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In Vivo Effects of Adriamycin or *N*-Trifluoroacetyladriamycin-14-valerate on a Mouse Lymphoma*

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Abstract—*The antitumor activity of adriamycin (ADR) and its analog* N-*trifluoroacetyladriamycin-*14*-valerate (AD*32*) was studied* in vivo *in a solid form of the transplantable mouse B cell lymphoma DBA*3*. At the selected single dose of* 15 mg/kg *of ADR, and* 80 mg/kg *of AD*32*, both drugs had comparable effects in overall tumor control. These two dosages were used throughout the experiments, which were designed to elucidate some of the antitumor mechanisms of action of the tested compounds. The recovery kinetics of clonogens were studied using the agar diffusion chamber technique. In ADR-treated tumors, maximal reduction of the survival fraction of colony-forming cells (DC-cfu) was observed at* 18 hr *post-injection. Over the next* 6 hr *the number of DC-cfu increased* 3.9*-fold and returned to pre-treatment levels. Since cell division or cell loss, measured with flow cytometry or with the* [^{125}I]*-iododeoxyuridine assay respectively, cannot explain rebound of clonogens in treated tumors, the changes of DC-cfu strongly suggests the presence of a repair of ADR-induced damage in cells left* in situ *over a* 24*-hr period. This event was shown at time-intervals when significant levels of ADR were still detectable by HPLC in tumor tissues. The appearance and disappearance of ADR-induced DNA–protein crosslinks and protein-associated DNA single-strand breaks, established by the DNA alkaline elution technique, coincided temporally with the rebound of DC-cfu, suggesting repair of ADR damage at the molecular level. In AD*32*-treated tumors, maximal decrease of the survival fraction of DC-cfu was present at* 12 hr *post-treatment. There was only a gradual return to pre-treatment levels over the next* 1.5 days. *While the rebound of DC-cfu cannot be explained by selective cell loss, flow-cytometric data showed a synchronous cell cohort passing through the cell-cycle phases at* 12–48 hr *post-treatment, contributing to cell replication. DNA alterations induced by AD*32 *were identical with those caused by ADR. In partial contrast to the findings with ADR, their removal could indicate replacement of damaged cells by newly generated undamaged cells and/or 'repair' of DNA. In conclusion, the results suggest that AD*32 *has several potential therapeutic advantages over its parent compound: (*1*) AD*32 *did not induce an efficient and clearly discernible repair of tumor cells, whereas a repair mechanism, responding to a high toxic dose of ADR, could be present in ADR-treated tumors; (*2*) the decrease in the number of DC-cfu in tumors treated with AD*32 *lasted over* 2 days, *as compared to only* 12 hr *in ADR-treated tumors; (*3*) synchronization of a cell cohort, which might be utilized in drug combination treatments, was induced by AD*32*.*

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Abbreviations: ADR, adriamycin; AMNOL, adriamycinol; AD32, *N*-trifluoroacetyladriamycin-14-valerate; AD41, *N*-trifluoroacetyladriamycin; AD92, *N*-trifluoroacetyladriamycinol; DC-SM, diffusion chambers filled with a semisolid matrix; DC-cfu, clonogens starting growth of colonies in a diffusion chamber; SDS, sodium dodecyl sulfate; AO, acridine orange; [^{125}I]-UdR, [^{125}I]-iododeoxyuridine.

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INTRODUCTION

THE ANTHRACYCLINE antibiotics daunorubicin and ADR are effective chemotherapeutic agents in a wide spectrum of human neoplastic diseases [1, 2]. As with other agents, eventually resistance to the drug develops. The effectiveness of these compounds is also limited by acute myelosuppression and cumulative cardiotoxicity [1–3]. The recently developed ADR analog AD32 [4] has better experimental antitumor activity than its parent compound [5, 6], shows significantly less toxicity and has a greater therapeutic ratio [5]. Also, studies in rabbits [1], hamsters [7] and phase I and II clinical studies [8] have shown markedly diminished cardiotoxicity.

There is ample evidence that neoplastic cells *in vivo* or *in vitro* have the capacity to 'repair' damage induced by X- or u.v.-irradiation (reviewed in [9]), and by various chemotherapeutic drugs such as nitrogen mustard [10], 5-fluorouracil [11, 12], cyclophosphamide [11, 13] and the nitrosoureas [14]. Cell damage can be influenced by conditions after these treatments. A delayed assay of tumor clonogens probably allows for the late entry of damaged cells into the mitotic cycle, thus permitting more time for their repair. This in turn results in increased survival of tumor clonogens. Injury to tumor cells may also become lethal if it is accumulated as a consequence of repeated treatments with chemotherapeutic drugs [9]. Both phenomena—referred to as 'repair' in an operational manner—require further validation by cytokinetic and/or subcellular analysis. While the effects of ADR on repair of u.v.- or X-ray-induced damage are well documented (e.g. [15, 16]), there is only limited information available on repair processes following ADR treatment by itself [9, 17, 18]. Our recent studies using a clonogenic assay have suggested repair in ADR-treated lymphoma cells *in vivo* [19]. In this paper we have extended our studies to AD32 and asked the question of whether damage-repair processes at the cellular or DNA macromolecular levels can be detected in tumors treated with AD32. Since this study compares some of the mechanisms of the antitumor action between ADR and AD32, we chose to select drug dosages which gave equivalent effects in the overall tumor control. The 2 doses were established in a graded-dose experiment with survival of tumor hosts as an end point, and applied in the studies. We have used the DC-SM clonogenic and the DNA alkaline elution assays in a solid tumor system as before, and also report the results of HPLC drug analysis, flow-cytometric cell-cycle distributions and cell loss estimates.

MATERIALS AND METHODS

Tumor system

DBA3 [19] is a poorly differentiated lymphocytic B cell lymphoma, maintained by serial transplants in isogenic mice of the DBA/2J inbred strain. In its solid form, a $\sim 1.0\ mm^3$ graft of tumor tissue implanted s.c. into the dorsolateral chest region established a tumor mass *in situ* of approximately $0.32 \pm 0.04\ cm^3$ on day 7 after the implant. The tumor spreads invasively and leads to lung and liver metastases on days 8–9, killing the host between days 9 and 12. This lymphoma also grows as an ascitic or leukemic form. Near exponential growth of the primary solid tumor is observed on days 3–9, followed by growth retardation on day 9 or thereafter. Primary solid tumors have been used in our present experiments.

Drug treatments

ADR (NSC-123127, Farmitalia, Milan, Italy), 5–20 mg/kg diluted with sterile saline, or AD32 (NSC-246131, prepared for us by Farmitalia), 20–140 mg/kg diluted with 20% Diluent 12 (polyethoxylated castor oil–ethanol; Pharmaceutical Resources Branch, Division of Cancer Treatment, NCI) in saline, were injected i.p. in a single dose into mice with day 7 tumors. Tumor-bearing controls were injected i.p. with saline or Diluent 12–saline. The control as well as drug-treated animals were killed by cervical dislocation 1 hr–4 days after the injection. Tumor control and toxicity of the above listed single doses of ADR or AD32 were performed at the start and repeated during current experiments, following recommended protocols [20]. DBA/2J females (The Jackson Lab., Bar Harbor, ME) 8–10 weeks old, 18–22 g, were used throughout the experiments.

Preparation of a single-cell suspension

The tumor tissue was excised, minced, suspended in TC Medium 199 with 10% fetal calf serum (GIBCO, Grand Island, NY) and gently passed through a 200 mesh wire screen. The dispersed cells were layered over Lymphocyte Separation Medium (Litton-Bionetics, Kensington, MD) and centrifuged at 260 *g* at 4°C for 15 min. Cells from the interface were separated, washed, resuspended in TC Medium 199 and used for the DC-SM clonogenic assay. This procedure removed cell debris, red blood cells, most of the infiltrating cells and cells with pyknotic nuclei, but did not change the composition of various subpopulations of tumor cells as monitored by the incidence of cells with various types and number of nucleoli [21, 22]. In a typical procedure the yields were $2–3 \times 10^7$ viable isolated single

cells/g (wet wt) of tumor tissue, and trypan blue exclusion was 95%. The incidence of cell aggregates monitored by light microscopy was $<1/100$ cells, and white blood cell contamination $\leqslant 1.5\%$. As described later, further modifications were used in the preparation of cell suspensions for flow cytometry and DNA alkaline elution.

Assay of clonogens

Mechanically dispersed cells were counted with a Coulter counter and their concentration adjusted. Diffusion chambers (Millipore Corp., Bedford, MA) were assembled according to an established procedure [23], sterilized and seeded with 78–565 tumor cells uniformly dispersed in a matrix of 0.3% agar. Chambers were sealed, implanted i.p. into DBA2J mice and removed 7 days later. The semisolid matrix, still in diffusion chambers, was fixed by a methanol–formalin–acetic acid stained with May–Grünwald Giemsa. Stained clots were made permanent by mounting with Cytomoung (LaMar Lab., Oceanside, LI) on microscopic slides under a coverslip. Cell colonies were counted in a low-power microscope and cell morphology evaluated with 25× or 40× objectives. Colonies formed in diffusion chambers were defined as aggregates of $\geqslant 50$ cells. A linear relationship was obtained between the number of seeded cells and counted colonies, with a highly significant correlation coefficient of the slope ($r = 0.932$, d.f. = 68, $P < 0.001$). Regression analysis placed the slope intercept close to 0 (y intercept = 0.6; y, No. of colonies), suggesting that each colony originated from a single cell. Since tumor cells in a colony seem to be decendants of a single cell, presumably a clonogen (DC-cfu), cloning efficiency of tested tumors was defined as the No. of colonies per 100 cells of the original inoculum. Cloning efficiency of control untreated tumors ranged from 13.6 to 50.7%, with the mean and S.D. of 31.5 ± 10.2.

HPLC analysis

For the anthracycline analysis, drug-treated tumor-bearing mice were killed at intervals ranging from 6 hr to 3 days after the injection. Upon removal, tumor tissues were placed in capped test-tubes (12 × 75 mm, Falcon, Oxnard, CA), frozen instantly in liquid nitrogen and stored at −75°C until further processing.

On the day of analysis the samples were gradually thawed, washed in ice-cold saline, blotted dry and weighed. The tissues suspended in 9 vol. of Tris-HCl buffer, pH 8.5, containing 3% SDS w/v, were individually homogenized in the Virtuis homogenizer. The homogenate was extracted 3 times with 2 vol. of ethyl acetate: 1-propanol (9:1, v/v). Known amounts of *N*-trifluoroacetyladriamycin-14-octanoate (AD28) [24] were added as internal standard to the pooled extract, which was then evaporated to dryness under vacuum. The residue was reconstituted in 200 μl of methanol. The methanol extracts were cooled to −20°C for 10 min to precipitate SDS prior to analysis. Aliquots of the methanol extract were used for HPLC analysis. Both reverse-phase and complementary normal-phase HPLC systems were employed in order to identify and quantitate various metabolites [25–27].

Tumor tissues obtained from five untreated animals (day 7 tumors) served as controls. The tumor tissue was divided into 2 portions: one portion was used as blanks and the other was used to study extraction efficiency of various anthracycline compounds known or suspected to be metabolites of AD32 and ADR. The study of extraction efficiency included several steps. An eight-component mixture, consisting of ADR, AMNOL, adriamycinone, AD92, AD41, AD60 (adriamycin-14-valerate), AD48 (13-dihydro-*N*-trifluoroacetyladriamycin-14-valerate) and AD32, was added in 1–5 nmol/g concentrations to the homogenized tissue. The compounds were extracted and quantified by HPLC and the percentage recovery of each compound was determined. The efficiencies were used to correct the concentrations of compounds determined in the tumor extracts. Such a procedure improves the quantification of anthracycline species [27].

Flow cytometry

Control or drug-treated tumor-bearing mice were killed at scheduled intervals ranging from 6 hr to 4 days after injection. Tumors were mechanically converted into a single-cell suspension as described above, omitting the use of Lymphocyte Separation Medium. Cell suspensions, prepared from three tumors obtained from mice treated identically, were pooled and used as two aliquots for the analysis. Following a wash in cold medium, the pellets of pooled cells were resuspended in medium and the counts adjusted to $1\text{–}3 \times 10^6$ cells/ml. In some instances the accumulation of metaphases in tumors was induced by a single i.p. injection of vincristine sulfate (Oncovin, Eli Lilly and Co., Indianapolis, IN), 1.5 mg/kg body wt. [19, 28, 29]. Mice were killed 3 hr after injection, and the tumor tissue processed as described above. The cells were made permeable by treatment with a solution containing 0.08 N HCl, 0.15 M NaCl and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), as described earlier [30, 31], and stained with chromatographically purified AO (6 g/ml; Polysciences Inc., Warrington, PA) in a solution containing 0.2 M Na_2HPO_4, 0.1 M citric acid

buffer (pH 6.0), 1 mM sodium EDTA and 0.15 M NaCl.

Simultaneous staining of DNA and RNA and its specificity have been previously described and discussed [30–32]. Briefly, under the present staining conditions AO intercalates into double-helical nucleic acids (predominantly DNA in this case), fluorescing green (530 nm) in blue light, as does the dye monomer [33], while it 'stacks' in polymeric form on single-stranded nucleic acids (in this case RNA) with a metachromatic shift in maximum emission to red (640 nm) [34]. Double-stranded RNA is rendered single-stranded by the combination of EDTA and AO [35]. The stoichiometry of the staining reaction has been described for DNA by others [36] and by us [31, 32], and for RNA by Bauer and Dethlefsen [37].

The green (DNA) and red (RNA) fluorescence measurements, and green fluorescence pulse-width (proportional to nuclear diameter) of individual AO-stained cells were obtained by use of an FC-200 flow cytometer (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, MA). Interactive computer analysis programs were used to obtain mean values and normalized histograms of fluorescence for populations and subpopulations as illustrated in Figs 3 and 4. The computer-drawn displays were obtained with a Tektronix 4010-1 graphics display terminal (Tektronix Inc., Beaverton, OR).

Cell loss

The net flow of intact and/or dead cells from the tumor was measured by the [^{125}I]-UdR labeling assay [38–40]. Three groups of tumor-bearing mice, 24–28 animals each, were put on drinking water containing 0.1% potassium iodide for 2 days. On day 5 after the tumor implant mice were injected i.p. in four doses over 12 hr with 0.2 μCi/g body wt of [^{125}I]-UdR (sp. act. > 2000 Ci/mM, New England Nuclear Corp., Boston, MA). Two days were allowed for equilibration of the label, and on day 7 after tumor implantation the labeling index of tumor cells in autoradiographs was > 97.0%. At this time mice were injected with ADR (AD32) or saline (Diluent 12–saline). Immediately before, as well as 6 hr–4 days after, the injection, groups of 4 mice were killed, the tumor tissue excised, trimmed of connective tissue and kept for 48 hr in three changes of 70% ethanol. This treatment eliminated the soluble derivatives of [^{125}I]-UdR from the tissue without any loss of the molecules incorporated into DNA [41].

The measurement of cell death by this assay was tested under 2 conditions where cell loss is known to occur: in the first, mice with 7-day old tumors, pre-labeled with [^{125}I]-UdR, were treated with L-asparaginase (Merck, Sharp and Dohme, Rahway, NH), 20,000 I.U./KG [42, 43]. In the second, mice with 7-day-old tumors were injected with the highly toxic [^{125}I]-UdR dose of 5.0 Ci/g body wt [44, 45].

The level of [^{125}I]-UdR re-utilization in our tumor system was also tested [38, 45]. Details of the experiments are in footnotes to Table 4. The radioactivity of tumor tissue was measured in a well-type gamma scintillation counter. The measured radioactivity was corrected for physical decay back to the time of injection of mice in the first series and re-calculated as counts/g of wet tissue. Any fluctuation of counting efficiency was monitored by a concomitant measurement of a standard ^{125}I sample. The results were re-calculated as the percentages of radioactivity retained in each tumor at death, where 100% was the radioactivity measured immediately before the drug or solvent injection.

DNA alkaline elution technique

The methods used for this assay were those of Kohn *et al.* [46, 47], with only minor modifications [27]. On day 6 after the tumor implantation mice were injected i.p. with [2-^{14}C]-thymidine (sp. act. > 50 mCi/mM), 0.1 μCi/g body wt every 2 hr for 8 hr, and this resulted in labeling > 96% of tumor cells as determined autoradiographically [22]. On day 7 mice were injected with ADR (AD32) or saline (Diluent 12–saline), and 1 hr–3 days later the tumor tissue was converted into a single-cell suspension, using the dispersion technique described above preceded by enzymatic treatments. Minced tumor tissues were suspended in 50 ml of 0.15% trypsin (GIBCO, Grand Island, NJ) and incubated with continuous gentle agitation at 37°C for 2 15-min periods. DNase, 6 μg/ml (Sigma Chemical Co., St. Louis, MO), was added for the last 10 min. The suspension was centrifuged at room temperature and suspended in TC Medium 199 with 10% fetal calf serum. Some points of the DNA alkaline elution and DC-SM assays were obtained using tumors of the same passage of transplants. Experimental cells were either kept at 0°C without irradiation, or irradiated with 300 rads. L1210 cells (Sloan-Kettering), labeled with [methyl-^{3}H]-thymidine (sp. act. > 50 mCi/mM; Amersham, Arlington Heights, IL), 0.1 μCi/ml for 24 hr and irradiated with 150 rads at 0°C, were used as internal standards; 7×10^5 experimental cells and 3×10^5 internal standards, 10^6 cells total, were placed onto each polyvinylchloride filter (Millipore Corp, Bedford, MA), washed and lysed. One half

of all samples were also incubated with 0.5 mg/ml of proteinase K in 2% SDS (BDH Chemicals Ltd., Poole, England)–0.025 M trisodium EDTA at room temperature for 1 hr. Elution was carried out at a flow rate of 0.035 ml/min and eluates were collected at 90-min intervals. The radioactivity of individual fractions and radioactivity on the filter was determined in a scintillation counter. Eight samples were always processed simultaneously, 4 of control untreated and 4 of drug-treated tumors (single date point). Of each 4, two samples were treated with proteinase K and two were not treated. Each pair included one sample irradiated with 300 rads and one unirradiated sample. Steps in data analysis and in computation of frequencies of DNA strand breaks or crosslinks have been summarized elsewhere [47].

RESULTS

Tumor control and toxicity studies

Comparative antitumor activity for i.p. single-dose treatment with ADR or AD32 is shown in Table 1. The day of treatment (drug or saline injection to mice with 7-day-old tumors) was considered as day 0 in our calculations. The mean survival time (MST), increase in life span (ILS), cumulative days of tumor control (CD) and survival (CDS) relative to saline-injected tumor-bearing mice showed optimal single doses corresponding to 15 mg/kg of ADR or 60–100 mg/kg of AD32, with no significant difference in measured parameters. Single i.p. injections of 15 mg/kg of ADR or 80 mg/kg of AD32 were used throughout the experiments as isoeffective doses. It should be noted that because of the nature of the experiments contemplated, the experimental protocol intentionally uses single- rather than multiple-dose treatment.

The comparative toxicity of a single dose of ADR or AD32 injected i.p. into normal DBA/2J mice was also studied. The MST after ADR treatments decreases in all instances, and the percentage of toxic deaths ranged from 19/48 days (5 mg/kg to 100/6 days (20 mg/kg), reaching LD 100/36 days for the dose of 15 mg/kg body wt. In contrast, no toxic deaths were seen within the same 60-day observation period with 20–100 mg/kg of AD32.

The effects of drugs on tumor clonogens

The growth of DC-cfu in diffusion chambers provided an endpoint for measuring drug effects at various intervals post-treatment. In control untreated tumors the initial increase in the number of DC-cfu through 24 hr was followed by a gradual decline on days 2–4 (Fig. 1A). These two phases correspond to exponential growth of primary tumors through days 8 or 9, followed by growth retardation on days 9–10 and later [22]. In ADR-treated tumors maximal reduction of the survival fraction was observed at 18 hr post-treatment (Fig. 1B). Over the next 6 hr the number of DC-cfu returned to pre-treatment levels. In AD32-treated tumors there was a bi-phasic drop in DC-cfu at 12 and 24 hr post-injection (Fig. 1C), followed by a gradual return to pre-treatment levels over the next day and a half.

Metabolism of ADR and AD32

The mean percentage recoveries obtained for the 8 anthracycline compounds were as follows: ADR, 40; AMNOL, 26; adriamycinone, 80; AD32,

Table 1. Effect of single-dose treatments with ADR or AD32 on DBA3S tumors

Treatment	Dosage (mg/kg)	Total No. of experiments/mice	MILS* (%)	MST† (days)	CD‡ (days)	CDS§ (days)
Saline	-	8/39	-	2.2 ± 0.9	1	11
ADR	10	4/19	55.6	3.5 ± 0.9	6	17
	10	1/5	70.4	3.8 ± 0.5	9	19
	15	4/21	145.7	5.5 ± 1.3	13	30
	20	1/5	115.3	4.8 ± 1.8	8	24
D12	-	4/18	-	2.4 ± 0.8	2	12
AD32	20	1/6	58.3	3.8 ± 0.8	3	20
	60	2/14	112.5	5.1 ± 1.0	6	26
	80	3/15	120.8	5.3 ± 0.9	7	27
	100	2/10	120.8	5.3 ± 1.2	7	27
	140	1/5	116.7	5.2 ± 1.1	6	28

Mice used in the experiments were females, 19.3 ± 1.6 g body wt. *t*-test: ADR: 5 and 15 mg/kg, $P < 0.001$, d.f. 38; 10 and 15 mg, $P < 0.01$, d.f. 24; 15 and 20 mg, not significant; AD32: 20 and 80 mg/kg, $P < 0.01$, d.f. 18; differences between 60, 80, 100 and 140 mg not significant. ADR, 15 mg, and AD32, 80 mg, not significant.

*MILS, median increase in life span = $100 \times (MST_t/MST_c - 1)\%$, where MST_t and MST_c are mean survival times of drug-treated and untreated mice respectively.

†MST, mean survival time ± S.D.

‡CD, cumulative days of tumor control = Σ days when tumor volume on day $t + 1 \leqslant$ tumor volume on day t (normalized to 5 mice/experiment).

§CDS, cumulative days of survival (normalized to 5 mice/experiment).

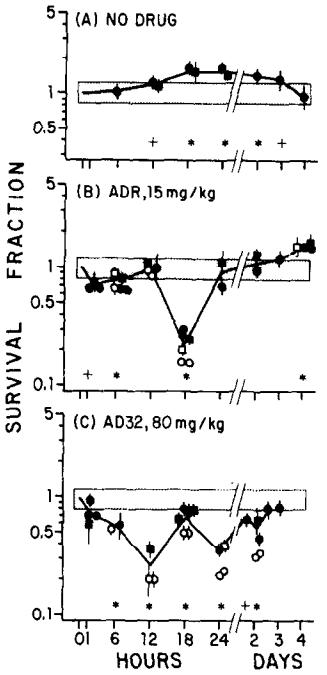

Fig. 1. Fraction of DC-cfu in control untreated tumors (A) compared with survival fractions of DC-cfu in tumors treated with a single dose of ADR 15 mg/kg *(B), or of AD32,* 80 mg/kg *(C), injected at time* 0. *Each point represents the mean ± S.D. of the survival fraction, based on DC-cfu counts in* 3–5 *DC-SM. Two independent sets of experiments are indicated by circles (○●) or by squares (□■). Initial densities of cells seeded into DC-SM differ by a ratio* 3 *(○□):* 1 *(●■). The hatched areas show* 2 *S.D. of the mean (=* 1.0*) for DC-cfu of untreated tumors at time* 0 *(seven separate experiments, total of* 47 *DC-SM). t-Test: differences between control tumors at time* 0 *and tumors assayed at particular time intervals: (*)* $P < 0.001$; *(+)* $P < 0.01$; *the rest is not significant. Data shown in panels (A) and (B) represent experiments published previously* [19].

83; AD41, 92, AD48, 57; AD60, 61; and AD92, 86. The concentrations of the compound detected in tumors were corrected accordingly. The concentration of other fluorescent anthracycline metabolites was expressed as equivalents of AD28. Several metabolites that were not identified were characterized by their retention times.

HPLC analysis of control tumor tissues showed no fluorescence signal. For ADR-treated tumors, only parent drug and AMNOL could be detected in any significant concentrations at 6 hr (Fig. 2). The concentration of ADR declined rapidly between 6 and 18 hr, after which time the decay was slower. Significant concentrations of ADR could still be detected 3 days after drug administration. In AD32-treated tumors, AD41 was found to be the major metabolite at 6 hr, with smaller quantities of AD92, ADR (Fig. 2) and AMNOL. In addition, 2 other small peaks were detected, both by reverse-phase and normal-phase HPLC (Table 2). The retention of these signals matched with those of 7-deoxy-13-dihydroadriamycinone (AD151) and 7-deoxyadriamyci-

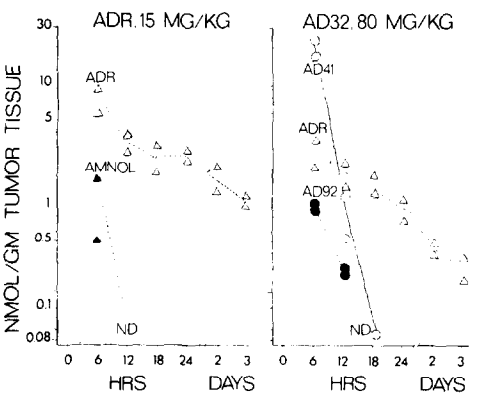

Fig. 2. Concentration of main anthracycline species in tumors following administration of ADR (15 mg/kg) *or AD32* (80 mg/kg). *Two tumors were analyzed per each time point. For technical detail see Materials and Methods. Minor metabolic species, isolated in the same experiment, are listed in Table* 2. *ND, not detected.*

*Table 2. Minor metabolic species** detected in tumors treated with ADR or AD32

Time post-drug injection	ADR, 15 mg/kg retention times (reverse-phase)	AD32, 80 mg/kg AMNOL†	AD112†	AD151†	retention times (reverse phase)
6 hr	–	2.899	1.869	1.960	5.17 ± 0.286
	5.87 ± 0.289	–	1.170	1.335	5.11 ± 0.57; 5.41 ± 0.63
12 hr	5.88 ± 0.099	–	–	–	–
	3.85 ± 0.056	–	–	–	–
18 hr	3.60 ± 0.69				
	5.80 ± 0.08	–	–	–	5.41 ± 0.146
	–	–	–	–	–
24 hr	5.54 ± 0.49				
	5.70 ± 0.11	–	–	0.145	–
	–	–	–	0.455	–
2 days	–	–	–	0.010	5.40 ± 0.057
	–	–	–	–	5.40 ± 0.334
3 days	–	–	–	–	5.40 ± 0.321

*Main metabolic species are shown in Fig. 2.

†Concentration of AMNOL, AD112 7-deoxyadriamycinone and AD151 7-deoxy-13-dihydroadriamycinone, are expressed as nmol/g equivalents of AD28 (internal standard).

none (AD112). The concentration of AD41 and AD92 rapidly declined over 12–18 hr. Despite the greater amount of AD32 used for treatment, levels of ADR derived from AD32 were always lower than ADR levels in tumors treated with ADR. Consistent with the known persistence of ADR in tissues [48], the levels of ADR derived from AD32 declined slowly and ADR was still detectable in tumor samples 3 days after the administration of drug.

Analysis of the cellular DNA and RNA content

The distribution of cells obtained in the scattergram illustrated in Fig. 3A are typical of specimens obtained from this tumor, regardless of the methods used to prepare a single-cell suspension. The cells outside the outlined contour (i.e. with low red fluorescence) represent either genuine intact cells with low RNA content, partially broken cells with little cytoplasm left or isolated nuclei. However, in contrast to intact cells, isolated nuclei or broken (dead) cells are susceptible to enzymatic degradation. As shown in Fig. 3, when cells were incubated for 20 min with 0.5% trypsin (GIBCO, Grand Island, NY) and 0.1 mg/ml DNase I (Worthington Biochemical, Freehold, NJ) the majority of low RNA cells disappeared, leaving only a small population of G0/G1 cells (most likely white blood cells) and an enriched population of high RNA cells whose distribution throughout the cell cycle and RNA pattern were typical of an actively growing tumor. As is shown in the DNA histograms in Fig. 3, the distribution of tumor cells with high red fluorescence selected by thresholds in Fig. 3A is similar to that obtained subsequent to enzymatic digestion in Fig. 3B. In the absence of enzymatic treatment there are also cells in S phase with moderate RNA values which may represent slightly damaged cells. These cells are not removed by thresholding but generally constitute a small proportion of cells analyzed (Fig. 3A).

Interactive computer programs were used to obtain the cell cycle distribution of tumor cells exposed to saline (D12-saline) or to drugs from the DNA histograms after exclusion of cells with low RNA content (see Fig. 3E). Figure 4 illustrates the fluctuations of cells between the various cell cycle compartments. Control untreated or ADR-treated tumors showed little variation in the percentage of G2 + M cells after various time intervals. However, ADR-treated tumors did have consistently greater percentages of cells in G0/G1 phase and fewer cells in S phase at each time point. AD32-treated tumors manifested an early increase of G2 + M phase cells which peaked 12 hr after drug injection. The percentage of G2 + M phase cells returned to near normal values by 24 hr and remained constant over the next 3 days. Coincident with the increase in G2 + M phase cells was the decrease in G0/G1 phase cells observed between 6 and 21 hr (Figs 3E and 4). By 24 hr the percentage of G0/G1 phase cells reached 70% (i.e. above the normal range). Over the next 24 hr cells in G0/G1 phase decreased and S phase cells increased by an equivalent percentage, and then by day 4 returned to normal levels. These results suggest that in AD32-treated tumors, a synchronous cohort of cells passes through G2, mitosis, G1 and S phases during the first 48 hr, and then a re-establishment of the 'normal' cell cycle distribution takes place 48 hr later. This view was reinforced by a subsequent experiment in which control tumor-bearing mice and mice treated with ADR or AD32 for 21 hr were injected with vincristine sulfate 3 hr before being killed. Figure 3 (C–F) illustrates the resulting increase in the percentage of G2 + M phase cells (scattergrams and solid-line histograms) vs the tumors obtained at an equivalent time from mice not treated with vincristine sulfate (dashed-line histogram). In each instance the percentage of G2 + M phase increased, though the increase was much more substantial in the AD32-treated tumors (i.e. from 22 to 24%; Table 3), whereas the lowest increase was found in tumors treated with ADR.

Rates of cell loss

As described in Materials and Methods, [^{125}I]-UdR was used to study the effects of ADR and AD32 on cell loss. The pooled results of two separate experiments are shown in Fig. 5. No significant difference were observed in the rates of cell loss over a 48-hr period between the tumors of untreated mice and mice treated with ADR or with AD32. Control experiments included studies of [^{125}I]-UdR re-utilization and of the ability of the assay system to measure cell death under conditions where it is known to occur. A very low level of reutilization (2.5%) was detected (Table 4). A positive control for the cell-loss assay demonstrated that cells killed by L-asparaginase or by toxic doses of [^{125}I]-UdR were removed efficiently from the tumors (Fig. 5).

DNA alterations as detected by the alkaline elution technique

These studies compare the *in vivo* effects of ADR and AD32 on the formation and frequencies of DNA lesions in DBA3S cells. Exposure to either drug resulted in protein-associated DNA breaks and DNA–protein crosslinks (Fig. 6). In both instances formation of DNA–protein crosslinks preceded the appearance of protein-associated

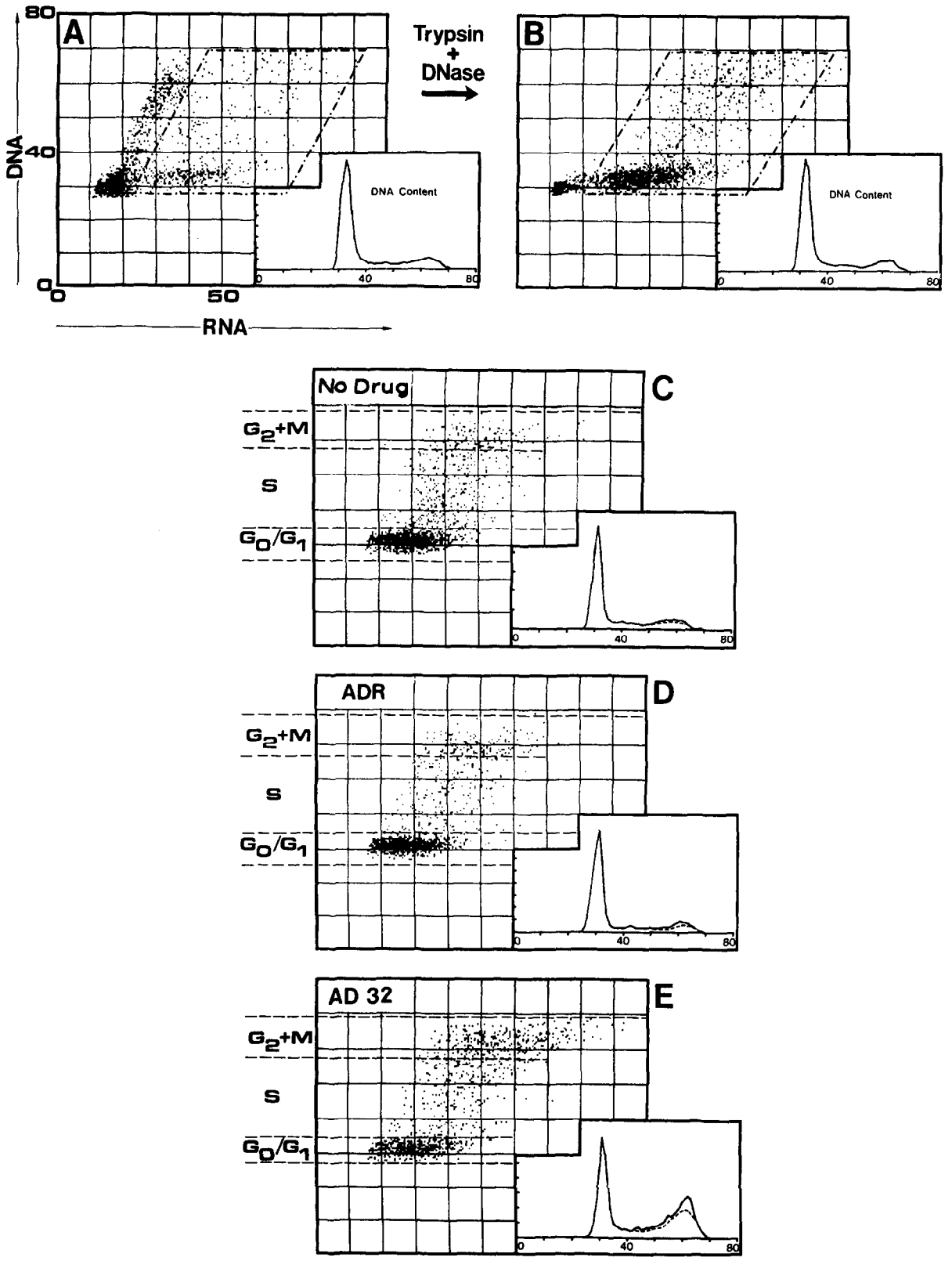

Fig. 3. (A) Tumor sample from ADR-treated (12 hr) *mice, mechanically dispersed into a single-cell suspension. Staining for DNA and RNA is described in Materials and Methods. Each dot represents an individual cell. The position of each dot relative to the axes represent the relative amount of DNA (ordinate) and RNA (abscissa) of the cell in arbitrary units. Approximately* 1.5×10^3 *cells are displayed in each scattergram. (B) An aliquot was removed from sample (A) and incubated for* 20 min *at* 37°C *with* 0.25% *trypsin and* 0.1 mg/ml *DNase. DNA histograms for both (A) and (B) were obtained by interactive computer thresholding of cells within the bounds of the parallelograms displayed. (C) Cell suspension of control untreated tumors were obtained from tumor hosts injected with vincristine sulfate* 3 hr *before death (solid histogram and scattergram) or left untreated (dashed histogram). (D) Same as (C) except mice treated with ADR for* 18 hr. *(E) Same as (C) except mice treated with AD32 for* 18 hr.

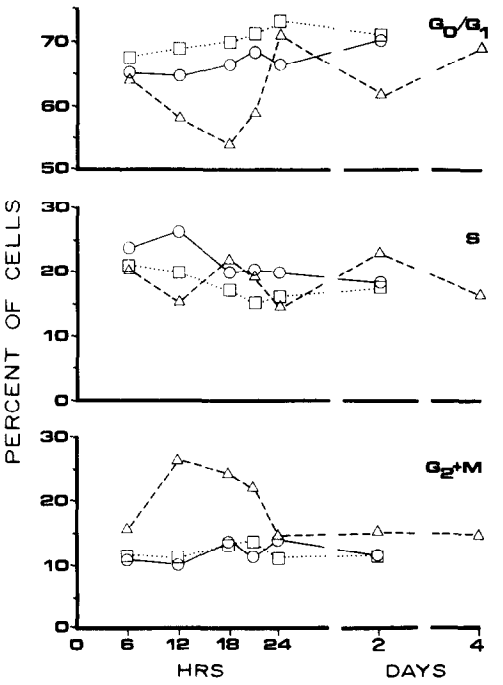

Fig. 4. Cell cycle analysis of control (saline-treated) tumors (○), tumors treated with ADR (□) or with AD32 (△) for varying lengths of time. Tumors from 3 mice treated in an identical way were pooled into 2 aliquots for each time point. The resulting data is the average of the 2 aliquots.

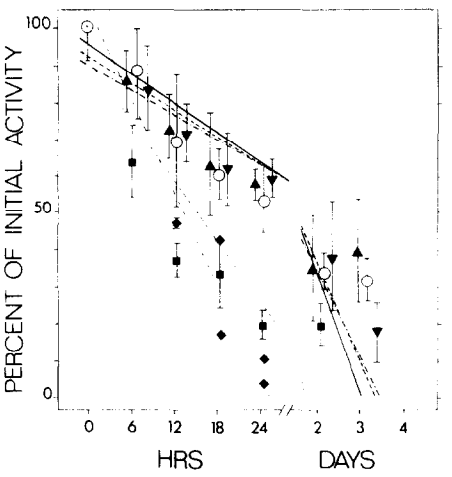

Fig. 5. Loss of [^{125}I]-UdR activity in tumors with time after the treatment. Counts are normalized to 100% at time 0, which was 48 hr after the administration of [^{125}I]-UdR to tumor-bearing mice. Differences between slopes for control untreated tumors (○——) and tumors treated with ADR, 15 mg/kg (▲– – –), or with AD32, 80 mg/kg (▼–·–·–), are not significant (d.f. 51, P >0.1 for both sets of data). Differences between slopes for ADR- or AD32-treated tumors and slopes for tumors treated with L-asparaginase, 20,000 I.U./kg (■– – –), or for tumors treated with 5 μCi/g body wt of [^{125}I]-UdR (◆–·–·–) are significant (d.f. 34–40; P <0.001, both tails). Each symbol represents the mean ± S.D. for a group of 4 mice assayed in two separate experiments (controls, ADR and AD32), or the mean ± S.D. for 3 mice (asparaginase and [^{125}I]-UdR). Data for ADR and [^{125}I]-UdR were published separately [19].

*Table 3. Effects of vincristine sulfate on the accumulation of G2 + M cells in tumors treated with ADR or AD32**

Sample	G2 + M cells (%) Control	Vincristine	Difference† (%)	Increase‡ (%/hr)
Control	11.2	15.2	4.0	1.3
ADR	13.6	17.0	3.4	1.1
AD32	22.0	34.0	12.0	3.0

*For technical details see Materials and Methods.
†Difference between vincristine-treated and untreated tumors.
‡Increase of G2 + M phase cells (%/hr of vincristine blockade).

Table 4. Reutilization of [^{125}I]-UdR label in tumors

No. of implanted mice	[^{125}I]-UdR-labeled cells* Viable	Heat killed	Unlabeled cells*—viable	[^{125}I]-UdR incorporation†
4	–	2×10^6‡	–	–§
4	2×10^6	–	–	1.00 ± 0.239
4	1.2×10^6	–	1.5×10^6	0.506 ± 0.153
4	–	1.2×10^6	1.5×10^6	0.125 ± 0.001
3	–	–	2×10^6	0.005 ± 0.007

*The day 5 tumors were labeled with [^{125}I]-UdR (see Materials and Methods), and on day 7 were converted mechanically into a single-cell suspension. Aliquots of the cell suspension were incubated at 60°C for 1 hr, and the rest kept at 0°C. Cell suspension of unlabeled cells was also obtained using day 7 tumors.
†The measured radioactivity was corrected for physical decay, recalculated as counts/g wet tissue and further normalized to a standard size of cell inoculi. The radioactivity of tumors originating from a cell suspension of labeled viable cells only was considered as to be equal to 100%.
‡Shown number of tumor cells in 0.2–0.3 ml of PBS was injected s.c. into the dorsolateral chest region of DBA/2J mice of the same sex and age as those used for regular tumor implants.
§No tumor growth over a 60-day observation period.

breaks (Fig. 7). There was, however, a considerable difference between both drugs in the timing of the appearance and disappearance of DNA lesions. Following a latent period of at least 12 hr, DNA-protein crosslinks appeared at 18 and 24 hr after the injection of ADR, whereas increased frequencies of protein-associated breaks were present at 24 hr. DNA lesions were no longer increased on day 2 and 3. A single-dose treatment with AD32 was followed by the appearance of DNA-protein crosslinks at 6–18 hr and protein associated breaks at 12–24 hr after the injection. DNA alterations were not detectable at significantly increased frequencies on day 2.

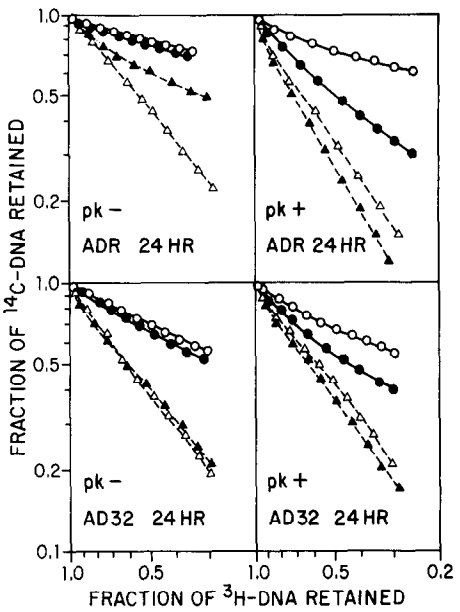

Fig. 6. Effects of ADR or AD32 on alkaline elution kinetics of DNA. ADR, 15 mg/kg, *or AD32,* 80 mg/kg, *were injected* 18 *or* 24 hr *earlier into tumor-bearing mice. Control (○, △) and cells from drug-treated tumors (●, ▲) were either left unirradiated (○, ●) or were irradiated with* 300 rads *(△, ▲) and lysed (see Materials and Methods). Cell lysates were eluted without (pk–) or with (pk+) proteinase K treatment. Elution data are summarized in Fig. 7.*

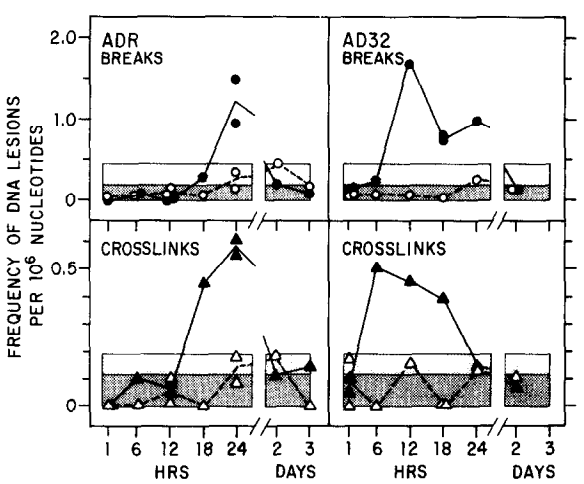

Fig. 7. Apparent frequencies of DNA lesions in drug-treated tumor cells. ADR, 15 *or AD32* 80 mg/kg, *were injected at time* 0. *Frequencies of DNA strand breaks (○), DNA protein-associated breaks (●), DNA-DNA crosslinks (△), and DNA-protein crosslinks (▲) were calculated* [47] *for tumors removed at shown time-intervals after the injection. For each assay, cells were pooled from* 2–3 *drug-treated or from a similar number of untreated (control) tumors. 'Background' values, indicated by the hatched area, were calculated using the means* [^{14}C]*-DNA ('experimental cells') at* 0.5 *of* [^{3}H]*-DNA vs the means, with* 1 *S.D. being added* [27]. *Some of the data showing ADR-induced lesions were published separately* [19].

DISCUSSION

The data presented above extend our earlier observation on ADR repair [19] by studies on metabolic fate of ADR and AD32 in treated tumors with HPLC and by characterizing the proliferation rate with flow cytometry. These studies were designed to compare the cytokinetic effects of the 2 drugs. Such a comparison was made in tumors treated with drug dosages equivalent in the overall tumor controls. A major aim of the present study was to examine whether *in vivo* repair also occurred in cells treated with AD32. Before the occurrence of repair processes could be established, the proliferation of surviving cells and/or the selective loss of killed cells from drug-treated tumors had to be considered. Either of these events could influence the interpretation of our results. Strong evidence, however, was obtained against their occurrence in experiments with ADR. As shown in Fig. 1, a maximal 4.4-fold reduction of the survival fraction of DC-cfu (relative to time 0 values) at 18 hr was followed by a 3.9-fold increase in clonogens over the next 6 hr. The first 24 hr post-treatment was also accompanied by significant changes of cell progression through the cell-cycle phases. The flow-cytometric analysis detected: (1) higher percentages of G0/G1 cells and lower percentages of S-phase cells in ADR-treated tumors as compared with untreated controls (Fig. 3); and (2) following a vincristine blockage, a decreased accumulation of cells (presumably arrested metaphases) in G2 + M phase (Table 2). The latter observation is in substantial agreement with previously reported prolongation of the cell doubling time in ADR-treated DBA3 tumors [19], and supports the notion that cell proliferation by itself could not explain the increase of DC-cfu at 24 hr. The failure to find greater cell loss in ADR-treated than in untreated tumors over a 48-hr period indicates that selective removal of damaged cells also was not a decisive factor. To account solely for an increase of DC-cfu at 24 hr post-treatment caused by the loss of 'sterile' damaged cells, the cell loss should have been at least 4-fold higher in ADR-treated than in untreated tumors.

In order to validate the interpretation of cell loss data, two types of control experiments were included: (1) experiments have shown a very low level of [^{125}I]-UdR re-utilization under conditions when dead labeled cells, amounting to 40% of total cell population, were in close contact with living unlabeled cells. Cell loss estimates in ADR (AD32)-treated tumors are slightly biased as a consequence of re-utilization of the label. The detected re-utilization, however, cannot account for any significant change of cell loss data. Should an extreme situation be considered when 'control tumors' have no re-utilization and 'ADR-treated tumors' re-utilize 12.5% of the label, then the regression analysis still shows a non-significant difference between the slopes (d.f. 51, $P > 0.1$). When cells were killed with a known cytocidal agent (L-asparaginase or toxic doses of [^{125}I]-UdR), most of the [^{125}I]-activity associated with labeled cells was removed from the tumor within 24 hr after the onset of treatments (Fig. 5). Thus the failure of ADR or AD32 to increase the rate of [^{125}I] loss from the tumor over the [^{125}I] loss in control untreated tumors indicates that the two agents do not exert any detectable cytocidal effects at early times (6 hr–2 days) after the drug injection.

Since cell division or cell loss cannot explain the increase in DC-cfu in ADR-treated tumors, the rebound of clonogens shown in Fig. 1 is interpreted as suggestive of repair of ADR-induced damage in cells left *in situ* over a 24-hr period. This event was registered at times when significant levels of ADR were still detectable in the tumor tissue.

Another objective of this study was to correlate changes in measured survival of DC-cfu with the incidence of macromolecular alterations. The results indicate that ADR induces *in vivo* an identical type of DNA alteration as was observed *in vitro* [18]. The appearance of DNA–protein crosslinks at 18 and 34 hr and of protein-associated breaks at 24 hr post-injection coincided with the time of the decrease and rebound of DC-cfu. These findings are consistent with the hypothesis [46, 48] that, following cell exposure to ADR, a repair enzyme—presumably a topo-isomerase—is activated and becomes attached to DNA strands, resulting in DNA–protein cross-linking. Later on, the endonuclease causes strand scissions (DNA breaks concealed by protein), and this event coincides with the above-described rebound of clonogens. The results also show that cell clonogenicity is regained at 24 hr, with macromolecular repair still under way. This can be explained by the completion of repair processes after cell explanation into the DC-SM system.

In AD32-treated tumors the HPLC analysis has shown rapid conversion of AD32 into AD41 and AD92. Both biologically active metabolites [49, 50] are present in tumor tissues 12–18 hr after treatment, along with ADR which appeared as a metabolite of AD32. Despite a more than 5-fold higher dose of AD32 over the dose of ADR used to treat tumor-bearing mice, the ADR concentration was 2–4 times less in AD32 than in ADR-treated tumors. The DC-SM assay detected a bi-phasic decrease of DC-cfu in treated tumors. The first decrease and rebound of DC-cfu over 18 hr after AD32 injection was coincident with high levels of drug metabolites and their decay (Fig. 2). This phenomenon is reproducible, but its relationship to the interaction of drug metabolites, to the disruption of the tumor matrix and subsequent manipulation of isolated cells requires further study.

While the second gradual rebound of DC-cfu, noticeable through day two and a half after treatment, cannot be explained by selective cell loss (Fig. 5), flow cytometry indicates participation of cell proliferation in the rebound phenomena. The results strongly suggest the presence of a synchronized cell population, first accumulated in the G2 + M phase over 18 hr, with a peak at 12 h after the injection. This is followed by their traverse through G0/G1 and S phases. Vincristine blockade further increased the size of the synchronized cohort in the G2 + M phase at 21 hr, presumably by mitotic arrest. In an asynchronous cell population, if it is assumed that (a) all cells are in the cycle, (b) the block by vincristine is 100% efficient and (c) the doubling time is known (45.6 ± 13.7 hr [19]), one would expect the cells to accumulate in mitosis at a known rate, corresponding to 2%/hr in this case. We presently observe that cells accumulate in G2 + M somewhat slower than expected in control and ADR-treated tumors (Table 3). The lower than expected value in control tumors is probably the result of several variables, e.g. the time required for vincristine to take effect [28]. Thus AD32-treated tumor cells accumulated in G2 + M at 225% the rate observed in untreated controls. A simple calculation shows that, at the interval between 18 and 24 hr, DC-cfu could increase 1.56-fold and that cell doubling could have accounted for the rebound of clonogens between days 1 and 2.5 (Fig. 1). DNA alkaline elution technique demonstrated that AD32 induces *in vivo* the identical type of DNA alterations as this compound and its metabolites do *in vitro*. It should be stressed, however, that unlike the situation in DNA3 lymphomas *in vivo*, no detectable conversion of AD32 into ADR was detected *in vitro* in L1210 cells [27]. Similarly, as in experiments with ADR, DNA–protein cross-

links appeared first (at 6 hr), followed by protein-associated breaks (at 12 hr after the injection). The appearance of DNA alterations coincided with the time of decreases in the survival fraction of DC-cfu. The disappearance of breaks and crosslinks on day 2 after AD32 treatment cannot be interpreted unambiguously. It may indicate replacement of cells with DNA alterations by a newly generated undamaged cell population and/or a selective removal of DNA alterations in individual cells. An answer to the question of a possible overlap will require more direct methods, such as assays of DNA repair replication.

While the improvement in therapeutic ratios of AD32 over its parent compound has been demonstrated [5, 6], this study suggested several facets of its improved effectiveness as observed in primary tumors: (1) the decrease in the number of DC-cfu in AD32-treated tumors continued for over 2 days, as compared to 12 hr in ADR-treated tumors; (2) AD32 did not induce an efficient and unambiguously discernable repair, as was the case in ADR-treated tumors; (3) AD32 synchronized the lymphoma cells in their progression through the cell cycle. Should further studies show these cells to be clonogens, the synchronizing effect could be utilized in drug combination treatments. Furthermore, low toxicity of a single dose of AD32 observed here would allow the use of repeated injections of the drug with presumably beneficial effects.

HPLC analysis showed the presence of ADR in the tumor tissue as a metabolite of AD32. ADR persisted at detectable levels through day 3 after the treatment, whereas the other two major metabolites, AD41 and AD92, decayed relatively rapidly over 18 hr. While the improved effectiveness of AD32 over ADR cannot be explained by the sole presence of ADR as a metabolite in AD32-treated tumors, ADR biological interactions with AD41 and AD92 should be considered and further explored.

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