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 μg/ml, 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 been 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 polysobutylcyanoacrylate 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. (Amerham, 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 Plurulie Plurie SE 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 polyalkylmethacrylate 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% Plurie SE 6800). The reaction was then chemically started by adding 10 mg of a free radical initiator (potassium persulfate), and the copolymerization was maintained during 1 h at 90°C. After filtration on a 10–20-μm glass filter, the suspension was dialyzed as described previously (34) to remove the remaining monomers and other impurities. For all experiments the suspensions were buffered at pH 8.2 (0.01 m sodium acetate-0.1 m NaOH), adjusted to 2 × 10¹³ particles/ml, and then sterilized at 121°C during 120 min.

The nanosizer analysis (Coulter Electronics, Hialeah, FL) indicated that the particles were very sharply distributed in size with a mean diameter ranging from 270 to 350 nm (SD ±55 nm) batch-to-batch with a polydispersity 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 × 10¹³ particles/ml.

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3 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 deuterated dimethyl sulfoxide and IR spectroscopy. Hydrogen ion titration measurements indicated approximately 1 to 1.5 × 10^10 carboxyl groups on the specific surface of each particle. The molecular weight of the methacrylic polymers was estimated by liquid exclusion chromatography on CPG-Ultrolitegel (Waters Associates, Milford, MA) to be 450,000.

B-ADR stock suspensions were obtained by adding appropriate amounts of Adriamycin under aseptic procedures to the control nanosphere suspension in order to obtain various concentrations ranging from 0.2 to 1 mg/ml of suspension. The suspensions were then kept at 4°C overnight in order to complete the binding. Aliquots of each B-ADR stock suspension were then freeze-dried in 2-ml polypropylene centrifuge tubes and stored at −30°C until use without any appreciable decomposition. Radioactive B-ADR suspensions were obtained by the same procedure but using [14C]-ADR and nonradioactive ADR in order to obtain the desired specific activity and drug load per nanosphere.

The surface potential (ζ 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 × 10^12 nanospheres/ml) but found to be negative (−56.8 ± 3.4 mV). Each stock suspension was determined using a microelectrophoresis technique (36) and was found to be negative (−56.8 ± 3.4 mV). Each stock suspension was determined using a microelectrophoresis technique (36) and was found to be negative (−56.8 ± 3.4 mV). Each stock suspension was determined using a microelectrophoresis technique (36) and was found to be negative (−56.8 ± 3.4 mV). Each stock suspension was determined using a microelectrophoresis technique (36) and was found to be negative (−56.8 ± 3.4 mV). Each stock suspension was determined using a microelectrophoresis technique (36) and was found to be negative (−56.8 ± 3.4 mV). 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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 and 0.04 µg/ml in continuous exposure), had a significant effect on cell proliferation but was more cytostatic than cytotoxic. Indeed, after 4 days of incubation, the percentage of growth inhibition, compared to control, was 81% for 0.0 µg/ml and 94% for 0.04 µg/ml of added Adriamycin.

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 marked inhibition related to nanosphere density was observed when cells were exposed to 0.05 µg/ml under identical conditions (98.5 ± 0.7%). Using the
INTRACELLULAR SUSTAINED-RELEASE FORM OF ADRIAMYCIN

Fig. 3. Acid-insoluble [methyl-3H]thymidine incorporation following 2 days of exposure of U-937 cells to F-ADR or B-ADR. Cells were incubated with 0.02 or 0.05 µg/ml F-ADR or B-ADR. For each concentration of B-ADR, an identical number of 800 nanospheres/cell was obtained by mixing appropriate number of nanospheres (at 0.5 fg/nanosphere) with control nanosphere suspension. After 2 days, the radioactive precursor incorporation was performed as described in “Materials and Methods.” Each value shown is the mean ± SD (bars) of two separate experiments using two culture wells per point.

Fig. 4. Percentages of growth inhibition compared to control after a 24-h exposure of U-937 cells to F-ADR or B-ADR followed by an additional incubation in drug-free medium. Experiments were performed as described in “Materials and Methods.” Data shown represent the observed inhibitions after 3 days. Class A, F-ADR; Class B, B-ADR, 160 nanospheres (at 0.5 fg)/cell for 0.02 µg/ml and 400 nanospheres (at 0.5 fg) for 0.05 µg/ml; Class C, B-ADR, 160 nanospheres (at 0.5 fg) plus 640 control nanospheres/cell for 0.02 µg/ml and 400 nanospheres (at 0.5 fg) plus 400 control nanospheres/cell for 0.05 µg/ml; Class D, B-ADR, 800 nanospheres (0.1 fg)/cell for 0.02 µg/ml and 800 nanospheres (at 0.25 fg)/cell for 0.05 µg/ml. □, 0.02 µg/ml; △, 0.05 µg/ml). The values given are means ± SD (bars) of two separate experiments, each one performed in duplicate.

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 × 10^4 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 an 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 low density of 800 nanospheres/cell.
experiments demonstrated that significant amounts of intracellular vacuoles containing nanospheres. Drug accumulation by electron microscopy of U-937 cells incubated with B-ADR showed pinocytosis as a lysosomotropic complex. Transmission electron microscopy (TEM) after freeze-fracture showed a porous structure for nanospheres, ADR ionic binding could exist inside particles leading to a slow-release process.

addition, since electron microscopy after two washing steps following drug incubation; (b) an incubation in a medium containing neuraminidase after cell exposure to B-ADR, in order to unbind nanospheres from the glycocalyx, did not modify the apparent intracellular Adriamycin concentration; (c) it has been demonstrated previously that comparable nanoparticles were well internalized into a variety of cells such as fibroblasts, macrophages, or monocytes (26–28). Particularly, we showed by flow cytometry that similar nanospheres loaded with ethidium bromide were internalized by normal human monocytes within less than 2 h (44, 45).

Using radioactive ADR incorporation, we observed that the amount of ADR accumulated into cells was more important with B-ADR than with F-ADR during a short-term period. Since the uptake mechanisms of the two forms are different (simple diffusion for F-ADR and endocytosis for B-ADR), one possible explanation for this fact may be that the endocytosis of a single particle brings, at once, from 0.1 to 0.5 fg of ADR into the cells. Moreover, after a longer incubation period, the intracellular ADR content was not different for cells exposed to B-ADR or F-ADR. This fact could be explained by a progressive saturation of the endocytotic process with time. However, drug efflux studies clearly demonstrated that intracellular levels of Adriamycin were more sustained in cells exposed to B-ADR than in cells exposed to F-ADR. For F-ADR the efflux was monophasic but it was biphasic for B-ADR. For B-ADR, the similarity of the initial 5-h period with F-ADR may be due to an exodus of free drug released from the nanospheres internalized first or drug weakly bound to the carrier. In contrast, the second slower phase could represent efflux of drug released from the nanospheres internalized later or drug located inside the particle and more firmly bound. Furthermore, the net intracellular drug accumulation at any time should be viewed as the difference between influx and efflux rates. Consequently, the higher intracellular levels of drug obtained during a 1-h incubation with B-ADR could be attributed also to a decreased efflux process from the cells.

It appears that a sustained intracellular level for Adriamycin is a determining factor of cell toxicity. Indeed, continuous exposure experiments have indicated that B-ADR inhibited cell proliferation more strongly than F-ADR. Furthermore, B-ADR has produced a constant cytotoxic effect during the entire incubation period in contrast with F-ADR which was only cytostatic. This fact has been confirmed by the more pronounced inhibition of DNA synthesis when cells were incubated with B-ADR. The same effects have been observed during the 24-h exposure experiments. In addition, drug-free nanospheres have demonstrated no influence on cell growth and DNA synthesis.

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 polyethylacrylate 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 i 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 intracellular Adriamycin were present in the cells after incubation with B-ADR. Furthermore, the possibility that Adriamycin determined in the cell pellet may be due more to adhesion of nanospheres to the cell membrane than to endocytosis is unlikely for several reasons: (a) we were unable to observe a significant number of nanospheres on cell membrane by scanning electron microscopy after two washing steps following drug incubation; (b) an incubation in a medium containing neuraminidase after cell exposure to B-ADR, in order to unbind nanospheres from the glycocalyx, did not modify the apparent intracellular Adriamycin concentration; (c) it has been demonstrated previously that comparable nanoparticles were well internalized into a variety of cells such as fibroblasts, macrophages, or monocytes (26–28). Particularly, we showed by flow cytometry that similar nanospheres loaded with ethidium bromide were internalized by normal human monocytes within less than 2 h (44, 45).

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The finding of a significant influence of the number of nanospheres per cell ("density"), irrespective of apparent local drug concentration, suggests that the amount of Adriamycin in the cell is not the only parameter involved in the enhancement of its antitumor activity. For a same amount of drug, the antitumor activity for B-ADR was significantly increased when nanospheres uniformly loaded at 0.1 fg were used. In contrast, nanospheres at 0.5 fg, mixed with drug-free particles in order to maintain an identical nanosphere density per cell, have a
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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Enhancement of Adriamycin Antitumor Activity by Its Binding with an Intracellular Sustained-Release Form, Polymethacrylate Nanospheres, in U-937 Cells

Alain Astier, Benedicte Doat, Marie-Jeanne Ferrer, et al.


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