A Differential Interaction of Daunomycin, Adriamycin, and N-Trifluoroacetyladriamycin 14-Valerate with Mouse Peritoneal Macrophages

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ABSTRACT

The interaction of three anthracycline drugs, daunomycin, Adriamycin, and N-trifluoroacetyladriamycin 14-valerate, with mouse peritoneal macrophages was explored. As assessed by drug-specific cytofluorescence, Adriamycin and daunomycin accumulated slowly within macrophages, first staining the nucleus and then the cytoplasmic inclusions that were induced by the drug treatment. N-Trifluoroacetyladriamycin 14-valerate distributed rapidly into the cells, was excluded from the nucleus, and induced numerous cytoplasmic inclusions. Electron microscopy revealed that the cytoplasmic inclusions were vacuoles containing some amorphous material and not the classical autophagic vacuoles containing organelles and membrane lamellae. All the drugs induced cell shrinkage with time and brought about cell death within 24 hr. Loss of cell function and viability was dose and time dependent; i.e., a 6-hr incubation with daunomycin or Adriamycin, 2.5 μg/ml, brought about a 50% reduction in the phagocytic capacity of the treated macrophages. The damaging potency towards macrophages (daunomycin > Adriamycin > N-trifluoroacetyladriamycin 14-valerate) is in the reverse order to the in vivo therapeutic efficiency.

INTRODUCTION

Adriamycin and daunomycin, anthracycline antibiotics, exhibit significant antitumor activity in experimental tumors (6, 7), as well as in human neoplasms (7, 26). Both drugs have been shown to intercalate between adjacent base pairs of the DNA double helix, to inhibit DNA and RNA polymerase-mediated reactions, and to inhibit labeled precursor incorporation into nucleic acids (3, 5, 7, 19). The antitumor activity, however, is not limited to analogs with a high affinity to DNA; AD32,4 which does not bind to calf thymus DNA (25) and which does not exhibit drug-specific nuclear fluorescence (17), has a superior therapeutic activity in several rodent tumor systems (16, 22). Evidence is accumulating for interaction of anthracycline drugs with structures other than those associated with the nucleus and nucleic acid synthesis. It has been suggested that the antimitotic effect of daunomycin could involve perturbation of spindle fiber function (4), a suggestion that found support in the finding that at concentrations above $2.5 \times 10^{-3}$ M daunomycin perturbs the magnesium ion-induced tubulin polymerization and inhibits in vitro microtubule assembly (21). Moreover, Adriamycin was shown to exhibit affinity to phospholipids, especially to negatively charged ones (9).

In an attempt to characterize anthracycline drug-membrane interactions, we have recently shown a differential interaction of various Adriamycin derivatives with octanol, phospholipid bilayers, and erythrocyte membranes (13). The study clearly showed that all the tested drugs, Adriamycin, daunomycin, AD32, adriamycin 14-octanoate, and adriamycin 14-acetate, intercalated to a variable degree into the lipid domains of membranes where they exerted an effect on the thermotropic behavior of the lipid bilayer. The last-mentioned 3 drugs changed the osmotic fragility of human erythrocytes, and except for Adriamycin all the positively charged drugs led to biconcave-cup shape transformation.

Mantovani (18) had recently shown that daunomycin was at least 4 times more toxic to macrophages than was Adriamycin and suggested that the fact that the former was less effective than Adriamycin as a cancer chemotherapeutic agent in vivo may stem from a greater destruction of macrophages by daunomycin and a reduction in the contribution of macrophages to host immunity. In view of the above and since in all the criteria that we have used daunomycin showed a higher membrane-drug interaction than did Adriamycin, it was of interest to further explore the distribution and cytoxic effect of these drugs on mouse peritoneal macrophages. In addition we tested the intracellular localization of AD32 and its effect on macrophage function and viability. Cytotoxicity of anthracycline drugs towards nonproliferating cells may be highly relevant to the nontoxic side effects exerted by these drugs that limit their use.

MATERIALS AND METHODS

Anthracycline Drugs. Adriamycin, daunomycin, and AD32 were kindly supplied by Farmitalia, Milan, Italy. Media. Dulbecco’s modified Eagle’s medium (medium) was supplemented with penicillin, 100 units/ml, and streptomycin, 100 μg/ml. Medium, heat-inactivated newborn calf serum (serum) and PBS were obtained from Grand Island Biological Co., Grand Island, N. Y.
Peritoneal Macrophages. Peritoneal macrophages were aseptically collected from male BALB/c mice weighing 20 to 25 g. Peritoneal exudate cells suspended in medium were allowed to attach (5 × 10^6 cells in 0.15 ml, 60 min, 37°C) on either 25-mm-diameter Corning coverglasses or coverglasses coated with a thin sheet of Parlodion (for electron microscopy) (14). The coverglasses were placed in 35 × 10-mm Falcon tissue culture dishes. Subsequent to cell attachment, the plates were thoroughly rinsed in PBS to remove nonadhering cells, and the macrophages were cultivated for 24 hr at 37°C in 2 ml of 20% serum in medium in a CO2 incubator (5% CO2-air).

Intracellular Distribution of Anthracycline Drugs. Cell monolayers on coverslips were rinsed with PBS and exposed to the drugs (10 /μg/ml) dissolved in 20% serum-medium for the specified time. Monolayers were subsequently washed with PBS and inverted on a drop of PBS, and the circumference of the coverglasses was fixed to a microscope by warm wax. The viable cells were examined and photographed with a Carl Zeiss large-fluorescence microscope (Ultraphot), equipped with a No. BG12 excitation filter and No. 53 barrier filter. Photomicrographs were taken on Kodak Plus-X pan film (Tri-X 400 Asa) with an exposure time of 45 sec.

Electron Microscopy. Monolayers cultivated on Parlo- dion were allowed to interact with the drugs dissolved in 20% serum-medium as specified. At the end of incubation with the drugs, the cells were processed and embedded in epoxy resin as described (14). Thin sectioning was obtained with a Sorvall Porter-Blum MT 2-B ultramicrotome, and the sections were analyzed in a Philips 300 electron microscope (Ultraphot), equipped with a No. BG12 excitation filter and No. 53 barrier filter. The viable cells were examined and photographed with a Carl Zeiss large-fluorescence microscope (Ultraphot), equipped with a No. BG12 excitation filter and No. 53 barrier filter. Photomicrographs were taken on Kodak Plus-X pan film (Tri-X 400 Asa) with an exposure time of 45 sec.

Yeast Cell Phagocytosis. Stationary yeast cells (Saccharomyces oviformis) grown on agar slants were suspended in (Ca2+-Mg2+-free) PBS and boiled for 60 min. Subsequent to the washing in PBS, the yeast cells were suspended (30 × 10^4/ml) in 20% serum-medium as specified. Adriamycin monolayers were rinsed in PBS and incubated with the drugs dissolved in 20% serum-medium for the specified time. The monolayers were gently washed, and 1 ml of yeast cell suspension was added for an incubation period of 30 min. Monolayers were rinsed, fixed in 2% glutaraldehyde in PBS for 30 min at 22°C, and stained with Giemsa. Yeast cells ingested per 100 macrophages were enumerated in quadruplicate samples. Each time point had a separate control (100%). AD32 dissolved in ethanol, with a final concentration excluding trypan blue. A 24-hr exposure to the drugs resulted in a remarkable loss of cell viability. Macrophages treated with either 2.5- or 10-μg/ml doses of Adriamycin, daunomycin, or AD32 died and detached from the plate.

In an attempt to gain a better understanding of the drug-inflicted cell damage, we have resorted to electron microscopy. Fig. 2 shows macrophages treated for 30 min and 4 hr with either daunomycin or AD32. Subsequent to a 30-min incubation period with daunomycin or AD32, the macrophages did not show gross morphological changes as compared to control macrophages (not shown). During a 4-hr incubation period, the macrophages became extensively vacuolated (Fig. 2, b and d). The origin of these vacuoles is not easily deduced since lysosomes, mitochondria, and rough endoplasmic reticulum did not show significant divergence from the control. Occasionally, dead cells with a total disorganization of the nuclei and cytoplasmic structure were observed.

That the vacuolated spread cells of the kind shown in Fig. 2, b and d, were still functional was assessed by their capacity to phagocytize yeast cells. Fig. 3 represents monolayers of macrophages that were exposed for 30 min to yeast cells subsequent to a 6-hr treatment with relatively high doses of Adriamycin, daunomycin, or AD32 (10 μg/ml). Adriamycin treatment of macrophages (Fig. 3b) resulted in a partial shrinkage and rounding of a fraction of the cells. Close inspection of the rounded cells indicates that many of the shrunken cells ingested yeast cells. The spread macrophages showed a high degree of yeast ingestion. A 6-hr treatment with Adriamycin (10 μg/ml) reduced the number of ingested yeast cells to 50% of that of nontreated macrophages, and the fraction of macrophages void of yeast cells increased from 7 to 34%. Daunomycin, under the same conditions, had a higher inhibitory activity on macrophage phagocytosis. Fig. 3c shows that a high proportion of macrophages shrank to a higher extent than observed after Adriamycin treatment and that even the spread cells had a reduced capacity for phagocytosis. A 6-hr treatment with daunomycin (10 μg/ml) resulted in a 65% inhibition of yeast cell ingestion; 47% of the cells were void of yeast cells. The loss of phagocytic capability was both time and dose dependent.
The rate of loss of phagocytic activity with the drug. At a 9-hr incubation period, AD32-treated macrophages did not lead to substantial cell shrinkage (Fig. 3d), and the time with Adriamycin (•, 2.5 µg/ml), daunomycin (A, 2.5 µg/ml), and AD32 (□, 10 µg/ml). The corresponding open symbols denote percentage of macrophages devoid of yeast cells. +, nontreated macrophages. For details, see “Materials and Methods.”

Chart 1 presents the time course and extent of reduction of phagocytosis of macrophages treated with Adriamycin or daunomycin (2.5 µg/ml). The rate of loss of phagocytic capacity due to drug treatment increased with time implying complex cellular processes that determine the loss of function and not an instantaneous effect resulting from drug-membrane interactions. At a concentration of 10 µg/ml, the percentage of inhibition was higher at all time points. Daunomycin was notably more potent than Adriamycin in its effect on phagocytosis of macrophages (Chart 1) and also in inflicting morphological changes indicative of cell damage Fig. 3, b and c).

Treatment of macrophages with AD32 (10 µg/ml) for 6 hr did not lead to substantial cell shrinkage (Fig. 3d), and the phagocytic capacity of the macrophages was only slightly reduced (Chart 1, 19% decrease). Thus, the rapid entry of the drug and its induction of intracellular vacuolation (Figs. 1 and 2) did not affect macrophage phagocytic function at least for 6 hr. At 24 hr of exposure to the drug, a 78% inhibition of phagocytosis was observed (Chart 1), 85% of the glass-adherent cells being void of yeast cells. A significant fraction of macrophages was still glass adherent after 24 hr culture in the presence of AD32, whereas in the presence of Adriamycin or daunomycin about 95% of the macrophages detached from the coverslips on which they were plated.

The viability of macrophages exposed to the 3 anthracycline drugs was assessed by use of the dye exclusion method. Macrophages treated with Adriamycin, daunomycin (2.5 to 10 µg/ml), or AD32 (10 to 25 µg/ml) were 98 to 100% trypan blue negative up to a 5-hr incubation period with the drug. At a 9-hr incubation period, AD32-treated cultures were still 95 to 99% trypan blue negative, Adriamycin-treated cultures were already 15% to 15% trypan blue positive (with very few dye-positive detached cells), and daunomycin-treated cultures exhibited up to 30% dye-positive cells with a massive detachment of dye-positive cells from the coverglasses.

**DISCUSSION**

AD32-specific cytofluorescence appeared rapidly in macrophages as opposed to Adriamycin and daunomycin cytofluorescence (Fig. 1). The differential rate of intracellular localization is most probably due to the remarkable difference these drugs exhibit in drug-membrane interactions. The partitioning of AD32 into octanol and into the lipid domains of phospholipid and biological membranes is orders of magnitude higher than that of Adriamycin and daunomycin (13). Subsequent to partitioning into the membrane, the drugs partition into intracellular organelles. Adriamycin and daunomycin exhibited a high affinity to the nucleus, and this organelle was the first to fluoresce. With time numerous fluorescent cytoplasmic vesicles and vacuoles appeared, indicating that other cellular structures attract the drug and concentrate it within its boundaries. The pattern of intracellular localization and the size of the cytoplasmic inclusions suggest concentration into lysosomes and autophagic vacuoles as a possible site of destination. The entry and distribution of many polybenzenoid hydrocarbons and basic dyes such as acridine orange were followed in living cells by fluorescence microscopy, and it was found that these drugs permeate the cell passively and concentrate within lysosomes (1, 2, 8). The basic drugs concentrate also to variable extent within cell nuclei and mitochondria.

AD32, an Adriamycin derivative in which the glycosidic amino group was converted to an amide, did not show affinity to the nucleus (Fig. 1, f to j). This is in line with the lack of nuclear staining observed with this drug in WI-38 fibroblasts (17). In the latter cells the cytofluorescence was associated with spherical cytoplasmic structures (probably not mitochondria), reminiscent of lysosomes. On the level of the fluorescence microscope, AD32-specific cytofluorescence in macrophages did not seem to be confined to discrete intracellular organelles. With time of exposure to the drug, the macrophages developed numerous nonfluorescent cytoplasmic inclusions (Fig. 1, f to j). It is possible that this is due to the fact that AD32, being water-insoluble, remained within the limiting membrane and did not distribute into the interior of the inclusions.

A closer inspection of the ultrastructural changes that macrophages undergo subsequent to the exposure to the drugs revealed that the cytoplasmic inclusions either are electron lucent or contain amorphous material. No distinct structures could be identified within the vacuoles, and neither could one infer their origin. A similar type of vacuole was observed upon exposure of the macrophages to chloroquine (10, 11) and to hashish compounds (23). With both drugs distinct autophagic vacuoles were observed in addition to the electron-lucent toxic vacuoles.

Studying vacuole formation resulting from exposure of macrophages and L-cells to chloroquine, Fedorko et al. (11) arrived at the conclusion that both the Golgi and smooth endoplasmic reticulum vesicles (representing protolysosomes or primary lysosomes of these cells) were the most probable candidates for providing the limiting membranes of these vacuoles. Drug incorporation then results in the tendency of the vesicles to fuse with one another and with other membrane-limited cytoplasmic organelles. The appearance of toxic vacuoles marks, as in the case of the exposure of macrophages to hashish compounds (23), an irreversible cell damage leading to cell death.

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Treating macrophages with high concentrations of Adriamycin or daunomycin results in the shrinkage of a certain proportion of the cells (Fig. 3). The shrinking pattern is reminiscent of that obtained after macrophage treatment with high concentrations of colchicine (12). This observation could be in accord with the findings of Na and Timasheff (21) that daunomycin leads to dissociation of tubulin polymers and to inhibition of in vitro microtubule assembly.

The 3 anthracycline drugs affect the phagocytic capacity of macrophages to a variable degree. Since the phagocytic event requires intactness of various functional cell entities, it was taken as a parameter measuring the degree of damage inflicted on the macrophages by the drugs. The drug that causes the least damage to the phagocytic capacity of macrophages was AD32. Within 6 hr Adriamycin and daunomycin decreased the phagocytic capacity by about 50% (Chart 1). The decrease stemmed from both elevation of the number of cells that did not ingest yeast cells and some reduction in the extent of ingestion in the phagocytizing cells. The heterogeneity in the response of the cultured cells to the drugs is remarkable. After 24 hr, however, all the cultured cells were affected. A significant differential effect of Adriamycin and daunomycin on the phagocytic capacity of macrophages is in line with their differential rate of penetration and extent of accumulation in various cells (24).

Using the capacity of macrophages to accumulate $^{86}$Rb as a parameter of cell viability, Mantovani (18) has shown that the damage to the uptake mechanism is highly dose and time dependent.

It appears that all the anthracycline drugs can affect cell viability by a mechanism similar to that described for non-DNA-intercalating drugs. In addition, these drugs contain a quinone structure that could be involved in the generation of free radicals and lipid peroxidation events that lead to membrane damage (15, 20).

The fact that AD32 is the least damaging to macrophage function and viability could be of relevance in in vivo systems where the effectiveness of chemotherapy is in part determined by the intactness of host defense mechanisms. It is therefore of interest that the therapeutic effectiveness of the 3 drugs is in the reverse order of the damaging effect inflicted in vitro on macrophages, i.e., AD32 > Adriamycin > daunomycin.

REFERENCES


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