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Immunology

Immobilized doxorubicin increases the complement susceptibility of human melanoma cells by protecting complement component C3b against inactivation

(complement resistance/monoclonal antibody R24/immunomodulation/cancer therapy)

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ABSTRACT **Human melanoma cells resistant to killing by monoclonal antibody R24 plus human complement became susceptible after treatment with doxorubicin (adriamycin). Treatment with doxorubicin prevented the rapid degradation of surface-bound complement component C3b that has been identified as a protective mechanism of complement-resistant melanoma cells. Doxorubicin caused the increased complement susceptibility as free drug and after immobilization onto glass beads to prevent cellular uptake. Immobilized doxorubicin was more effective than free drug, causing enhanced complement susceptibility at concentrations where the free drug was no longer active. In contrast to free doxorubicin, which exhibited a direct cytotoxic effect leading to cell death within 4 days, immobilized doxorubicin did not affect cell viability. These findings suggest that combination therapy of the complement-activating monoclonal antibody R24 with the complement-enhancing drug doxorubicin may be a promising approach for the treatment of melanoma.**

Nucleated cells such as tumor cells are a natural target of complement. It is well established that nucleated cells vary in their susceptibility to complement-mediated lysis. Recently, we identified a protective mechanism of human melanoma cells against complement attack. We found that complement component C3b is rapidly inactivated on resistant cells (1). In that study we used a murine monoclonal IgG3 antibody for sensitization. The antibody, R24, binds to the G_{D3} ganglioside antigen of human melanoma cells (2, 3) and efficiently activates human complement (4).

Here we report that human melanoma cells resistant to R24 and human complement can be converted into susceptible cells by pretreatment with doxorubicin (former generic name, adriamycin). This conversion into susceptible cells by doxorubicin treatment is accompanied by the protection of C3b against rapid inactivation. Doxorubicin also exerts this effect of enhancing complement susceptibility when immobilized on glass beads, thereby preventing cellular uptake. Immobilized doxorubicin is devoid of direct cytotoxic effects and enhances susceptibility to complement even at concentrations where free doxorubicin is no longer active.

R24, in addition to being a complement activator (4), also efficiently mediates antibody-dependent cellular cytotoxicity (5). These biological effector functions of R24 are considered to be responsible for the encouraging results of a phase I clinical trial with R24 in melanoma patients (6). Combination therapy with a monoclonal antibody and a drug that enhances the biological effector function of the antibody may become a promising means of cancer therapy.

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MATERIALS AND METHODS

Materials. The sources of the R24 monoclonal antibody, of the two monoclonal antibodies to human C3, and of the human melanoma cell lines SK-MEL-170 and SK-MEL-93-2 have been described (1).

Immobilization of Doxorubicin. Ten milligrams of doxorubicin hydrochloride (Sigma) in 10 ml of 0.1 M sodium borate buffer (pH 8.0) was mixed with 1.5 g of dry carbonyldiimidazole-activated glycerol-coated controlled-pore glass beads [74–125 μm diameter, 130 m^2 of total (external plus internal) surface area per g; Pierce] and gently agitated on a rotating wheel at 4°C for 72 hr. Subsequently the beads were washed with borate buffer and reacted with 100 ml of 0.1 M hydroxylamine (pH 8.0) for 1 hr at 4°C to eliminate unreacted imidazolylcarbamate groups. The beads were washed extensively (5 days) with borate buffer, acetonitrile, and methanol at 4°C until free doxorubicin could no longer be detected. The final product had ≈30 μg of doxorubicin covalently attached per 100 μl of packed wet beads and was stored in the dark at 4°C until use. The degree of substitution was determined by incubating 100 μl of doxorubicin beads for 24 hr at 4°C in 1 ml of 0.6 M HCl/ethanol (1:1, vol/vol). This acid treatment splits the glycosidic bond, releasing the red aglycone (adriamycinone), which was determined spectrophotometrically at 233 nm using an $E^{1\%}_{1cm}$ of 673 (7). The absorption coefficients for doxorubicin and adriamycinone were found to be identical. Control beads were treated only with hydroxylamine. Fig. 1 shows the structure of the immobilized doxorubicin.

Treatment of Cells with Doxorubicin. If not otherwise stated, confluent SK-MEL-170 cells were harvested from flasks, washed, and incubated overnight at 37°C in aliquots of 5×10^6 cells in a total volume of 1 ml of complete culture medium containing either 60 μg of free doxorubicin or 15 μg of immobilized doxorubicin (50 μl of beads). Prior to cytotoxicity testing, free doxorubicin was removed by washing, and doxorubicin beads were removed by differential sedimentation after Vortex mixing.

Other Methods. Determination of complement cytotoxicity by dye exclusion, indirect C3 immunofluorescence, binding of ^{125}I-labeled C3 and ^{125}I-labeled monoclonal anti-C3 antibodies to melanoma cells, extraction of ^{125}I-labeled C3 from melanoma cells with subsequent NaDodSO$_4$/PAGE and autoradiography, and determination of G_{D3} surface antigens on melanoma cells by binding of ^{125}I-labeled R24 antibody were performed exactly as described (1). Intracellular doxorubicin was extracted and measured by spectrofluorometry at 580 nm, using 470-nm light for excitation (8).

RESULTS

Free or Immobilized Doxorubicin Enhances Susceptibility to Complement. The human melanoma cell line SK-MEL-170 is

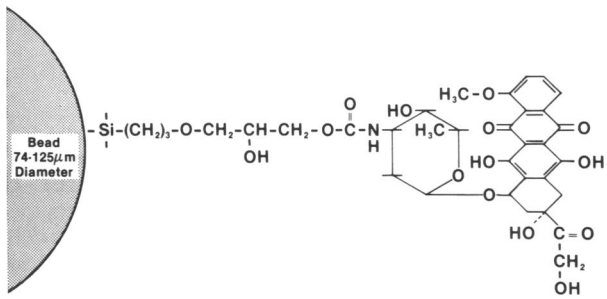

FIG. 1. Structure of immobilized doxorubicin.

resistant to killing by R24 antibody plus human complement. After optimal sensitization and in the presence of excess complement, the final cytotoxicity barely exceeded 30%. Pretreatment of SK-MEL-170 cells with free or immobilized doxorubicin increased the final degree of cytotoxicity to ≥70% (Fig. 2). From the dose–response curves shown in Fig. 2, it is apparent that immobilized doxorubicin is ≈4 times as effective as free doxorubicin. Fifteen micrograms of immobilized doxorubicin per ml caused maximal complement susceptibility, whereas the same amount of free doxorubicin showed only a marginal effect. Under these experimental conditions (15 μg of immobilized doxorubicin per ml), 0.75 μg (5%) of the doxorubicin was removed from the beads and taken up by the cells (Table 1). By repeated exposure of the same doxorubicin beads to fresh batches of cells, the amount of released doxorubicin could be reduced to well under 1% of the doxorubicin input and not-detectable intracellular levels without significantly reducing the complement-enhancing effect of the immobilized doxorubicin. These results indicate that the observed effect of immobilized doxorubicin is not due to cellular uptake of released drug.

Treatment with free or immobilized doxorubicin caused no change in the antigen expression as determined by the binding of radiolabeled R24 antibody (data not shown), indicating that the increased complement susceptibility of doxorubicin-treated cells was not a consequence of increased antigen expression.

Fig. 3 shows that 10–12 hr of exposure to free or immobilized doxorubicin was necessary to achieve maximal enhancement of complement susceptibility. Fig. 4 shows that the enhanced complement susceptibility was persistent after treatment with free doxorubicin, whereas it was reversible after treatment with immobilized doxorubicin. Eight hours after removal of the immobilized drug, the cells had regained the original resistance to R24 plus complement.

Free or Immobilized Doxorubicin Increases C3 Binding and Decreases C3 Degradation. Pretreatment of the resistant SK-MEL-170 cells with free or immobilized doxorubicin increased the amount of cell surface-bound C3 as judged by indirect immunofluorescence (Fig. 5). Without sensitization of the cells with R24 antibody, no C3 fluorescence was observed, indicating that doxorubicin treatment did not render the cells activators of the alternative complement pathway. Fig. 5 further demonstrates that the bond between doxorubicin and the beads is stable. Free doxorubicin was taken up by the cells and massively accumulated in the nuclei; however, in cells exposed to the immobilized compound, no intracellular or intranuclear doxorubicin was visible. ^{125}I-labeled C3 was used to quantitate the apparent increase of C3 binding to doxorubicin-treated SK-MEL-170 cells (Fig. 6); the effects of free and immobilized drug were virtually identical, resulting in levels of C3 binding comparable to those seen with susceptible cell lines (1). The effect of doxorubicin on the degradation of cell-bound C3b was assessed by extracting radiolabeled C3 from the cells (Fig. 7) and by identifying the various forms of C3 on the cells with monoclonal antibodies (Fig. 8). It is evident that doxorubicin inhibits the rapid degradation of C3b on SK-MEL-170 cells in a concentration-dependent manner. Consistent with above findings (see Fig. 2), immobilized doxorubicin was ≈4 times as effective as free doxorubicin.

Table 1. Effect of preexposure of doxorubicin beads to fresh batches of cells on the ability to enhance complement cytotoxicity and on the amount of released drug

No. of preexposures	Doxorubicin uptake (per 5 × 10^6 cells)		Complement-mediated cytotoxicity, % (mean ± SD, n = 3)
	μg	% of input	
0	0.75	5.0	73 ± 2
1	0.1	0.7	71 ± 2
2	<0.05	—	65 ± 3
3	<0.05	—	65 ± 3

Fifteen micrograms of immobilized doxorubicin was exposed 0–3 times to fresh batches of 5 × 10^6 SK-MEL-170 cells by incubation for 1 hr at 37°C in a total volume of 1 ml of complete medium. The preexposed doxorubicin beads were then used to treat SK-MEL-170 cells by the standard procedure described in *Materials and Methods*. Subsequently, complement-mediated cytotoxicity and intracellular doxorubicin were determined.

Immobilized Doxorubicin Is Not Cytotoxic. Immobilized doxorubicin at 15 μg/ml, a concentration sufficient to cause maximal complement susceptibility, is devoid of cytotoxic activity, whereas free doxorubicin, even at the concentration of 15 μg/ml, which has only a marginal effect on complement

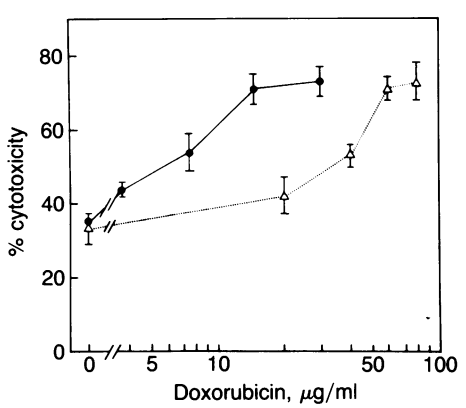

FIG. 2. Complement susceptibility of SK-MEL-170 cells after exposure to various concentrations of free (△) or immobilized (●) doxorubicin. Data are means ± SD (3 determinations).

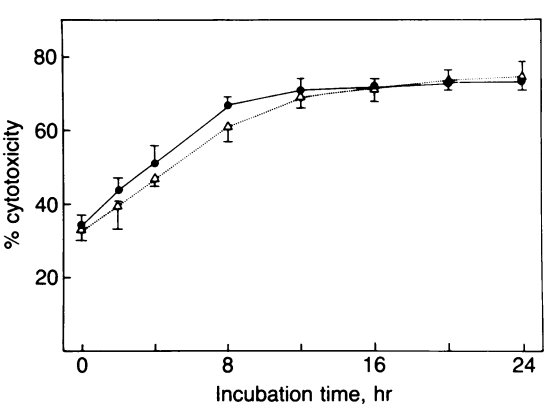

FIG. 3. Effect of incubation time with free (60 μg/ml; △) or immobilized (15 μg/ml; ●) doxorubicin on complement susceptibility of SK-MEL-170 cells. Data are means ± SD (3 determinations).

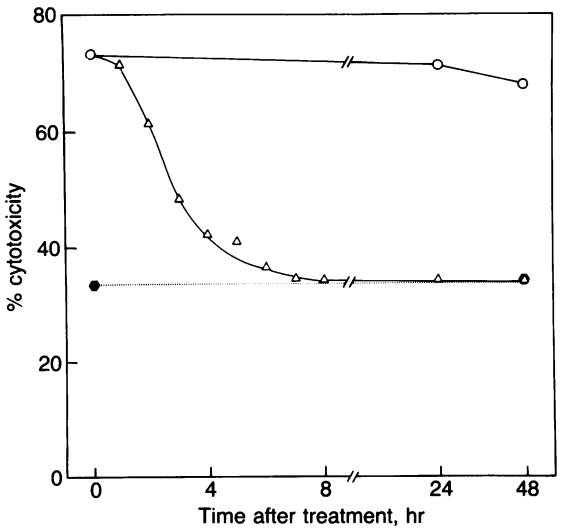

FIG. 4. Persistence of the enhanced complement susceptibility of SK-MEL-170 cells after treatment with free (60 μg/ml; ○) or immobilized (15 μg/ml; △) doxorubicin. After treatment, the drug was removed and the cells were kept in complete medium at 37°C. Complement cytotoxicity was determined at time intervals as indicated. The cytotoxicity for untreated cells (●) is indicated.

susceptibility, leads to cell death within 4 days after exposure to the drug (Fig. 9).

DISCUSSION

In this paper we show that pretreatment with the chemotherapeutic drug doxorubicin converts human SK-MEL-170 melanoma cells, which are resistant to killing by monoclonal antibody R24 plus human complement, into susceptible cells. The final degree of cytotoxicity of doxorubicin-treated SK-MEL-170 cells (≥70%) is identical to the degree of cytotoxicity of SK-MEL-93-2 cells, which are susceptible to R24 plus complement (1). Doxorubicin treatment of the complement-susceptible SK-MEL-93-2 cells had no effect on the final degree of cytotoxicity (data not shown). These results suggest that the doxorubicin-enhanced susceptibility of complement-resistant cells is due to the specific inhibition of a protective mechanism present only in resistant cells. Rapid inactivation of cell-bound C3b is a protective mechanism of resistant melanoma cells (1). Here we show that doxorubicin treatment prevents the rapid inactivation of C3b on SK-MEL-170 cells. Therefore, protection of C3b from inactivation seems to be the ultimate effect of doxorubicin treatment responsible for the increased susceptibility to complement.

Enhanced complement susceptibility after doxorubicin treatment has also been reported, by another group of investigators, for hepatoma and mastocytoma cells after sensitization with polyclonal antisera (9–13). They reported

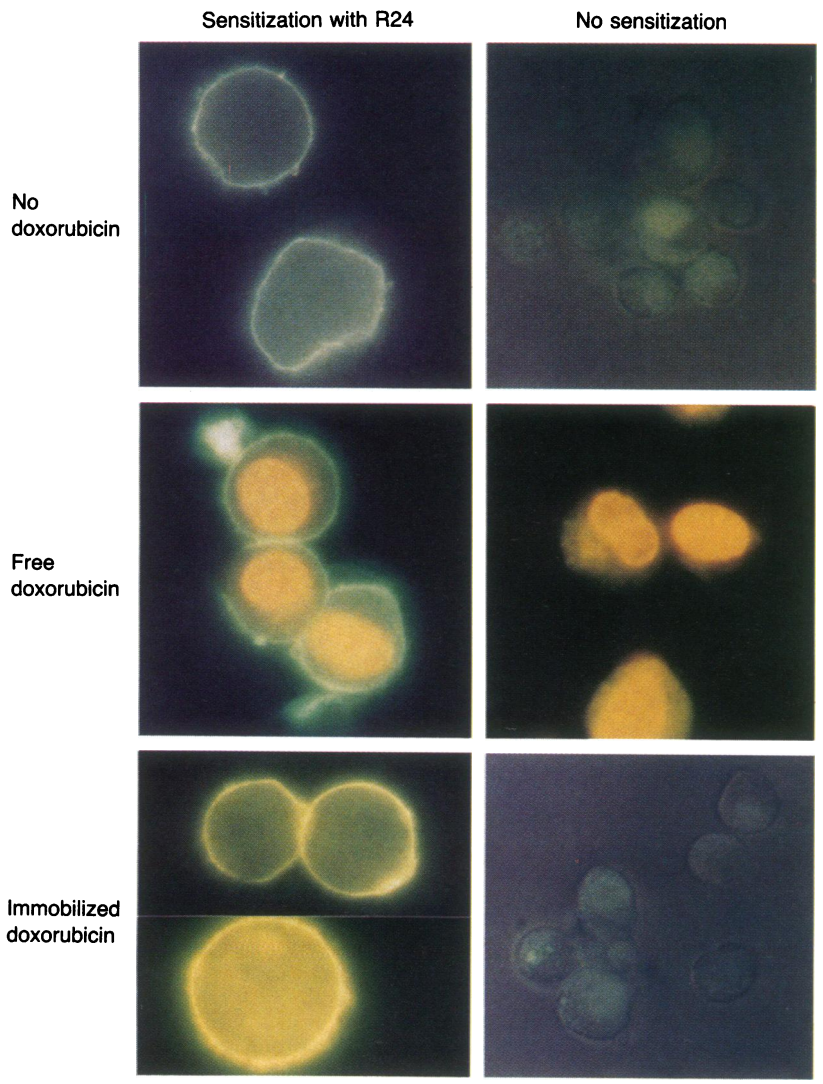

FIG. 5. Indirect immunofluorescence (green) detection of C3 on SK-MEL-170 cells sensitized with the R24 antibody (*Left*) and without sensitization (*Right*). Cells were treated with 15 μg of immobilized (*Bottom*) or 60 μg of free (*Middle*) doxorubicin per ml or were not treated with the drug (*Top*). In cells treated with free doxorubicin, the drug is visible by its yellow fluorescence.

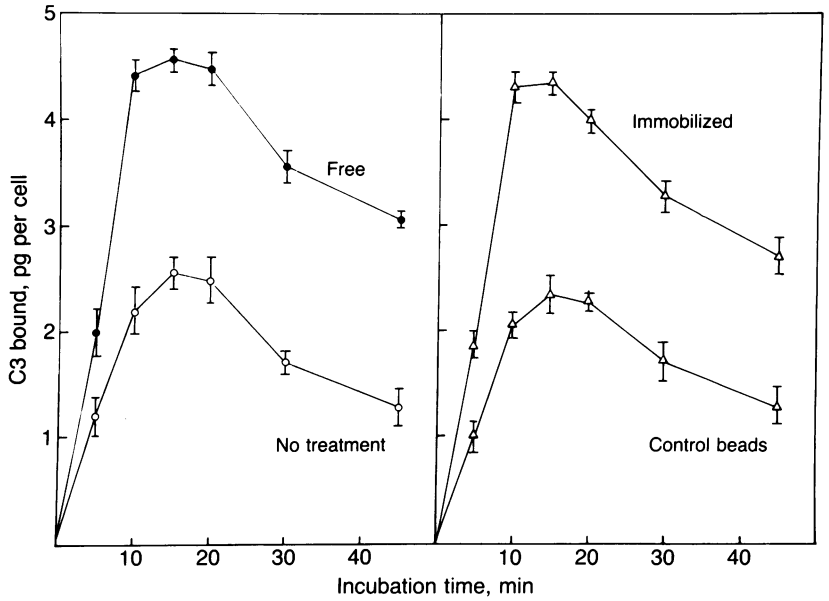

FIG. 6. Effect of free (60 μg/ml) and immobilized (15 μg/ml) doxorubicin on the amount of C3 bound to SK-MEL-170 cells after complement activation. Data are means ± SD (3 determinations).

differences in lipid composition (11, 12) and membrane fluidity (13) after doxorubicin treatment, which they suggested are responsible for the increased susceptibility to complement. In agreement with our results, they found no effect of doxorubicin treatment on antigen expression, but they did not observe any changes in the binding of C3 and C4 (10). However, they did not examine the kinetics of binding of the complement components, nor did they identify the forms of C3 and C4 present on the cells.

The higher effectivity of immobilized doxorubicin suggests that its constant extracellular availability may be responsible for this effect, since free doxorubicin is rapidly taken up by cells (ref. 14; see also Fig. 5). This conclusion makes it likely that doxorubicin's site of action to achieve the enhanced complement susceptibility is on or close to the cell membrane. If indeed a direct contact of doxorubicin with the cell membrane has to occur in order to effect the enhanced complement susceptibility, the effectivity of the immobilized doxorubicin is 3–4 orders of magnitude higher than that of the free drug, since only ≈0.02% of the immobilized doxorubicin molecules are present on the outer surface of the beads, whereas the vast majority is bound to internal surfaces of the porous beads not accessible to the cell surface. Assuming that the doxorubicin is distributed evenly throughout the volume of the beads, the surface concentration (which would be the local concentration at the point of contact with the cell membrane) is at least 500 μg/ml.

The effect of the immobilized doxorubicin is not due to "bleeding" and cellular uptake of freed drug. The amount of intracellular drug after exposure to doxorubicin beads is so low that it cannot be responsible for the observed complement-enhancing effect—even if it were supplied as free drug. Furthermore, immobilized doxorubicin is active at concentrations at which free doxorubicin shows no effect.

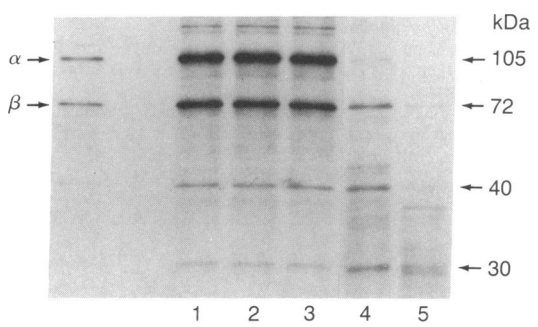

FIG. 7. Forms of C3 extracted from complement-susceptible SK-MEL-93-2 cells and complement-resistant SK-MEL-170 cells after treatment with free or immobilized doxorubicin. The cells were sensitized with R24 antibody and were incubated with human serum supplemented with ^{125}I-labeled C3. Extracts of cells after 15 min of incubation were subjected to NaDodSO$_4$/PAGE with subsequent autoradiography. The dominant bands from susceptible SK-MEL-93-2 cells not treated with doxorubicin (lane 1) and from optimally treated (60 μg of free or 15 μg of immobilized doxorubicin per ml) SK-MEL-170 cells (lanes 2 and 3, respectively) are the α′ (105-kDa) and β (72-kDa) chains of C3b. On untreated SK-MEL-170 cells (lane 5) and on cells treated with a suboptimal concentration of free doxorubicin (20 μg/ml, lane 4), the C3b degradation products iC3b, C3dg, and C3d are present. The α and β chains of purified human C3 are shown in lane at far left.

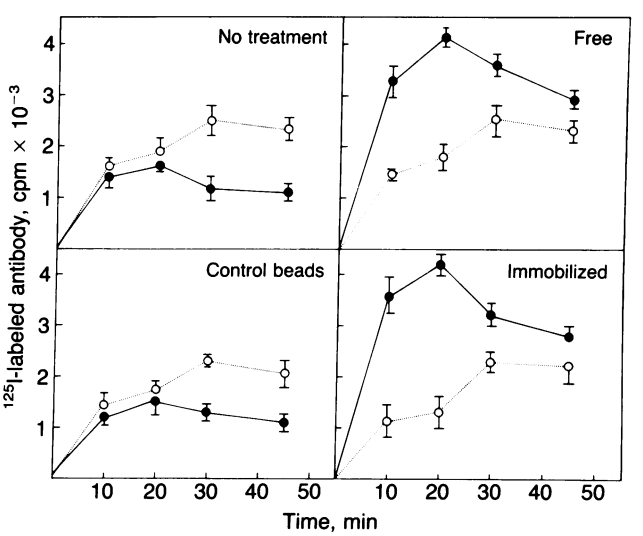

FIG. 8. Use of monoclonal antibodies to C3 to characterize forms of C3 on SK-MEL-170 cells treated with 60 μg of free (*Upper Right*) or 15 μg of immobilized (*Lower Right*) doxorubicin per ml. Results obtained with untreated cells (*Upper Left*) and with cells treated with control (no doxorubicin) beads (*Lower Left*) are also shown. One antibody binds to an epitope on C3c, thereby detecting C3b and iC3b (●). The other antibody binds to a neoantigen present on the C3b degradation products iC3b, C3dg, and C3d (○). Sensitized cells were tested after various times of incubation with human serum at 37°C. Data are means ± SD (2 determinations).

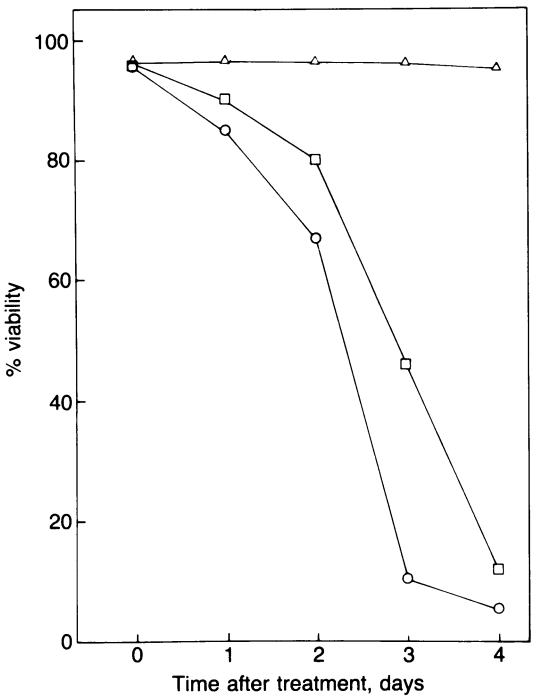

FIG. 9. Effect of 60 μg (○) and 15 μg (□) of free and 15 μg (△) of immobilized doxorubicin on viability of SK-MEL-170 cells. After treatment, the drug was removed and the cells were kept in complete medium at 37°C. Viability of cells was determined by dye exclusion with 0.4% (wt/vol) trypan blue in a hemacytometer.

Other workers have immobilized doxorubicin by covalent linkage to various insoluble supports (15–18). These investigators showed that immobilized doxorubicin still exerts its direct cytotoxic effect and that it was more effective than free doxorubicin. We wish to emphasize that our study is very different from those investigations. We are concerned not with the direct cytotoxic activity of doxorubicin but with its activity to enhance complement-mediated cytotoxicity. The effect of enhanced complement susceptibility occurs much faster than the direct cytotoxic effect of the drug. Maximal complement susceptibility is reached after 10–12 hr of incubation with free or immobilized doxorubicin. At that time virtually 100% of the cells are viable. Four days more (in the absence of doxorubicin) was required to show the maximal direct cytotoxic effect of free doxorubicin, and immobilized doxorubicin was devoid of direct cytotoxic effects. This is in contrast to the findings of others, who described direct cytotoxic effects of immobilized doxorubicin (15–18). Most likely, this difference is due to the different cell line used in this study.

Preliminary work strongly suggests that doxorubicin causes enhanced complement susceptibility by formation of oxygen radicals (19). It is likely that these radicals not only will alter the specific target(s) responsible for the protection of C3b against inactivation and for the enhanced complement susceptibility but also will damage many other cellular components. The altered lipid composition (11, 12) and membrane fluidity (13) of cells exhibiting enhanced complement susceptibility after doxorubicin treatment may therefore be only coincidental and not be primarily responsible for the enhanced susceptibility.

The primary target of the doxorubicin action that causes the persistence of the cell-bound C3b and the enhanced complement susceptibility is unknown. Possible candidates are complement-regulatory membrane proteins, such as complement receptor 1 (CR1) or decay-accelerating factor (DAF), or cell-derived C3b-cleaving proteases. However, the presence of these proteins has not been demonstrated on melanoma cells. Whatever the responsible target molecule altered by the action of doxorubicin might be, cells exposed to the immobilized drug apparently can repair the damage and thus revert to the complement-resistant state within 8 hr after removal of the drug. In contrast, the enhanced complement susceptibility of cells after treatment with free doxorubicin is irreversible. The inability of the cells to repair the doxorubicin-induced damage responsible for enhanced complement susceptibility is most likely due to the accumulation of the drug in the nucleus. Doxorubicin intercalates into DNA, which will interfere with cell metabolism and thus not only prevent the repair of the membrane damage but lead eventually to cell death.

Our findings suggest that the combination of the complement-activating R24 monoclonal antibody with doxorubicin may prove to be an efficient therapy for treatment of melanoma. This combination might cause antitumor effects by enhancement of complement-dependent killing. However, the clinical usefulness of doxorubicin is limited by its relatively high toxicity. In our system, the direct cytotoxic effect of the drug seems to be due to the intracellular accumulation of free drug. Therefore, combination therapy of the R24 antibody with immobilized doxorubicin might achieve strong antitumor effects with reduced general toxicity of doxorubicin due to a substantially decreased cellular uptake. For this, a biocompatible form of immobilized doxorubicin will be needed.

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