. However, this may be due to two compensating effects of the two thiol-containing compounds. For example, GSH has been described to inhibit doxorubicin-induced hydroxyl radical production by beef heart submitochondriar particles (37), thereby acting as a potent hydroxyl radical scavenger. On the other hand GSH increased the destructive effect of doxorubicin-iron complexes on erythrocyte ghosts from 25 to 95% (21), in this case acting as an effective reductant for doxorubicin-iron complexes. Thus, it is not clear whether the semiquinone radical is formed by a nonenzymatic process or by a membrane-bound or secreted oxidoreductase.

The ability of immobilized doxorubicin to enhance complement susceptibility suggests cell membrane components as target for the attacking oxygen species. However, both superoxide anion radical and hydrogen peroxide, the latter derived from spontaneous dismutation of the superoxide anion radical, are sufficiently stable to diffuse away from their sites of formation. Most probably, the superoxide anion radical does not cross the cell membrane in substantial quantities since it can only pass through a specific "channel" (24), but hydrogen peroxide crosses all cell membranes easily. Thereby, hydrogen peroxide may generate hydroxyl radicals in different parts of the cell wherever a transition metal is available. The very reactive hydroxyl radical could then react with molecules in its immediate vicinity.

First evidence for the cell membrane as main target was
the increased activity of doxorubicin after immobilization. A 3-fold lower amount of immobilized doxorubicin as compared to free doxorubicin is sufficient to effect maximal enhancement of complement susceptibility. An intracellularly located target, however, should be more sensitive to free than to immobilized doxorubicin, since free doxorubicin is taken up by cells to a very high extent (29–31) leading to an intracellular accumulation of doxorubicin by more than two orders of magnitude as compared to the extracellular concentration (31). Furthermore, intracellular doxorubicin has been shown to generate activated oxygen species in different compartments of the cell: in microsomes (6–8, 50), sarcosomes (6, 51), mitochondria (9, 35–37, 52) and in nuclei (53). Rapid inactivation of free doxorubicin in intracellular compartments as an alternative explanation for the greater activity of immobilized doxorubicin is unlikely. No significant metabolism has been demonstrated in different cells under in vitro conditions during incubation periods similar to those in our experiments (30, 31, 54–57). Most probably, the higher activity of immobilized doxorubicin in comparison to free doxorubicin is a consequence of the constant extracellular availability and of a shorter diffusion distance from the extracellular sites of oxygen radical generation to target molecules on the cell membrane. Due to its cellular uptake and subsequent accumulation in the nucleus, free doxorubicin is only for a limited time in close contact with the cell membrane. Hydroxyl radicals generated in the nucleus are unlikely to be responsible for free-radical-induced injury of the cell membrane since in the intracellular environment the hydroxyl radical diffuses only 10–20 Å before being destroyed by an H atom donor.

Further support for cell membrane components as main target derives from the observation that by increasing the cell concentration the complement-enhancing effect of free doxorubicin is decreased by a much higher extent than that of immobilized doxorubicin. The simplest explanation is that by increasing the cell concentration free doxorubicin is faster removed from the medium as a result of an enhanced cellular uptake leading to a shorter period of availability for cell surface interaction. In contrast, the extracellular concentration of immobilized doxorubicin remains constant. Only at very high cell concentrations (>10⁶ cells/ml), the activity of immobilized doxorubicin starts to decrease.

At present, the specific target molecule(s) of doxorubicin action responsible for the increase of complement susceptibility is not known. However, we have shown that both free and immobilized doxorubicin inhibit the degradation of C3b (2). Whether this involves the damage of membrane-bound protective proteases, or complement regulatory membrane components such as the CR1 complement receptor or the decay accelerating factor (58), or other changes in the environment of deposited C3b, is currently under investigation.

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