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The Immunodepressive and Hematotoxic Activities of *N*-Trifluoro-Acetyl-Adriamycin-14-Valerate*

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Abstract—*The immunodepressive and hematotoxic activities of* N-*trifluoroacetyl-adriamycin-14-valerate (AD32), a compound having a better antineoplastic effectiveness than Adriamycin (AM) in animals, were investigated in mice. AD 32 was found to induce marked but relatively transient reductions in bone marrow stem cells with an effect in general comparable to that of AM. Both cellular and humoral immune reactivities could be inhibited by the drug and the antitumoral effectiveness of AD 32 was reduced in previously immunodepressed hosts.*

INTRODUCTION

THE REMARKABLE clinical activity of adriamycin (AM) in a wide range of neoplastic conditions [1, 2] has led to widespread efforts in the search of analogs possessing higher antitumoral capacity and comparable or lower toxicity especially as regards cardiotoxicity, the major limiting side-effect of this drug. Among the derivatives so far developed, *N*-trifluoroacetyl-adriamycin-14-valerate (AD 32) has been shown to display a clearly higher neoplastic efficacy than AM on a series of animals tumors [3–5] although a definitive comparative evaluation of the toxicities of the two agents, which may have at least partially different modes of action [6], is still not possible. The antineoplastic effectiveness of this drug is currently under clinical investigation [7, 8].

In recent years compelling evidence has been gathered in support of the conclusion that the different interaction with the immune system of AM and its earlier analog daunomycin (DM) plays a major role in giving to the former agent a better antineoplastic effectiveness [9–11]. On this basis and in view of the increasing awareness that the immunodepressive activity of antitumorals may be an important determinant of their therepeutic capacity [12], it was of interest to characterize the immunodepressive and hematotoxic activities of AD 32 employing conditions in which the effects of AM had previously been investigated.

MATERIALS AND METHODS

Animals

Female C3H, $B6D2F_1$ and CDF_1 mice of both sexes (20 ± 2g body weight), obtained from Charles River, Calco, Italy, were used.

Drugs

AM, a generous gift of Farmitalia, Milan, Italy, was freshly dissolved in saline and injected intravenously (i.v.). Two lots of AD 32, one obtained from Dr. D. M. Israel (Sidney Farber Cancer Center, Boston, Mass.), and the second from Dr. F. Arcamone (Farmitalia, Milan, Italy), were used; since no significant differences in the *in vivo* activity were detected between the two lots, results have been pooled. The drug was freshly di-

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ssolved in Tween 80, 10% in saline as previously described [3] and injected i.v. Imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno) (DTIC) was kindly provided by Dr. H. J. Wood, Jr. (DCT, NCI, Bethesda, Md) and dissolved in saline before i.p. administration.

Tumor

The L1210 Ha leukemia (originally obtained from Dr. E. Mihich, Roswell Park Memorial Inst., N.Y.) was maintained by weekly i.p. passages in compatible DBA/2 (H-2^d) mice whereas $CD2F_1$ mice (10 per group) were used for the chemotherapy experiments. For tumor allograft resistance studies, 10^7 L1210 Ha leukemia cells were transplanted i.p. into histoincompatible C3H (H-2^k) hosts (10 per group) after removal by osmotic lysis of contaminating erythrocytes from the ascites and checking by trypan blue exclusion that tumor cell viability was >95%.

Response to sheep erythrocytes (SRBC)

$B6D2F_1$ mice were given i.p. injections of 4×10^8 SRBC on day 0 and spleen hemolytic plaque-forming cells (PFC) were counted by the technique of Jerne and Nordin [13] on day 4 unless otherwise stated. For inducing a secondary response, an equal SRBC challenge was administered 10 days after primary immunization and the number of direct and indirect PFC was evaluated 4 days later, using a modification [14] of Jerne's technique. Goat antiserum to mouse γ-globulin (Meloy Labs., Inc., Springfield, Va., U.S.A.) at a final dilution of 1:800 was used for development of indirect PFC. Control mice were given a 10% solution of Tween 80 in saline. Data are presented as geometric means (in parenthesis ±S.E.) after logarithmic transformation as recommended by Dresser and Wortis [15].

Response to SIII

The type 3 pneumococcal polysaccharide SIII was kindly supplied by Dr. P. J. Baker (NIAID, NIH, Bethesda, Md.); $B6D2F_1$ mice were given 0.5 μg of SIII i.p. on day 0 and the response was assessed on day 5. Splenic PFC specific for SIII were detected by the technique of Cunningham and Szenberg [14] using SRBC sensitized with SIII by the chromium chloride procedure [16]. Control mice were given a 10% solution of Tween 80 in saline. As for the anti-SRBC response at least three experiments with eight animals per experimental group were performed.

Skin grafting technique

The classical technique described by Billingham [17] was followed: tail skin from C3H was grafted onto $CD2F_1$ mice, under Nembutal anesthesia. Criteria for graft rejection were as previously detailed [17].

Assay of bone marrow stem cells

The procedure originally described by Till and McCulloch as detailed elsewhere [10], was used. Bone marrow cells from normal or drug-treated donor $B6D2F_1$ mice (4 per experimental group) were obtained by repeated flushing of femur shafts with ice-cold Hank's balanced salt solution, cell viability was checked microscopically by dye exclusion and 3×10^5 cells were injected i.v. into previously supralethally irradiated (800 rad whole body of X-rays) syngeneic recipients (8 per group). Animals were sacrificed 7 days later, their spleens fixed in Bouin's solution, and the number of colonies (CFU) was counted under a dissecting microscope.

Statistical analysis

Statistical significance was assessed by Duncan's test [18]; dose-response curves were calculated using linear regression analysis.

RESULTS

Effect of AD 32 on primary and secondary responsiveness to SRBC

The effect of single AD 32 doses on the immune responsiveness to SRBC, an antigen requiring T–B lymphocyte cooperation, was firstly investigated. Table 1 shows that this

*Table 1. Effect of AD 32 on primary anti-SRBC response**

Drug (mg/kg)	Day of treatment	PFC/spleen	Controls (%)
—	—	36,160† (26,280–35,990)	100
40	−2	29,250 (23,250–32,810)	97
40	0	12,620‡ (8450–19,470)	40
40	+2	197§ (110–450)	0.6
80	−2	9230§ (7240–15,370)	30
80	0	<10§	<0.1
80	+2	<10§	<0.1

*$B6D2F_1$ mice were given 4×10^8 SRBC on day 0; drug was administered i.v. at different times relative to immunization; test was performed on day 4.
†Geometric mean; Number in parenthesis: ±S.E.
‡$P<0.05$ vs control.
§$P<0.01$ vs control.

*Table 2. Immunodepressive activity of AD 32 evaluated at different times after immunization**

Drug (mg/kg)	PFC/spleen on days 4	7	10‡
—	41,130† (36,400–48,720)	14,010 (12,190–16,100)	13,140 (8110–17,070)
40	390§ (240–550)	360§ (250–550)	4102§ (3400–4970)
80	<10§	<10§	2100§ (1200–3540)

*B6D2F$_1$ mice were given 4×10^8 SRBC i.p. on day 0, drug was administered i.v. on day 2 and the assay performed at times indicated.
†Geometric mean; number in parenthesis: ±S.E.
‡Total spleen PFC (direct and indirect).
§$P<0.01$ vs control.

drug was most immunodepressive when administered after antigenic challenge, in these conditions a 99% reduction in peak response being obtained also with 40 mg/kg i.v., a dose antitumorally ineffective when single injections are used [4]. Higher drug doses were, however, capable of reducing primary PFC levels also when given 2 days before antigen. When the kinetics of the response was followed (Table 2), it was found that a true depression and not merely a shift in the peak response followed AD 32 injection, since PFC values on day 7 and 10 after SRBC were still significantly reduced in respect to vehicle injected, SRBC stimulated controls. The dose-respose curves (Fig. 1) for the immunodepressive activity of AD 32 and AM in these conditions best fitted an exponential as indicated by linear regression analysis performed after logarithmic transformation of the results. The doses reducing by 50% peak PFC in the spleen were 5 and 2mg/kg i.v., respectively.

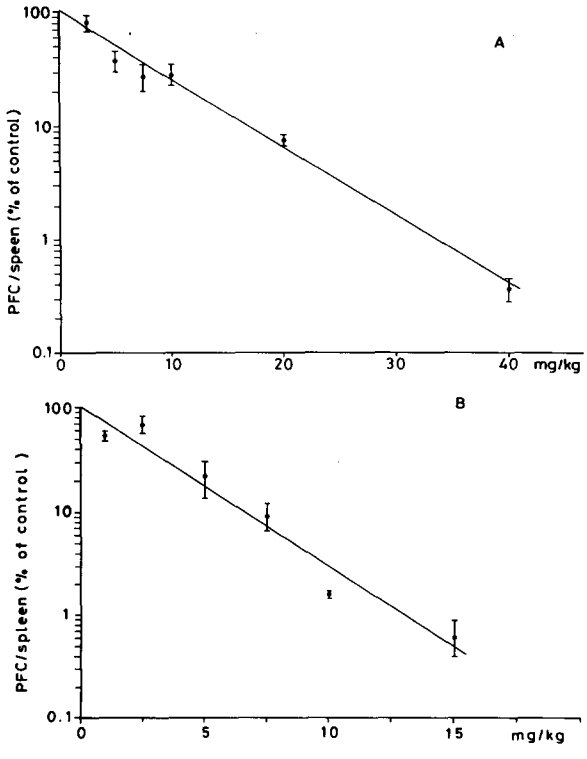

Fig. 1. Dose-response curve of AD 32 (A) and AM (B) given on day 2 relative to SRBC immunization: test was performed on day 4.

When the effect of AD 32 on secondary anti-SRBC response was investigated administering the drug 2 days after the second antigen injection and counting antibody-producing cells 48 hr later, a marked immunodepression was again observed, although the reduction in PFC levels detected was significantly lower than seen after a primary stimulation. Table 3 also shows that at all doses tested, drug injection produced a stronger reduction of direct, IgM than of indirect, non IgM PFC.

Effect of AD 32 on primary response to SIII

The effect of the drug on the immune response to a T-independent antigen as SIII [19] was next investigated; for these experiments AD 32 was injected in mice 2 days after an optimal immunizing challenge and counting specific anti-SIII PFC on day 5, i.e., at peak control response. Table 4 shows that in comparison to the anti-SRBC response, antibody production to SIII was markedly less sensitive to AD 32 depression. In fact, a dose of 40 mg/kg which resulted in over 99% decrease in the spleen levels of primary anti-SRBC PFC, gave only modest and not significant reductions in anti-SIII PFC, significant reductions in the latter response being observable only with doses of 80 mg/kg or above.

Effect on allograft responsiveness

In subsequent experiments, the effect of AD

*Table 3. Effect of AD32 on secondary anti-SRBC response**

Drug (mg/kg)	Direct IgM PFC/spleen	Control (%)	Indirect Non-IgM PFC/spleen	Control (%)
—	14,940† (13,190–16,920)	100	15,008 (14,450–15,600)	100
40	1510‡ (1150–1980)	10	4330‡ (3720–5040)	29
80	900‡ (700–1170)	6	3000‡ (2375–3790)	20
120	850‡ (570–1260)	5.7	2540‡ (1700–3800)	17

*B6D2F$_1$ mice were given 4×10^8 SRBC i.p. on days −10 and 0; drug was given on day 2 and the assay performed on day 4.
†Geometric mean; Number in parenthesis: ±S.E.
‡$P<0.01$ vs control.

*Table 4. Effect of AD32 on anti-SIII response**

Drug (mg/kg)	PFC/spleen	Control (%)
—	2120† (1520–2965)	100
10	2070 (1910–2240)	97
40	1440 (1330–1550)	68
80	734‡ (590–900)	35
120	458‡ (400–520)	22

*B6D2F$_1$ mice were given 0.5 μg of SIII i.p. on day 0; drug was administered on day 2 i.v. and assay performed on day 5.
†Geometric mean; number in parenthesis: ±S.E.
‡$P<0.05$ vs control.

*Table 5. Effect of AD 32 on allogeneic tumor rejection**

Drug (mg/kg)	MST†	D/T‡
—	>90	0/10
10	11	1/20
20	10.2±0.5	3/10
40	9.7±0.5	8/10
60	7.8±0.4	8/10
80	7.4±0.5	10/10
100	6.6±0.3	10/10

*C3H mice were treated with drug 24 hr before transplantation of allogeneic 10^7 L1210 Ha tumor cells. Normal mice treated with AD 32 (100 mg/kg) and not injected with tumor survived over 90 days.
†Mean survival time in days ±S.E.
‡Dead over total number of tumor transplanted mice.

32 in models of immune reactivity mediated predominantly or exclusively by cellular responses was examined. Table 5 shows that when 10^7 L1210 Ha leukemia cells were transplanted into H-2-incompatible C3H hosts, all mice were capable of rejecting the tumor graft surviving indefinitely thereafter. If however, these animals had been injected 24 hr before with AD 32, their capacity to resist the tumor transplant was clearly impaired since a significant proportion (30–40%) of lethal takes was observed already with drug doses of mg/kg and 80–100% progressive growths were found with doses of 40 mg/kg or above. In contrast, when the capacity of single AD 32 doses to prolong the survival of C3H (H-2^k) tail skin grafts in CD2F$_1{}^d$ (H-2$^{d/d}$) recipients was investigated, no evidence of significant immunodepressive activity could be obtained. In fact, the mean day of graft rejection was 11.9±0.9 in control mice and 11.6±0.8 in animals injected 24 hr after grafting with 100 mg/kg AD 32, i.e., a dose highly immunodepressive in other systems.

Chemotherapeutic activity of AD 32 in immunodepressed hosts

In order to obtain insights on the possible importance of the effect of AD 32 on the immune system in its antineoplastic activity, the increase in lifespan (ILS) and proportion of long-term survivors given by this drug were evaluated in normal and immunodepressed CD2F$_1$ mice transplanted with the compatible, highly immunogenic L1210 Ha leukemia [20]. Immunodepression was induced with either 400 rad X-rays or injection of 180 mg/kg i.p. DTIC given 1 or 5 days before transplant, respectively. DTIC has previously been shown to induce profound and long-lasting inhibition

of both cellular and humoral responses in mice [21]. As shown in Table 6, the chemotherapeutic activity of single i.v. doses of AD 32 was markedly decreased in animals pretreated with either DTIC or radiation, in comparison to normal hosts, as evidenced not only by the clear reductions in the % ILS values but also by the absence of long-term survivors in these immunodepressed mice. In the conditions employed, previous experiments had shown that these combined treatments of AD 32 and X-rays or DTIC were perfectly tolerated by normal mice with 100% over 90 days survival.

Table 6. *Effect of AD* 32 *in immunosuppressed hosts*

400 rad	DTIC	AD 32 (mg/kg)	ILS*(%)	LTS†(%)
−	−	40	60	50
−	−	60	106	70
+	−	40	23	0
+	−	60	34	0
−	+	40	30	0
−	+	60	72	0

DTIC (180 mg/kg i.p.) was given 5 days and 400 rad X-rays 1 day before L1210 Ha leukemia transplantation (10^5 cells i.p.) to $CD2F_1$ mice. Long term survivors were excluded from % ILS calculation. AD 32 was given i.v. 1 day after tumor transplant.

*Increase in lifespan.

†Percentage of mice surviving over 60 days.

Effect on bone marrow stem cells

The effect of single i.v. injections of AD 32 on bone marrow stem cell numbers in $B6D2F_1$ mice is presented in Fig 2A; for these studies bone marrow cells were collected from donor mice 24 hr after drug injection and counting of spleen colonies in the irradiated recipients was performed 7 days after cell transfer. It may be seen that AD 32 administration produced marked reductions in stem cell numbers, the dose-response curve observed in these conditions being again exponential but with a less steep slope ($\alpha = 0.033$) than that observed for the inhibition of primary PFC response ($\alpha = 0.139$). For AM (Fig. 2B), a similar dose-response curve was obtained ($\alpha = 0.149$) and the doses reducing by 50% the number of colony forming units were estimated to be 4.8 mg/kg for AM and 21 mg/kg for AD 32.

The kinetics of the effect on bone marrow was followed after administration of 120 mg/kg AD 32, i.e., a dose previously shown [4] to be optimal in various mouse tumor systems

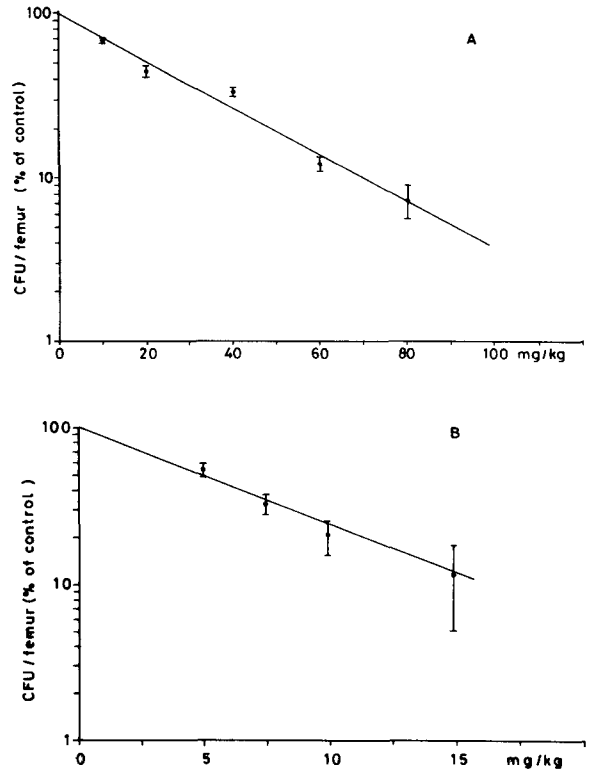

Fig. 2. *Dose-response curve of AD* 32 (*A*) *and AM* (*B*) *on the survival of hemopoietic stem-cells. Drug was given to cell donor animals* 24 hr *before sacrifice.*

when employed as a single treatment, and of 15 mg/kg AM, i.e., the highest single dose of this compound not followed by excessive toxicity ($\leqq$ 20% early deaths).

In these experimental conditions maximal depression was reached 24–48 hr after treatment, with CFU values which were 5–7% of controls for AD 32 and AM respectively; recovery was relatively rapid thereafter and CFU counts in control range were detectable 10–12 days after injection with both drugs (Table 7).

Table 7. *Time kinetics of the bone marrow CFU depression induced in* $B6D2F_1$ *mice by a single dose of AM or AD* 32*

Day after drug treatment	CFU/femur (per cent of control) AM	AD 32
0.25	N.T.†	13.2±2.6
1	7.3±2.4	5.3±3.0
2	5.2±1.9	3.0±1.5
4	26.9±3.7	10.2±4.4
7	54.6±1.5	70.0±5.0
11	82.7±7.2	92.0±8.9

*Donor mice were given 15 mg/kg of AM or 120 mg/kg of AD 32 i.v. at different times before sacrifice.

†N.T. = Not tested.

DISCUSSION

The results obtained in the present study show that AD 32, a novel AM analog with better antineoplastic effectiveness in animals [3–5], possesses a definite immunodepressive activity in mice. This conclusion is easily reached for humoral responsiveness in view of the findings that single injections with this agent at doses having modest antitumoral effect could profoundly reduce the response to both T-independent and T–B lymphocyte dependent stimuli, such as SIII and SRBC, respectively. The inhibition of these humoral reactivities by AD 32 is analogous in a number of respects (e.g., optimal activity after antigenic challenge, exponential dose-response curve, lower sensitivity of the SIII response) with that previously observed for AM [10], the most notable difference being the higher activity of the latter compound on a mg/kg basis.

The marked depression of antilymphoma allograft resistance, a predominantly cell-mediated reactivity [22], seen after AD 32 would indicate that this agent can also interfere with cellular immunity. Recent data [23] on the effect of antitumoral drugs on natural killer (NK) cells, a lymphoid cell population that has been suggested to represent a surveillance mechanism in the control of tumor growth [24], showed that antitumorally effective AD 32 doses decrease the NK activity. However, that cell-mediated immune reactivities may be more resistant than humoral responses to AD 32 inhibition can be suggested both by the inability of otherwise immunodepressive single drug doses to delay the rejection of H-2 incompatible skin graft, a result which parallels previous observations with other chemical immunodepressants [25, 26], and by the findings obtained in immunodepressed tumor-bearing hosts. Cellular mechanisms have been shown to play the major role in the complex immunoreactivity against tumors [22]. The fact that the antineoplastic effectiveness of AD 32 was reduced in previously immunodepressed mice indicates that host reactivity against the neoplasm is not totally abrogated by antitumorally effective drug doses and can thus synergize with the direct cytocidal effect of the compound.

Experimental evidence is available [12, 20, 27] in favour of the conclusion that a different qualitative and quantitative interaction with host defence is a determinant factor in giving AM its higher *in vivo* antineoplastic activity compared to its analog DM. The superiority of AD 32 over AM previously described and best seen when repeated rather than single treatments are employed [4, 5] could in principle have been attributed to a lower overall immunodepressive potency of AD 32. The complex of these results showing that AD 32 is more immunodepressant in a number of systems than AM at doses displaying comparable antitumoral activity would not, however, seem to support this hypothesis. Rather, the observation that AD 32 was more effective than AM in experimental tumors of low (e.g., 3LL carcinoma), or undetectable immunogenicity (e.g., L1210 Cr leukemia) [4], suggests that this chemical and/or its metabolites possesses a greater direct cell killing capacity than AM.

When single treatments are employed, AD 32 is administered at doses 8–10 times higher than optimal AM doses and in these conditions AD 32 is significantly more effective on various neoplasms [4, 5]. In comparing the effect of these drugs on bone marrow stem cells, a limiting cell population with cytotoxic antitumorals, a 8:1 dose ratio was thus used. In these conditions, not only the nadir in CFU values, but also recovery times from depression were comparable for both drugs suggesting a similar hematotoxic capacity for these anthracyclines. These findings are thus in agreement with those of Parker *et al.* [5] who showed that similar reductions in DNA synthesis in the bone marrow of tumor-bearing mice were induced by AD 32 doses 10 times greater than AM. Therefore the higher antineoplasic activity of AD 32 does not seem to be obtained at the expense of a substantially greater toxicity for the hematopoietic system than for AM. The basis for the reported lower toxicity of AD 32 thus remains to be elucidated, although a different cardiotoxicity [3, 5] could be an important factor.

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