Cell Surface Effects of Adriamycin and Carminomycin Immobilized on Cross-Linked Polyvinyl Alcohol

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ABSTRACT

Previous reports have claimed Adriamycin to be cytotoxic to cultured tumor cells when the drug is covalently immobilized on a solid support, thus suggesting a cell surface mechanism of action for the drug. Although these previous reports attempted to rule out released drug or endocytosis of drug-support particles as alternative explanations for the observed cytotoxicity, a more thorough analysis is necessary to substantiate fully the cell surface idea. In the present work, the stability of the drug-support linkage was increased by use of cross-linked polyvinyl alcohol as the support and cyanuric chloride or a diazonium salt for attachment of the drug. Different anthracycline orientations were tested by coupling Adriamycin at the amino sugar and carminomycin at the d-ring. The Adriamycin cross-linked polyvinyl alcohol and carminomycin cross-linked polyvinyl alcohol preparations had much lower drug release rates than did the earlier used carbamate-linked Adriamycin cross-linked agarose materials. All three immobilized drug preparations inhibited the growth of L1210 or S180 clones following 2- or 20-h incubation with cells at 37°C. The results strongly support the concept that immobilized anthracyclines can be cytotoxic to cultured cells, for at least two different orientations of the drug on the support.

INTRODUCTION

The anthracycline antibiotics represent one of the most important classes of antitumor drugs in current clinical use. The original proposal to explain the cytotoxic action of these drugs was intercalation with DNA in the cell nucleus with subsequent inhibition of DNA and RNA synthesis (1). However, some of the newer anthracyclines do not appear to function by intercalation. For example, N-trifluoroacetyl adriamycin-14-valerate does not bind appreciably to DNA and thus presumably acts by a mechanism that does not involve nucleic acid intercalation (2). In addition, there is a growing body of evidence to show that the anthracyclines also exert a variety of actions on the cell surface (3-6).

One way of testing for cell surface actions of these drugs is to couple them covalently to a solid support so that the drug-support particles are too large to enter the cell. Then, when the coupled drug is incubated with cultured cells, any observed cytotoxic effects are assumed to be due to interaction between immobilized drug and the cell surface. For this conclusion to be tested, it must be shown that (a) the rate of release of drug from the support is too small to account for the cytotoxicity, (b) the drug-support particles are not taken into the cells by endocytosis or, if taken up, are not cytotoxic, and (c) the functional group on the drug molecule used for coupling is not required for the cytotoxic action.

Several preliminary reports have appeared (7-9), describing the coupling of Adriamycin to solid supports and the resulting cytotoxic effects of the adducts on cultured cells. In these reports, the authors attributed the cytotoxicity to interaction between the coupled Adriamycin and the surface of the cells. However, in retrospect, it is apparent that several important methodological questions were not completely answered, so that a definitive conclusion, attributing the cytotoxicity to the immobilized anthracyclines, could not be made.

In one of the preliminary studies (7, 8), Adriamycin was coupled to polyglutaraldehyde microspheres, presumably through the amino sugar group on the drug. The polyglutaraldehyde microspheres had a mean diameter of 0.45 μm. Therefore, some of these drug-support particles should have been taken up by endocytosis by the roughly 10-μm-diameter CCRF/CEM or L1210 cells. Such uptake was in fact noted in one of the published studies (7). After incubation with the immobilized drug, the cells were extracted and assayed by fluorescence emission for intracellular Adriamycin. It is not clear how well the drug-containing microspheres were separated from the cells; so, the lack of evidence for intracellular Adriamycin was suggestive but not rigorously established. The release rate data also were not suitable for ruling out a free drug effect. Released drug was measured by spectrofluorimetry at a detection limit of 1 pmol (0.5 ng). Release rates were reported as a percentage of bound drug rather than as the more useful ng of drug released per time per g of dry support. The amount of bound drug was estimated by difference rather than by direct measurement, so that the data on drug released at various times were of uncertain accuracy. And finally, a reported release of 0.00010 to 0.0006% in 24 h from 10⁶ M Adriamycin beads would produce a 1 to 6 nm solution of free Adriamycin. The higher concentration is sufficient to cause inhibition of cell growth. Hydrolysis of the polyglutaraldehyde Adriamycin linkage also may have occurred at a low rate.

In the other preliminary study (9), Adriamycin was coupled through the amino sugar to 1,1'-carbonyl-dilimidazole-derivated cross-linked 6% agarose to give a relatively stable N-alkyl carbamate linkage. The sensitive detection methods used in the present study have shown that the cytotoxicity during the first incubation of immobilized drug with cells was due to released drug and that low levels of drug probably were released, possibly due to hydrolysis of the carbamate linkage, during the first incubation with cells at 37°C. This conclusion was also reached in the earlier study. However, the presence of intracellular drug...
could be measured at nontoxic extracellular concentrations as low as 1 nM. Thus, the presence of intracellular drug below this concentration (i.e., undetectable) should not be cytotoxic (9), although nontoxic pharmacological effects could not be excluded. Therefore, it is doubtful that released drug accounted for the cytotoxicity during the second incubation of immobilized drug with cells. The diameter of the derivatized agarose beads ranged from 40 to 210 μm when wet, according to the manufacturer, so cellular uptake by endocytosis could be ruled out. It should be noted that linkages between the support and the drug, that may be viewed as quite stable for immobilization of enzymes or for attachment of affinity ligands, may still have excessive leakage rates for these immobilized drug cell surface studies.

The present work presents a more rigorous experimental approach for the preparation and testing of the covalently coupled anthracyclines. Two types of supports, 3 different linkages, and 2 different points of attachment on the anthracycline molecules were used. Most of the work was done using CL-PVA as the support, although derivatized 6% agarose from the earlier studies also was examined further. Both Adriamycin and carminomycin were coupled to the polyvinyl alcohol, using linkages that were of greater stability than the ones used in the earlier studies (7-9). The measurements of drug release rates were carried out much more rigorously and with higher detection sensitivity than reported for the previous works. Preliminary reports of portions of this work have appeared elsewhere (10, 11).

MATERIALS AND METHODS

Materials. Adriamycin, high purity without any additives from the National Cancer Institute, was used without further purification. Carmi- nomycin, received in solid form mixed with mannitol as a gift from Bristol Laboratories, also was used as received. HPLC assay of the Adriamycin showed only one peak, while that of carminomycin showed 2 peaks with the unknown impurity equivalent to about 5% of the carminomycin. The polyvinyl alcohol was fully hydrolyzed Elvanol 71-30, having a degree of polymerization of about 17,000, and was a gift from E. I. du Pont de Nemours. Terephthalaldehyde (Aldrich) was recrystallized from methanol (m.p. 113-114.5°C); cyanuric chloride (Aldrich) was recrystallized first from anhydrous benzene and then from anhydrous acetone and vacuum dried (m.p. 139.5-141.0°C); p-Nitrobenzyl chloride (Eastman) was used as received. Reacti-Gel 6X (Pierce Chemical Co.) was used as the support for coupling through the carbamate linkage to cross-linked 6% agarose. Two lots of [14C]Adriamycin hydrochloride of 7.5 to 12.2 mCi/mmol specific activity were obtained through Dr. John Douros of the National Cancer Institute. Each lot gave a single peak, which eluted at the Adriamycin retention time, when assayed by HPLC. The eluents were collected into 40 fractions of 1 ml each and quantified by liquid scintillation counting.

Preparation of Support and Coupling of Drugs. CL-PVA was prepared by the acid-catalyzed reaction of terephthalaldehyde with polyvinyl alcohol, based on the procedure of Manecke and Vogt (12) (Chart 1). Methanol was added to block unreacted aldehyde groups. Following overnight reaction, the solids were filtered and washed with water and methanol until free of chloride ion and unattached terephthalaldehyde. The polymer particles were vacuum dried, ground, and sieved. The fraction between 90 and 106 μm was used for attachment of drug or for control studies with cultured cells.

For attachment of Adriamycin, cyanuric chloride was used to activate the CL-PVA (12) (Chart 1). The CL-PVA was heated to 80-90°C in 10% aqueous sodium hydroxide, cooled to room temperature, and drained of excess liquid. A solution of 10 g of cyanuric chloride in 90 g of dry acetone was added to the CL-PVA. After 10 min, the coupling was stopped by addition of 40 ml of 20% acetic acid. The activated support was washed with acetone to remove unattached cyanuric chloride and used immediately for coupling of Adriamycin (Chart 2). Adriamycin hydrochloride (37.5 mg) was dissolved in 5 ml of water and mixed with 25 ml of 0.2 mM sodium bicarbonate-carbonate buffer (pH 8.9). The Adriamycin solution was added to 7.4 g (wet weight) of the activated CL-PVA, covered with Parafilm and aluminum foil, and gently agitated for 4 h at room temperature. During this period, the pH changed only slightly to 8.6. After overnight refrigeration, washing of Structure 8 (Chart 2) was started to remove loosely attached Adriamycin.

For attachment of carminomycin, p-nitrobenzylchloride was used to activate the CL-PVA (12) (Chart 1). The CL-PVA (7.1 g dry weight) was swelled in water, filtered, mixed with 130 ml of 5% sodium hydroxide, and heated to 95°C. A 28-g portion of p-nitrobenzylchloride was added, and the slurry was stirred for 1 h. After cooling and filtration, the support was washed repeatedly with acetone and with water, then subject to Soxhlet extraction with acetone for 1 day to remove the final traces of unattached nitrobenzylchloride, and vacuum oven dried. An aliquot (1.5 g) of the nitrobenzyl-derivatized CL-PVA was reduced with sodium dithionite to the corresponding amine in 70 ml of 10% sodium hydroxide (Chart 2). The solid product was washed extensively with methanol and then water, followed by vacuum drying.
After diazotization, the support was placed in 0.2 M bicarbonate-carbonate buffer (pH 8.9), and about 2 mg of carminomycin, dissolved in 95% ethanol, were added (Chart 2). The slurry was agitated gently for 2 h at room temperature. A few drops of sodium hydroxide solution were added after 1 h to raise the pH from 8.6 to 8.9. After overnight refrigeration, washing of Structure 9 (Chart 2) was started to remove loosely attached carminomycin.

Adriamycin also was coupled to cross-linked agarose beads activated with 1,1′-carbonyldiimidazole, as described previously (9) (Chart 3). The wet beads had a diameter of 40 to 210 μm and a concentration of imidazolyl groups >50 μmol/mg of gel. For this coupling, 10.5 mg of Adriamycin hydrochloride were dissolved in water, and the pH quickly adjusted to 7 with dilute sodium hydroxide, followed by addition of 3.0 ml of 0.1 M borate buffer (pH 8.0). The solution was chilled to 4°C, mixed with 1 g of drained Reacti-Gel, and mildly agitated for 2 days at 4°C. Addition of 10 ml of 0.05 M hydroxylamine in 0.05 M borate buffer (pH 8.0) was used to block unreacted imidazolyl groups. Then, washing was started to remove loosely attached or adsorbed Adriamycin.

In all of the studies, the anthracyclines were protected from light by wrapping the containers with aluminum foil to prevent photodegradation (13).

Washing and HPLC Assay of Washes. With Adriamycin, the washing to remove loosely bound or adsorbed drug was done with methanol, acetonitrile, 0.02 M phosphate buffer (pH 7.4), and 3 M NaCl. Carminomycin was washed with the same materials, except water was used instead of acetonitrile. The initial washes were monitored for absorbance at 485 nm, using a Perkin Elmer Model 320 dual-beam spectrophotometer, and for fluorescence, with a Perkin Elmer Model 650-10S dual monochromator fluorescence spectrophotometer. When no drug could be detected in the washes using absorbance/fluorescence, the washes were assayed by HPLC (2 Waters Model 6000A pumps, U6K injector, and 720 gradient controller). The Perkin Elmer Model 650-10S fluorescence spectrophotometer, equipped with a Hitachi flow cell plus a Fisher Series 5000 recorder, served as the detection system. A Waters reversed-phase phenyl column was operated with a mobile phase consisting of the following: primary, 75% aqueous ammonium formate (pH 4.0) and 25% acetonitrile; secondary, 100% acetonitrile, gradient linear from 100% primary to 50:50 primarysecondary over 17 min (14). Fluorescence excitation was at 470 nm, and emission was at 548 nm for both Adriamycin and carminomycin.

Testing of Immobilized Drug with Cells. Suspensions of cultured L1210 murine leukemia or S180 sarcoma cells were grown in Fischer's medium containing 10% horse serum to a cell concentration of 0.8 to 1.0 x 10^8 cells/ml. About 50 ml of cell suspension were added to 50 to 250 mg of ethanol-sterilized immobilized drug (either fresh or recycled preparations) and incubated for 2 or 20 h at 37°C. Following the incubation period, the 50 ml of filtrate were divided, with 1 ml used for colony formation testing and 49 ml for extraction of any intracellular drug. Colony-forming efficiency was assessed by seeding soft agar with...
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A known number of cells and then calculating the percentage of cells that formed colonies after 10 to 14 days (15). The cloning efficiencies of untreated S180 and L1210 cells were 60% and 90%, respectively, with a ±20% day-to-day variability. Several replications of triplicate or quadruplicate experiments yielded a variability in relative survival between treated and control cells of ±10%. Thus, the differences in survival reported below are significant and reproducible. The cells to be tested for the presence of intracellular drug were separated from the drug-support particles by centrifugation of the cell-support mixture at 1000 × g over Ficoll:Hypaque. Only the support particles passed through the Ficoll, while the cells collected at the buffer:Ficoll interface. The drug-support particles could be washed and used again. The cells were collected, lysed in 0.5 ml of 2% sodium carbonate, and extracted with 2 ml of 9:1 (v/v) ethyl acetate:propyl alcohol. The lysis-extraction was repeated 3 to 4 times, and the organic layers were combined and evaporated with nitrogen to dryness. This procedure was 95 to 99% efficient at extraction of anthracyclines (16). After addition of 200 µl of 70% ethanol, 100 µl were injected for HPLC analysis. For the cellular extract assays, the HPLC system consisted of 2 Altex Model 110A pumps, an Altex 420 controller and 905-42 injector, an American Instrument Co. Fluoro-Monitor filter fluorimeter peaked at 360-nm excitation (Comb 7-60 filter) and passing emission wavelengths above 485 nm (Wattten No. 8 filter), and a General Electric Blacklite lamp. The column, mobile phase, and gradient elution were similar to those used for the HPLC assay of the washes.

RESULTS

Drug Release Rates. Washing of the immobilized drug preparations was carried out over several months time, during which the rate of release of drug gradually diminished. For the final washing periods, methanol was dripped continuously through packed beds of the drug-support particles at room temperature for 72 to 96 h at a rate of approximately 5 ml/h. The cumulative washes during the 72 to 96 h were collected and vacuum rotary evaporated to about 200 µl. An aliquot of the 200 µl was assayed by HPLC to quantify the amount of released drug. The minimum detectable quantity of drug was defined as twice the noise level of the Perkin Elmer fluorescence detector. By this criterion, 0.1 to 0.3 ng of drug could be detected. The release rate was calculated as the drug that appeared in the total wash solution divided by the washing time and the weight of dry support. The release rate results are summarized in Table 1. The pre-37°C results in Table 1 were assumed to be the base rates and possibly to continue during incubation of the drug-support particles with cells, since no further reduction was observed with additional washing with methanol. Methanol, rather than water, was used in the final washing and release rate measurements, because the release rates were at least as high in methanol as in water, and the particles sometimes underwent reversible color changes during prolonged (1 week) aqueous washing. Some of the pre-37°C washed drug-support particles subsequently were incubated in methanol for 6 to 7 h of additional time at 37°C to simulate the effects of the elevated temperature when used in the cell cytotoxicity studies. As shown in Table 1, considerable drug was released during the first 15 min and during the entire 6- to 7-h incubation at 37°C. The post-37°C results in Table 1 represent the base rates following prolonged washing after the 37°C treatment. Both the pre-37°C and post-37°C drug-support particles were used in cell cytotoxicity experiments. HPLC analysis showed that about 90% of the released material had the 13-min retention time of the Adriamycin standard, as compared to a retention time of 15 min for the aglycone. Control runs with soluble Adriamycin in methanol were carried out to correct for the conversion of Adriamycin to aglycone during vacuum evaporation of methanol washes.

The possibility of CL-PVA acting to sequester noncovalently attached Adriamycin during the immobilization for slow diffusional release during washing and cell testing was examined using 14C-labeled Adriamycin. In a separate experiment, radiolabeled plus unlabeled Adriamycin was agitated with underivatized CL-PVA for 3.5 h and then given the same 6 months of washing as the covalently attached preparation received. A control sample of underivatized CL-PVA also was carried through the shaking, months of washing, drying, and burning to CO2 for counting. It took 6 months of washing to remove 90% of the 14C radioactivity added to the underivatized CL-PVA preparation. This verified the need for prolonged washing to allow time for the noncoupled Adriamycin to diffuse out of the CL-PVA matrix. Similar diffusion rates were observed for the agarose support.

Loading of Drug Support. An aliquot of the post-37°C Adriamycin:cyanuric chloride CL-PVA preparation was hydrolyzed to release the aglycone (adriamycinone) and thereby obtain an estimate of the amount of immobilized drug. To accomplish this, the drug-support material (100-mg dry weight) was incubated for 48 h at 60°C in 0.2 N HCl. Mild acid hydrolysis had been shown previously to give complete cleavage of the aglycone—sugar bond of soluble Adriamycin (17). During hydrolysis, the aglycone precipitated. The solid was collected, washed with water to remove HCl, dissolved in methanol, and scanned spectrophotometrically. The absorption spectra were essentially identical for the material hydrolyzed from the drug-support material and an aglycone standard prepared from soluble Adriamycin. HPLC analysis of the drug-support hydrolysis product showed a single peak at 15-min retention time, identical to that for the aglycone standard. The highly purified aglycone standard was used to obtain an extinction coefficient of 1.34 × 104 M⁻¹ cm⁻¹ at 485 nm in methanol. This compares well with the value of 1.25 × 104 M⁻¹ cm⁻¹ at 485 nm for Adriamycin in methanol. From the absorbance of the drug-support hydrolysis product, the loading was calculated to be 0.89 mg of Adriamycin per 100 mg (dry weight) of the Adriamycin:cyanuric chloride CL-PVA preparation.

The concentration of immobilized Adriamycin within the support particles was calculated from the loading measurements and a knowledge of the particle size and weight. The mean weight of particles of underivatized CL-PVA was determined by weighing a known number of dry particles on a Cahn Model 29 electrobalance. The mean weight of 2.3 µg for each pyramid-
shaped particle of about 150 μm in length and 150 μm in height (1.1 × 10⁻⁶ ml) was equivalent to a loading of 0.020 μg of Adriamycin per particle. This loading utilized only 0.1% of the available CL-PVA hydroxide groups and was equivalent to a concentration of 34 μM Adriamycin (i.e., 0.034 mmol/ml of support) distributed throughout the total volume of each dry CL-PVA particle. Little swelling of the particles was observed in water, so that the 34 μM also was the approximate concentration of immobilized drug in the hydrated particles. The CL-PVA particles underwent attrition during the derivatization and washing procedures. The mean particle size was reduced to about 50 μm, but no particles under 20-μm shortest dimension were observed by microscopic examination.

The concentration of covalently immobilized Adriamycin also was checked by preparing a small sample of CL-PVA cyanuric chloride 14C-labeled Adriamycin. Both the mol ratio of cyanuric chloride to CL-PVA (0.36 for labeled versus 0.79 for nonlabeled syntheses) and the mol ratio of Adriamycin hydrochloride to cyanuric chloride (0.23 for labeled versus 1.2 for nonlabeled syntheses) used in immobilizing the Adriamycin were less for the 14C-labeled Adriamycin preparation. After several months washing to remove loosely attached drug, the 14C-labeled Adriamycin CL-PVA preparation was burned in a Packard Model 306 sample oxidizer. The resulting 14C-labeled carbon dioxide was absorbed and then counted with a liquid scintillation spectrometer. The results showed 1.59 mg of immobilized labeled Adriamycin plus 1.87 mg of immobilized nonlabeled drug. This was equivalent to 0.22 mg of Adriamycin per 100 mg of dry support or a concentration of 8.6 μM distributed throughout the CL-PVA particles. This was less than the 34 μM preparation by a factor of 4.0, approximately as predicted by the stoichiometry. Therefore, the labeled and nonlabeled Adriamycin-loading experiments gave reasonably consistent results.

An alternative method for expressing the concentration of immobilized Adriamycin is to assume the bound drug was distributed homogeneously throughout the culture medium. With 200 mg of drug support in 50 ml of culture medium, the 1.78 mg of immobilized Adriamycin would have had a concentration of 66 μM if distributed homogeneously in the medium. The immobilized 14C-labeled Adriamycin would have had a concentration of 16 μM if distributed homogeneously. Of course, in the polymeric particles, not all of the attached drug will be accessible to cellular interaction, since only a small fraction will be on the surface of the particles. This aspect is discussed later.

Effect of Drug-Support Particles on Cells. The Adriamycin: cyanuric chloride CL-PVA preparation (200 mg) was incubated at 37°C with 5 × 10⁴ S180 cells for 2 and 20 h. As shown in Chart 4, only 80% of the cells were capable of forming colonies on soft agar after the 2-h incubation. This decreased to 40% after the 20-h incubation. The CL-PVA, without attached drugs, had no effect on the ability of either S180 or L1210 cells to form colonies, as shown by separate control tests. Thus, the Adriamycin: cyanuric chloride CL-PVA particles were cytotoxic to the S180 cells under the test conditions. The data in Fig. 4 also show that no intracellular Adriamycin was detected when about 4.9 × 10⁶ of the drug-support incubated cells were extracted and assayed for Adriamycin. However, the Chart 4 results were obtained with pre-37°C washed drug support (Table 1), and the data shown were for the second incubation of the drug-support particles with S180 cells. Intracellular Adriamycin was found in the cells used in the first incubation. Although no intracellular Adriamycin was detected after the incubation with the second batch of cells, some drug probably was released. An estimate of the amount of released drug and the possibility that this amount could account for the observed cytotoxicity are discussed in the next section. The Chart 4 experiments were repeated with post-37°C washed drug-support particles. The results (Table 2) show that no intracellular Adriamycin was detected on the first incubation of the drug-support particles with the cells. This is clear evidence that, with pre-37°C drug-support preparations, the appearance of intracellular drug following the first incubation of the drug-support particles with cells was the result of drug release enhanced by the 37°C temperature of the cell test procedure.

The cytotoxic effects of the incubation of 200 mg of the diazonium: carminomycin CL-PVA particles with S180 or L1210 cells are given in Chart 5. These drug-support particles were the pre-37°C washed type and thus had not been conditioned at 37°C during the washing procedure. However, these particles did not show any evidence for intracellular carminomycin after incubation of the drug-particles with either the first or second batch of cells. The unusual time dependence of the results of Columns F and G of Chart 5 may be due to desensitization or
cytotoxicity must occur as a result of action on the cell surface. Possible mechanisms include, for example, modifications of cell membrane fluidity, changes in membrane transport systems for ions or nutrients, or inhibition of the rate of synthesis or degree of activity of membrane messenger molecules. It is conceivable that the immobilized Adriamycin may interact with a protein that protrudes from the cell surface into the surrounding solution, and that such interaction mediates the action of the drug. Several recent publications touch on this possibility. For example, a recent study using Fourier transform IR spectrometry to examine the interaction of dissolved Adriamycin and a human erythrocyte cell line suggested that Adriamycin may interact with cell membrane proteins (18). Recent photoaffinity labeling studies also demonstrate interaction of the drug with cell surface proteins (19). It may also be possible for Adriamycin to change the oxidation-reduction potential across the cell membrane by modifying the activity of an oxidation-reduction enzyme located on the outer surface of the membrane (20). Tritton and Hickman (21) have reviewed a large variety of potential mechanisms for action of antitumor agents on the cell membrane.

Some mechanisms might be favored by a long spacer arm between the immobilized drug and the support. In the present study, the linkage between CL-PVA and the immobilized drug was about 7 Å and 13 Å, respectively, for the Adriamycin and carminomycin preparations. These distances are based on a mean bond length of 1.4 Å. However, long flexible arms of the CL-PVA may have made the linkages appear to be much longer. The degree of cross-linking of the PVA utilized only 20% of the PVA hydroxyl groups or about one cross-link for every 340 repeating units (Chart 1). Therefore, there probably were relatively long polymeric chains of about 14,000 mean molecular weight of PVA that were anchored at one end, with the other end free to move about in solution and containing several Adriamycin or carminomycin groups. This would provide sufficiently long spacer arms for significant contact between the bound drug and the cell membrane. In future experiments, it may be desirable to vary the degree of crosslinking, with a constant Adriamycin loading, both with and without added spacer molecules, to see what effect changes in the drug-support geometry may have on the observed cytotoxicity.

Other important considerations are (a) the contact time between the cells and the immobilized drug and (b) a pharmacologically meaningful way for describing the concentration of immobilized drug. In considering the contact time, it is important to understand that the cells were grown in suspension. The cells encountered the immobilized drug by Brownian motion or by gentle agitation of the cell-support slurry. Visual microscopic examination showed that the cells did not remain attached to the polymer beads. However, no estimates are available on the mean contact time of the cell-support contacts.

The second problem referred to above is that of determining a pharmacologically meaningful concentration for immobilized drugs. Thus, we have found it useful to define 3 terms: (a) "total distributed," (b) "accessible distributed," and (c) "effective" concentrations. The total distributed concentration is defined by treating the total quantity of immobilized drug as if it was distributed uniformly throughout the solution. For example, 200 mg of Adriamycin CL-PVA (0.89 mg of drug per 100 mg of dry support) incubated with 50 ml of cell suspension gives a total distributed concentration of 1.78 mg/50 ml or 66 μM. In the earlier study...
with the agarose support (9), the total distributed concentration was 8.5 μM. However, a significant fraction of the total immobilized drug is on the interior of the support particles and thus not available for interaction with cells. Because of this, we define the accessible distributed concentration as the amount of drug on the surface of the support divided by the volume of solution. With the spherical agarose beads (9) or pyrimidal CL-PVA particles, it is estimated that the accessible distributed concentration was less than 0.01% of the total distributed concentration. The estimate of 0.01% was based on the assumptions that an Adriamycin molecule takes up 1.0 sq nm of surface area and 0.5 cu nm of volume. The number of Adriamycin molecules that could fit on the surface of a 100-μm-diameter agarose bead (3.1 x 10^10) was divided by the number of molecules that could fit in the volume of one bead (8.4 x 10^10) to give a ratio much less than 0.01%. The accessible distributed concentration was less than 0.85 nM with the earlier agarose bead preparation (9) and less than 6.6 nM with the CL-PVA material. Yet, it requires about 50 nM free Adriamycin to give 40 to 50% cell kill. The immobilized Adriamycin thus appears to be more potent than the free drug. The effective concentration is defined as the quantity of the immobilized drug divided by the volume of the particles and thus represents the actual concentration or density of immobilized drug both on the surface as well as in the interior of the particles. With the CL-PVA preparation, the effective concentration was 34 nm. Thus, at the point of contact between a cell and the drug-carrying support, the effective concentration is very high. Essentially then, the supports act to focus the action of the drug at high concentration at sites on the cell surface. By this explanation, the immobilized drug is capable of being more potent than the free, diffused drug in exerting its toxic action on the plasma membrane.

The second possible cause of the observed cytotoxicity is that of released drug, acting either on the cell membrane or within the cell. The rate of release of immobilized drug is a very important variable, and thus we have attempted to measure it very accurately. From Table 1, it is clear that drug released during the first incubation of the Adriamycin carbamate agarose preparations with cells was the source of the observed intracellular Adriamycin in the earlier study (9). The observed intracellular Adriamycin was equivalent to incubation of the cells with about 200 nM free drug. In good agreement, we find that the release rates from Table 1 for 50 mg of the agarose preparation for 2 h at 37°C would produce 12 nM released Adriamycin. In the earlier (9) study, the drug-support had not received a 37°C treatment prior to the initial incubation with the cells. However, if the cell-exposed Adriamycin-derivatized polymer was recycled with a fresh suspension of cells, the polymer retained its cytotoxic activity but did not release detectable intracellular drug. This suggests that either exposure to the cells or to a 37°C treatment (or both) was responsible for releasing the initially detectable intracellular Adriamycin. In the present work, the post-37°C release rates of 0.2 and 21 ng/h/g dry support for Adriamycin cyanuric chloride CL-PVA and Adriamycin carbamate agarose, respectively, would have resulted in concentrations of released Adriamycin of 0.01 nm or 0.3 nm after 2 h and 0.1 nm or 3 nm after 20 h of incubation of 200 mg of the CL-PVA or agarose supports, respectively, at 30°C in 50 ml of medium. None of these concentrations, with the possible exception of 3 nm, would be expected to exert a measurable intracellular cytotoxic effect on L1210 or S180 cells. In earlier tests (9), 1 nm free Adriamycin did not cause noticeable cell cytotoxicity but did give detectable intracellular Adriamycin after incubation with cells and subsequent extraction and assay for intracellular drug. The assumption is made that a second incubation at 37°C, this time in aqueous medium with cells, would not give a significant increase in release rates over the post-37°C values. When the drug-support particles were recycled and placed in fresh medium with a new batch of cells and again incubated for 2 or 20 h, no intracellular drug was detected. This is strong evidence that there was a major decrease in drug release rates between the first and second 37°C incubations. One potential source of enhanced drug release that needs to be checked further is the stability of the drug-support linkages in the presence of the horse serum component of the cell growth medium. Preliminary data did not show any enhanced Adriamycin release on addition of horse serum, but the numerous enzymes present in horse serum necessitate further testing.

The present work provides strong support in favor of a direct cytotoxic effect on the cell surface by immobilized Adriamycin and camarinomycin. The findings answer the methodological questions about one of the previous studies (9) but leave unanswered the question about the source of the cytotoxicity in the other (7, 8). Additional studies to provide evidence as to the molecular mechanism of action as well as better documentation of the coupling linkages are under way. It may turn out that this cell surface effect also is a major contributor to the mode of cytotoxicity for free as well as immobilized anthracyclines. If so, then being able to limit the number of actions, through immobilization, may be therapeutically useful in reducing the toxicity associated with this class of compounds. Preliminary work appears to support this idea (22).

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