Enhancement of Adriamycin Antitumor Activity by Its Binding with an Intracellular Sustained-Release Form, Polymethacrylate Nanospheres, in U-937 Cells

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ABSTRACT

We investigated the antitumor activity of Adriamycin on a monocytic-like cancer cell line U-937 after its binding on polymethacrylate nanospheres (diameter, 270–350 nm). Compared to free Adriamycin (F-ADR), nanosphere-bound Adriamycin (B-ADR) exhibits a 3-fold enhancement of cytotoxicity, as determined by cell growth inhibition and DNA synthesis, after continuous exposure to 0.02 and 0.04 µg/ml. The 90% growth inhibition concentration was 0.051 µg/ml for F-ADR and was 0.018 µg/ml for B-ADR (P < 0.001). Furthermore, the nanosphere densities per cell play an important role since for the same drug concentration the higher the density increases, the better the activity is. Indeed, after 4 days of incubation in a medium containing 160 nanospheres at 0.5 fg/cell, the cell counts were 62.8 ± 12.8% (SD) of the initial inoculum and they were only 16.1 ± 0.1% after incubation in a medium containing 80 nanospheres at 0.1 fg/cell (P < 0.001). A comparable enhancement of activity regarding the nanosphere densities was observed after a 24-h exposure to 0.02 and 0.05 µg/ml. Short-term uptake studies showed that B-ADR accumulation was higher with B-ADR than with F-ADR. In addition, the efflux kinetics was modified. For cells exposed to F-ADR for 4 h, the efflux half-life was 23.7 ± 7.7 h and the area to infinity under the efflux curve was 8.6 ± 2.8 µg/mg protein × h -1. For cells exposed to B-ADR, the efflux half-life increased to 85.9 ± 19.2 h and the area to infinity under the efflux curve to 29.6 ± 6.6 µg/mg protein × h -1 (P < 0.001). Electron transmission microscopy and previous findings have revealed that B-ADR was well internalized into cells. Our data support the hypothesis that B-ADR acts as an intracellular drug release complex after endocytosis. The findings regarding the number of nanospheres per cell and dose-effect relationships are consistent with mechanisms of drug actions extending to membrane domains.

INTRODUCTION

Cellular resistance to anthracyclines has been reported to be mostly related to a reduced intracellular drug accumulation including reduced influx and/or increased efflux (1–3). However, it is now widely recognized that unidirectional drug influx is a simple diffusion process and that no difference is detected in the influx of either Adriamycin or daunorubicin into several sensitive and resistant cell lines (4–6). Thus, resistance was attributed solely to a more active drug efflux mechanism out of resistant cells and has become part of the much larger topic of pleiotropic drug resistance (7–11). Several methods have been proposed to overcome this pleiotropic resistance (12–15).

A possible way of overcoming resistance, involving the use of a carrier, was pioneered by Trouet et al. (16). Surprisingly, this original concept of an intracellular sustained-release form has not been widely studied and the drug carrying has principally developed with the aim of drug targeting (17, 18). Particularly, liposomes (19–21) or microspheres (22–25) have been used as carriers for various antineoplastic drugs. Other studies have demonstrated greater interest of ultrafine particles (nanoparticles or nanospheres) with diameters of approximately 250 to 350 nm (26–29). Furthermore, it has been shown that the binding of antineoplastic drug to nanoparticles may modify their distribution pattern in tissues (30, 31). It has also been claimed that the binding with polyalkylcyanoacrylate nanoparticles increased the anticancer activity of actinomycin D (32) and 5-fluorouracil (31) in Sarcoma 180-bearing mice. The possibility of reducing the host toxicity of Adriamycin by fixing it on polyisobutylcyanoacrylate nanoparticles has been investigated by Couvreur et al. (33) but the cytotoxic activity of these macromolecular carriers on cancer cells was not studied. The present study was initiated to compare the cytotoxic effects of B-ADR3 to those of F-ADR on the monocytic-like cancer cell line U-937. We report here that the ionic binding of Adriamycin to polymethacrylate nanospheres with diameters from 270 to 350 nm enhances the cytotoxicity of this drug and increases its intracellular drug accumulation.

MATERIALS AND METHODS

Chemicals. [methyl-3H]Thymidine (43 Ci/mmol) and 14C-Adriamycin (56 mCi/mmol) were purchased from Amersham Corp. (Amersham, England). RPMI 1640 and fetal calf serum were furnished by Boehringer (Indianapolis, IN). Adriamycin and daunomycin were supplied by Roger Bellon Laboratories (Paris, France) in pharmaceutical form (10 mg/vial). The methacrylic monomers (methyl methacrylate, 2-hydroxypropyl methacrylate, and methacrylic acid) were obtained from Merck (Darmstadt, West Germany) and purified before use by vacuum distillation. The emulsifier Plurisol PE 6800 was furnished by BASF (Paris, France). The other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation and Characteristics of Nanosphere Suspensions. The method recently described by Rolland et al. (34, 35) was used to prepare the polyisobutylmethacrylate nanosphere suspensions. Briefly, the monomers (methyl methacrylate, 3 ml; 2-hydroxypropyl methacrylate, 1.5 ml; methacrylic acid, 0.5 ml) were introduced under constant stirring into 95 ml of an aqueous solution of emulsifier (0.0001% Pluriol PE 350). The suspension was dialyzed as described previously (34) to remove the remaining monomers and other ionic impurities. For all experiments the suspensions were buffered at pH 8.2 (0.01 M sodium acetate-0.1 M NaOH), adjusted to 2 x 1012 particles/ml, and then sterilized at 121°C during 120 min.

The nanosizer analysis (Coulter Electronics, Hialeah, FL) indicated that the nanoparticles were very sharply distributed in size with a mean diameter ranging from 270 to 350 nm (SD ±55 nm) batch-to-batch with a polydispersion index <1, corresponding to a monodispersed system. Scanning electron microscopy (Jeol JSM, Japan), after cathodic gold impregnation, showed that the obtained particles were uniformly spherical. The concentration of nanoparticles in suspension was based on dry weight analysis and was calculated to be 2 x 1012 particles/ml.

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1 The abbreviations used are: B-ADR, nanosphere-bound Adriamycin; F-ADR, free Adriamycin; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.
The presence of hydroxyl and carboxyl groups on the nanospheres was determined by Nuclear Magnetic Resonance in D2O solution. The molecular weight of the methacrylic polymers was estimated by gel exclusion chromatography on Superose 6 (Pharmacia, Uppsala, Sweden) using a 200-ml void volume column and standard proteins as markers. The chromatograms were recorded at 280 nm. The SEC eluent was a 0.1 M sodium borate buffer (pH 9.2) containing 0.1% sodium azide and 0.001% sodium dodecyl sulfate. The flow rate was 0.7 ml/min, and the temperature was controlled at 25°C. The molecular weight was calculated based on the elution volume using a calibration curve with standard proteins. The molecular weight of the nanospheres was determined to be approximately 100,000, indicating that they are stable and can remain intact for prolonged periods.

In Vitro B-ADR Stability Study. Ten ml of B-ADR suspension in buffered RPMI medium ranging from pH 5 to pH 8 (1 ml of stock solution at 1 mg/ml plus 9 ml of medium) were centrifuged at 3000 x g for 5 min at 4°C and the supernatant was discarded.

Radioactive counts were corrected for radioactivity trapped in extracellular water according to the method using [14C]sucrose and H2O incorporation. Intracellular ADR was expressed in ng/mg of cellular proteins.

Drug Exposure. A stock solution of F-ADR (100 µg/ml) was obtained by reconstitution of freeze-dried drug and subsequent serial dilutions with sterile double-distilled water under aseptic procedures. Aliquots of stock solution were stored in 5-ml polystyrene tubes at —30°C in order to obtain the desired specific activity and drug load per nanosphere. The surface potential of B-ADR freeze-dried nanospheres was determined using a microelectrophoresis technique (36) and was found to be negative (—56.8 ± 3.4 mV). Each stock suspension contained the same number of nanospheres (2 x 10^10 nanospheres/ml) but the amount of Adriamycin bound was 0.5 µg/nanosphere (0.92 x 10^-14 mol or 5.54 x 10^-10 molecules/nanosphere) for 1 mg/ml of suspension and 0.1 µg/nanosphere (0.03 x 10^-14 mol or 1.1 x 10^-10 molecules) for 0.2 mg/ml of suspension.

In Vitro B-ADR Stability Study. Ten ml of B-ADR suspension in buffered RPMI medium ranging from pH 5 to pH 8 (1 ml of stock solution at 1 mg/ml plus 9 ml of medium) were centrifuged at 3000 x g for 5 min at 4°C and the supernatant was discarded. The cells were then incubated at 37°C for 48 h, and at various times a 10-ml aliquot was removed from the culture and analyzed by HPLC. The drug exposure experiments, cells were then incubated at 37°C in a 5% CO2-air atmosphere up to 7 days. For short-term exposure, cells were incubated with F-ADR or B-ADR during 2 h and were then centrifuged at 200 x g for 5 min (at 4°C). The pellet was washed twice with ice-cold buffer (PBS, containing 3 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and 0.143 mM NaCl, pH 7.4). Cells were then resuspended in drug-free medium and incubated for up to 3 additional days. For both short-term and long-term experiments, cell counts were performed each day in duplicate and compared to control culture. Results were expressed as percentage of inhibition.

Short-Term Uptake. The cells were grown in RPMI medium buffered with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, up to a cell density of 1 x 10^5 cells/ml. The cells were incubated with radioactive ADR solution and suspension (final volume, 13 ml, 0.1 µCi/ml, concentration ranging from 0.2 to 1 µCi/ml; 1300 nanospheres/cell) at 37°C for 1 h. At each time, a 1-ml sample was removed from the tube and immediately centrifuged through 400 µl of a phthalate oil mixture (dibutyphthalate/dioctylphthalate, 35/15, d = 1.032) at 4000 x g for 90 s. All points were determined in duplicate. The aqueous medium was removed and the upper part of the tube was washed twice with water. The oil layer was discarded and the pellet was solubilized using 500 µl of 1 N NaOH and 100 µl of 1% Triton X-100 for 3 h at 60°C. The mixture was then poured into scintillation vials and the tubes were washed twice with 500 µl of water. The content of vials was neutralized by adding 500 µl of 1 N hydrochloric acid before the addition of 10 ml of aqueous scintillation fluid (Optiphase Safe; LKB, England).

Radioactive counts were corrected for radioactivity trapped in extracellular water according to the method using [14C]sucrose and H2O incorporation. Intracellular ADR was expressed in ng/mg of cellular proteins.

Efflux Study. For drug efflux studies, cells, at a density of 0.25 x 10^6 cells/ml in complete medium, were loaded with 0.5 µg/ml of F-ADR or B-ADR (4 x 10^10 nanospheres/cell) for 4 h. For each experiment 1 x 10^5 cells were used. After the drug-loading period, the cells were centrifuged and suspended in fresh medium. The cells were then incubated at 37°C for 48 h, and at various times a 10-ml aliquot was centrifuged at 200 x g for 5 min at 4°C for 5 min. The pellet was then washed twice with 1 ml of ice-cold PBS containing 10 mM sodium azide. For protein determination, the pellet was washed once with 1 ml of methanol and stored at 4°C until determination using the Lowry method modified by Hartree (40). For Adriamycin determination, the pellet was suspended in 1 ml of drug-free medium and the internal standard daunomycin was then added (0.1 µg/ml). ADR content was determined by HPLC. Each experiment was done in quadruplicate.

Adriamycin Inhibition of DNA Synthesis. At the end of the drug incubation period, 900 µl of cell suspension were harvested from each well, introduced into a 5-ml polypropylene tube, and incubated with 2 µCi of [methyl-3H]thymidine (specific activity 35 mCi/mmol) and 50 µg/ml of ADR for 1 h at 37°C. The cell suspension was then filtered through a 0.45-µm HAWP Millipore filter (Millipore Corp., Bedford, MA) and washed twice with 2 ml of ice-cold PBS. Two ml of ice-cold 10% trichloroacetic acid were then added onto the filter and the acid solution was drawn up after 1 min of contact. The filter was then rinsed twice with 2 ml PBS and poured into a scintillation vial after overnight drying at 60°C. The vial was then made 20 ml with scintillation fluid (Instarfluor, Packard, IL) and counted in a scintillation counter. The radioactivity was determined twice using a 1209 Rack Beta liquid scintillation counter (LKB, Bromma, Sweden) with a constant measured quenching (counting efficiency, 59%).

Electron Microscopy. U-937 cells in exponential phase were seeded at a density of 0.25 x 10^5 viable cells/ml and allowed to grow for one doubling time. Cells were then incubated at 37°C with 0.5 µg/ml of B-ADR (2 x 10^10 nanospheres/cell; 0.5 µg/nanosphere). After 1 and 2 h of incubation, 1.5 ml of the medium were centrifuged at 200 x g during 5 min (4°C) and the cells were washed once with 2 ml of ice-cold PBS. Ultrathin sections were obtained after glutaraldehyde/cacodylate buffer fixation and osmium tetroxide postfixation, followed by double staining with uranyl acetate and lead citrate. Sections were examined with a Philips 301 electron microscope. For scanning electron microscopy, the final suspension in 100% ethanol was filtered through a FHWP (Millipore Corp., Bedford, MA) and washed twice with 2 ml of ice-cold PBS. Two ml of ice-cold 10% trichloroacetic acid were then added onto the filter and the acid solution was drawn up after 1 min of contact. The filter was then rinsed twice with 2 ml PBS and poured into a scintillation vial after overnight drying at 60°C. The vial was then made 20 ml with scintillation fluid (Instarfluor, Packard, IL) and counted in a scintillation counter. The radioactivity was determined twice using a 1209 Rack Beta liquid scintillation counter (LKB, Bromma, Sweden) with a constant measured quenching (counting efficiency, 59%).

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Millipore filter (0.45 μm). The filter was then dried for 24 h under vacuum and a fragment was mounted on an aluminum stub with silver conducting paint. The sample was then electroplated with gold and viewed with a Jeol JSM scanning electron microscope.

Analysis of Data. Values are expressed as mean ± SD. The two-tailed Student t test, with application of Yates correction factor, was used to compare data. Dose-effect curves were studied using linear regression after variables transformation in log of the dose and in arcsin of the square root of the mortality fraction in order to obtain constant variance (41). Concentrations of drug that inhibit cell proliferation versus control by 90% and confidence limits were computed according to the method of Litchfield (42). Efflux half-lives were calculated from the terminal log-linear portion of the efflux curves using four points and the area under the intracellular concentration versus time curve to infinity were calculated by using the trapezoidal rule with extrapolation to infinity.

RESULTS

In Vitro B-ADR Stability Study. Ultracentrifugation experiments showed that the binding of ADR to nanospheres was stable above pH 6.5. Less than 2% of ADR was found in the supernatant for a pH ranging from 6.5 to 8. At a pH under 6.5, the ADR release was rapid, demonstrating an ionic binding. However, dialysis studies at pH 7.4 showed a slow unbinding process (half-life about 96 h), indicating that ADR may also be adsorbed on nanospheres. The relative importance of each process was not readily demonstrable but our results were compatible with the hypothesis that the binding is mainly ionic.

Effects after Continuous Exposure. The effect of continuous exposure to F-ADR and B-ADR on cell number is shown in Fig. 1. U-937 cells were unaffected by the presence of control nanospheres in culture medium and grew at the same rate as in drug-free medium. F-ADR, under the conditions used (0.02 μg/ml in continuous exposure), had a significant effect on cell proliferation but was more cytostatic than cytotoxic.

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Furthermore, 7% of the initial inoculum were still alive after 7 days of exposure to 0.02 μg/ml of F-ADR. In marked contrast, B-ADR was more cytotoxic since the cell count fell rapidly after 2 days of exposure and only 0.8% of the control was found after 4 days (about 10% of the initial inoculum). After 5 days, all the cells were killed for both concentrations. This result was confirmed by the dose-effect relationship. The concentrations of drug that inhibit cell proliferation versus control by 90% were 0.051 μg/ml for F-ADR (confidence limits, 0.048–0.055 μg/ml) and 0.018 μg/ml for B-ADR (confidence limits, 0.016–0.020 μg/ml; P < 0.001). The enhancement of Adriamycin cytotoxicity by binding on nanospheres was demonstrated to be more strongly dependent on the number of nanospheres per cell than on the drug concentration (Fig. 2). Indeed, after 4 days of incubation with 800 nanospheres at 0.1 fg/cell, the percentage of inhibition was 96.6 ± 0.5%. However, when cells were incubated with 160 nanospheres at 0.5 fg/cell (corresponding to the same Adriamycin concentration in medium), the percentage of inhibition was significantly decreased (90.4 ± 2.0%; P < 0.001). For the same Adriamycin concentrations, the F-ADR gave only 60 ± 6% of inhibition (P < 0.001). In addition, B-ADR (800 nanospheres/cell) gave a stronger inhibition of DNA synthesis (estimated by the incorporation of radioactivity into the acid-insoluble pool) than F-ADR. Fig. 3 shows that, for an identical Adriamycin concentration in medium (0.02 μg/ml), the radioactive counts after 2 days compared to control nanospheres were 86 ± 6% for F-ADR and 65 ± 4% for B-ADR (160 nanospheres at 0.5 fg plus 640 control nanospheres per cell; P < 0.01). For a concentration of 0.05 μg/ml, the effect was more marked: 43 ± 3% for F-ADR and 16 ± 1% for B-ADR (400 nanospheres at 0.5 fg plus 400 control nanospheres per cell; P > 0.001).

Effects after a 24-h Incubation. Fig. 4 shows the percentage of growth inhibition, compared to control, after a 24-h incubation followed by an extensive washing step and reincubation for 2 additional days in drug-free medium under the same conditions as those for continuous exposure experiments. For an exposure to 0.02 μg/ml with 160 nanospheres/cell (nanospheres at 0.5 fg), inhibition was 63 ± 8%. For the same concentration with 800 nanospheres/cell (160 nanospheres at 0.5 fg plus 640 control nanospheres), inhibition was 74 ± 6%. These values were not significantly different from those observed after exposure to 0.02 μg/ml of F-ADR. However, with 800 nanospheres at 0.1 fg/cell, the effect was improved (93.3 ± 3.9%, P < 0.01). A more markedly inhibition related to nanosphere density was observed when cells were exposed to 0.05 μg/ml under identical conditions (99.8 ± 0.7%). Using the
isotope incorporation into DNA, the same influence of the number of nanospheres per cell was found (data not shown).

Drug Accumulation and Efflux. The relationship between drug accumulation and the amount of drug in the medium was investigated during a 1-h exposure period with various concentrations of radioactive F-ADR and B-ADR ranging from 0.2 to 1 µg/ml (with a constant number of 1300 nanospheres/cell). The mean curves of three separate experiments are shown on Fig. 5. The accumulation rate was more important for B-ADR than F-ADR for all the concentrations tested. Moreover, the B-ADR uptake rate seemed to slow down after 30 min of incubation.

Cells treated with F-ADR or B-ADR (0.5 µg/ml; 4 × 10⁴ nanospheres/cell) for 4 h, rinsed, and thereafter incubated in drug-free medium were analyzed for drug retention. Fig. 6 shows the mean curves of three independent experiments. At the end of the incubation period, the intracellular amounts of drug were not different (F-ADR, 327 ± 105 ng/mg of protein; B-ADR, 427 ± 93 ng/mg of protein; P = 0.47). For cells exposed to F-ADR, the efflux kinetics were approximately log linear with a half-life of 23.7 ± 7.7 h and an area under the intercellular concentration versus time curve to infinity of 8.6 ± 2.8 µg/mg of protein/h. However, a marked difference was seen for B-ADR since, if the initial efflux rate was comparable until 5 h of incubation in drug-free medium, it was markedly reduced from this time, corresponding to a terminal half-life of 85.9 ± 19.2 h and an AUC to infinity increased to 29.6 ± 6.6 µg/mg protein/h (P < 0.001).

Transmission electron microscopy studies were done to determine if nanospheres were internalized into U-937 cells. A representative micrograph is shown in Fig. 7. Endocytosis vacuoles, containing a spherical and homogeneous particle compatible with a nanosphere, are found in cytoplasm even when cells are incubated at a low density of 800 nanospheres/cell.
INTRACELLULAR SUSTAINED-RELEASE FORM OF ADRIAMYCIN

Fig. 7. Electron micrograph of U-937 cells after a 2-h incubation in medium containing 0.5 μg/ml of B-ADR (2 × 10⁶ nanospheres/cell). Homogeneous particles, corresponding to internalized nanospheres (arrows), were observed in endocytotic vesicles. × 26,400; bar, 500 nm.

DISCUSSION

Intracellular sustained-release form for antineoplastic drugs is an interesting concept. Indeed, it is easily admitted that the pharmacokinetics at cellular level plays a fundamental role since intracellular drug concentration and duration of exposure are both required for an efficient drug-target interaction. In addition, increased drug efflux is the common mechanism involved in pleiotropic drug resistance. Consequently, we have tested the hypothesis that cancer cells could internalize drug carriers such as nanospheres, which should release Adriamycin more slowly.

Previous studies have indicated that polymethacrylate nanospheres exhibit methacrylic acid residues on their surface which could form ionic bonds with a variety of basic drugs such as Adriamycin, daunorubicin, or ethidium bromide (35). The physicochemical data presented in “Materials and Methods” seem to support this hypothesis. Furthermore, we showed that the binding for Adriamycin was stoichiometric and stable above pH 6.5. In addition, we demonstrated by ultracentrifugation that more than 99% of ADR remains bound on the nanospheres at physiological pH. However, dialysis experiments indicated that ADR binding on nanospheres is not entirely ionic. Despite this fact, it appears that ionic binding may be considered essential. Furthermore, the negative r potential exhibited by the ADR-loaded nanoparticles indicates that aggregation of nanospheres in medium is very unlikely. This fact was confirmed by scanning electron microscopy. Consequently, we have postulated that nanospheres loaded with Adriamycin could cross the cell membrane by endocytosis as described by Trouet et al. (43) for the Adriamycin-DNA complex. Finally, since the Adriamycin-nanosphere ionic bond is strongly pH dependent, the drug will be progressively released at the lower pH present in cell lysosomes during the action of acid hydrolases. In addition, since electron microscopy after freeze-fracture showed a porous structure for nanospheres, ADR ionic binding could exist inside particles leading to a slow-release process.

In this study, we present results suggesting that B-ADR could act as a lysosomotropic complex. Transmission electron microscopy of U-937 cells incubated with B-ADR showed pinocytic vacuoles containing nanospheres. Drug accumulation experiments demonstrated that significant amounts of intracellu-
lower activity. Furthermore, the dose-effect relationships suggest that B-ADR and F-ADR do not affect cells by the same mechanism since the slopes of the curves are different. This finding is consistent with the hypothesis that the cell surface is also a target for Adriamycin. Indeed, if B-ADR can interact with the cell surface before and during endocytosis, the density of drug-loaded nanospheres around the cell has a key role to play.

We performed preliminary studies on the cytotoxicity of B-ADR on cells without endocytotic activity (renal carcinoma cell line, RC-Pa). On these cells, B-ADR exhibited a 2-fold lesser activity than F-ADR. This finding is coherent with the hypothesis that the cell membrane could be also an important target in transducing the cytotoxicity of ADR, as claimed by several authors (46–51) using cellular models without endocytotic properties. Particularly, Rogers et al. (51) suggested that multiple membrane interaction at the cell surface may represent a novel mechanism of ADR toxicity. However, our results are compatible with an additive cytotoxicity mechanism involving membrane interactions and increased intracellular retention. Moreover, since the activity of B-ADR was about 3-fold higher in endocytotic cells than F-ADR, the relative importance of the two mechanisms seems to remain in favor of the enhancement of intracellular drug retention.

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