A Characterization of the Immunosuppressive Activity of Adriamycin and Daunomycin on Humoral Antibody Production and Tumor Allograft Rejection

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SUMMARY

The effects of adriamycin (AM) and its analog daunomycin (DM) on immunological responsiveness have been investigated in an effort to elucidate whether a differential interaction of the two drugs with the immune system could play a role in the higher antineoplastic activity of AM. It was found that AM induced a greater reduction in the number of antibody-producing cells after primary stimulation with sheep erythrocytes, whereas DM was more suppressive on the secondary response to the same antigen. Primary reactivity to the T-independent antigen S-Ill was reduced by AM, whereas DM was ineffective in the same conditions even at high doses. In addition, when a tumor allograft model was investigated, DM was significantly more immunosuppressive than was AM administered at equitoxic doses.

In contrast, these agents displayed similar activity in reducing bone marrow stem cells and in inhibiting DNA synthesis in this organ. The possibility that the different immunosuppressive capacity of AM and DM contributes to the greater antitumoral activity of the former is advanced.

INTRODUCTION

AM4 is an antineoplastic agent on which much interest is currently focused in view of its high activity in a wide range of human cancers (6). In several experimental tumor systems (9, 33, 39) and also in clinical neoplasms (28, 29, 41), AM has been shown to have greater therapeutic value than does its analog DM, from which AM differs only in the substituion of a hydroxyl group for a hydrogen in the acetyl moiety of DM. The reasons for the superior effectiveness of AM are still unclear. The biochemical mode of cytotoxic action is, in fact, generally believed to be similar for both drugs (27, 32), although differences in the extent of nucleic acid and protein synthesis inhibition have occasionally but contrastingly been observed in some systems (36, 40). In vitro, AM has been found to be inferior (23, 27, 31, 40) or equivalent (35) to DM in terms of reduction in mitotic and proliferative activities. A longer persistence of AM in tumor cells has been observed (36); however, other investigators (34, 40) have described opposite findings. In any case, this difference was present only with single injections, whereas after repeated doses the uptake of both antibiotics was reported to be similar (36). Differences in biotransformation and pharmacokinetics between the 2 analogs are also known, AM possibly being metabolized less than DM, at least in animals (1, 14, 42), and accumulating in tissues more than its analog (14, 42), although species differences may occur in this regard (2). Both these mechanisms possibly play a role in the different therapeutic activity of the 2 drugs.

In principle, a different qualitative and/or quantitative interaction of these drugs with the immune responsiveness of tumor-bearing hosts could also contribute to the differences in in vivo efficacy of AM and DM. This contention seemed plausible on the basis of the observation (34) that the greater antitumoral activity of AM was not detectable in previously immunosuppressed animals. In order to substantiate this possibility, a detailed comparative analysis of the effects of AM and DM on various types of immune reactivity was therefore undertaken.

In this report, the results obtained in evaluating primary and secondary humoral antibody production and tumor allograft rejection after administration of AM or DM are presented together with data on their effects on bone marrow stem cell numbers and DNA synthesis in this organ.

From the complex of these results it will be shown that AM and DM exhibit quantitative and qualitative differences in their immunodepressant activity, and evidence will be presented favoring the possibility that such differences can be an important factor in accounting for the superior therapeutic activity of AM over DM.

MATERIALS AND METHODS

Animals. C3H (H-2Kb) female mice (23 ± 1 g body weight), obtained from Charles River, Calco, Italy, were used for all experiments.

Drugs. AM and DM were supplied by Farmitalia, Milan, Italy. They were freshly dissolved in 0.9% NaCl solution and injected i.v.

Tumor. The L1210 leukemia was maintained by weekly i.p. passages in compatible DBA/2 (H-2b); after removal of...
erythrocytes by osmotic lysis, leukemia cell viability was assessed by trypan blue exclusion and 10^6 or 10^7 tumor cells were injected i.p. into histoincompatible C3H hosts. The number of leukemia cells in the peritoneal cavity was determined as previously described (25).

Response to SRBC. Mice were given i.p. injections of 4 × 10^6 SRBC on Day 0 and spleen hemolytic PFC were counted by the technique of Jerne and Nordin (22); unless otherwise stated, the response was assessed on Day 4.

For secondary response, a 2nd equal challenge was administered 10 days after primary immunization and the number of direct and indirect PFC was evaluated on Day 4 using the modification by Cunningham and Szenberg (13) of the technique of Jerne and Nordin. Goat antiserum to mouse γ-globulin (Hyland Laboratories, Los Angeles, Calif.) at a final dilution of 1:200 was used. Data are presented as geometric means (in parentheses ± S.E.) after logarithmic transformation as described by Dresser and Wortis (17). At least 5 mice/group were used in these studies.

Response to S-III. S-III was kindly supplied by Dr. P. J. Baker (National Institute of Allergy and Infectious Diseases, Bethesda, Md.); mice were given a single i.p. injection of an optimally immunogenic dose (0.5 μg) of S-III in 0.5 ml of 0.9% NaCl solution. Splenic PFC specific for S-III were detected by the technique of Cunningham and Szenberg (13); SRBC sensitized with S-III by the chromium chloride procedure (5) were used as indicator cells and the response was assessed on Day 5.

Assay of CFU. The classical approach of Till and McCulloch was followed. Bone marrow cells from normal or treated donor mice were obtained by repeated flushing of femur shafts with ice-cold Hanks’ balanced salt solution; cell viability was checked microscopically by dye exclusion; and 2 × 10^4 cells were injected i.v. into previously supralethally irradiated (800 rads of X-ray) syngeneic recipients. The animals were then sacrificed 8 days later, their spleens were fixed in Bouin’s solution, and the number of colonies of the technique of Jerne and Nordin. Goat antiserum to mouse γ-globulin (Hyland Laboratories, Los Angeles, Calif.) was injected 2 days after antigen inoculation and when the finding (results not reported) that PFC counts on Days 6 and 7 were still lower than those measured on Day 4. Chart 2 shows the dose-response curves obtained when AM or DM was injected 2 days after antigen inoculation and when the response was evaluated (on Day 4). It can be seen that, over

### Table 1

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Drug</th>
<th>PFC/spleen</th>
<th>% controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>–2 AM 35,820^a</td>
<td>(33,205-44,130)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>–2 DM 23,784^b</td>
<td>(19,629-29,157)</td>
<td>66.49</td>
<td></td>
</tr>
<tr>
<td>0 AM 28,279^b</td>
<td>(24,787-32,238)</td>
<td>79.95</td>
<td></td>
</tr>
<tr>
<td>0 DM 11,849^b</td>
<td>(10,710-13,110)</td>
<td>33.08</td>
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<tr>
<td>+1 AM 17,301^b</td>
<td>(14,435-20,724)</td>
<td>48.3</td>
<td></td>
</tr>
<tr>
<td>+1 DM 7,199^b</td>
<td>(6,161-7,629)</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>+2 AM 11,831^c</td>
<td>(8,668-16,405)</td>
<td>33.31</td>
<td></td>
</tr>
<tr>
<td>+2 DM 5,587^c</td>
<td>(4,334-7,235)</td>
<td>15.6</td>
<td></td>
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<tr>
<td>+2 AM 8,238^d</td>
<td>(6,447-10,566)</td>
<td>23.0</td>
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</tbody>
</table>

* Geometric mean. Numbers in parentheses, ± S.E.
* p < 0.05 versus controls.
* p < 0.05 versus AM.

### RESULTS

**Effects of AM and DM on Primary and Secondary Immune Response to SRBC.** The timing of AM or DM injection relative to antigen inoculation giving the highest immunosuppression was investigated first. As shown in Table 1, no decrease in PFC was seen when single i.v. doses of these agents, 5 mg/kg, were injected before antigen, whereas a significant immunosuppressive activity was observed when this dose was administered on the same day of immunization. The highest PFC reductions were found, however, with treatments given after SRBC; injections on Day 1 or 2 gave equivalent results in this respect. With higher doses (15 mg/kg) a significant immunodepression was seen also when the drugs were administered before antigenic stimulation (Chart 1), although treatments given on the same day or after the SRBC inoculum were still more effective. In fact, injections of this drug dose on Day 2 gave PFC values that were reduced to 0.2 and 1.5% of controls with AM and DM, respectively. That AM and DM do indeed depress primary response and not merely delay its peak was indicated by the finding (results not reported) that PFC counts on Days 6 and 7 were still lower than those measured on Day 4. Chart 2 shows the dose-response curves obtained when AM or DM was injected 2 days after antigen inoculation and when the response was evaluated (on Day 4). It can be seen that, over

**DNA Synthesis in the Bone Marrow.** At time zero and at various times as indicated after drug injection, 4 mice/time point received i.p. 100 μCi of [3H]Tdr (The Radiochemical Centre, Amersham, England; specific activity, 16 Ci/m mole) and were sacrificed after a fixed interval of 1 hr. Bone marrow cells were expressed from both femurs by repeated flushing with Hank’s balanced salt solution; 1,000,000 cells constituted a sample and 3 samples/mouse were counted. Cell processing for radioactivity counting in a Packard Tri-Carb liquid scintillation spectrometer was performed by the filter well technique as described by Coutinho and Möller (12). Results presented are representative of 3 such experiments.

**Statistical Analysis.** Statistical significance was assessed by Duncan’s test (18). Dose-response curves were obtained by a nonlinear least-squares method using a BMD X 85 program (15). Curve parameters were compared by t test using the method of Bozeman et al. (7).
the dose range examined, both compounds gave curves best fitting an exponential and that AM was significantly more active than DM in reducing peak PFC's in the spleen. The calculated \( b \) values were in effect 3.41 (3.11 to 3.72) and 2.43 (2.15 to 2.71) for the AM and DM curves, respectively (\( p < 0.01 \)), and the doses reducing PFC numbers to 50% of controls were estimated to correspond to 2.02 and 2.85 mg/kg for AM and DM, respectively.

When the ability of these agents to impair secondary responsiveness to the same antigen was investigated, both drugs showed a remarkable activity but, at variance with what was observed for the primary response, DM was significantly more active than AM in suppressing the predominantly indirect PFC (Table 2). Qualitatively and quantitatively similar data were obtained when the drugs were injected 48 hr after boosting.

**Effect on Primary Response to S-Ill.** In order to ascertain whether these agents could also affect the immune response to a thymus-independent antigen, AM and DM were injected into mice stimulated with S-Ill, which does not require T-B lymphocyte cooperation in the induction of a primary response (20). After administration of AM, 5 mg/kg, significant reductions in peak anti-S-Ill PFC also were observed with injections 24 hr before antigenic stimulation although posttreatments were more effective, a finding analogous to what was previously found with SRBC (Table 3). On the other hand, no significant PFC reductions were seen with an equal DM dose. As shown in the same table, a dose-dependent effect was observed with AM, whereas no significant activity was detected with DM even at doses highly effective on the SRBC response. In comparison with the anti-SRBC response, antibody production to S-Ill was relatively less sensitive to interference by AM; a 5-mg/kg dose of this drug reduced primary anti-SRBC plaques by approximately 85%, whereas with the latter antigen the decrease from control values was in the 65% range.

**Effect of Allogeneic Tumor Rejection.** Table 4 shows that, when normal C3H mice were transplanted with 10⁶ cells of the allogeneic, H-2-incompatible L1210 leukemia, all animals were capable of rejecting this tumor challenge and surviving indefinitely. If the mice were pretreated 24 hr before leukemia inoculum with AM or DM, 7.5 or 15 mg/kg, progressive tumor growth was observed in all recipients; however, with both drug dosages, death occurred significantly earlier in mice given injections of DM than in those given equal doses of AM. Direct measurement of leukemia cell numbers in the peritoneal cavity (Chart 3) revealed that tumor growth was significantly faster in DM-pretreated hosts than in their AM-pretreated counterparts, a value of 110 x 10⁶ leukemia cells being reached in the former group on Day 4 and only on Day 6 in the latter. Pretreatment of the mice with AM or DM at a dose of 15 mg/kg was not followed by lethal takes when the allogeneic tumor inoculum was lowered to 10⁶ L1210 cells; however, measurement of the

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg i.v.)</th>
<th>PFC/spleen indirect</th>
<th>% controls</th>
</tr>
</thead>
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<tr>
<td>AM</td>
<td>7.5</td>
<td>7617* (6284-9255)</td>
<td>100</td>
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<tr>
<td>AM</td>
<td>15</td>
<td>1561* (1102-1832)</td>
<td>20.5</td>
</tr>
<tr>
<td>DM</td>
<td>7.5</td>
<td>436* (192-995)</td>
<td>4.9</td>
</tr>
<tr>
<td>DM</td>
<td>15</td>
<td>122* (95-205)</td>
<td>1.6</td>
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* Geometric mean. Numbers in parentheses, ± S.E.

### Table 3

<table>
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<tr>
<th>Day of treatment</th>
<th>Drug</th>
<th>mg/kg</th>
<th>PFC/spleen</th>
<th>% controls</th>
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</thead>
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<tr>
<td></td>
<td>AM</td>
<td>5</td>
<td>1963</td>
<td>50</td>
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<tr>
<td></td>
<td>AM</td>
<td>5</td>
<td>1785* (1570-2043)</td>
<td>45.7</td>
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<tr>
<td></td>
<td>AM</td>
<td>5</td>
<td>1172 (1062-1422)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>AM</td>
<td>10</td>
<td>527* (412-607)</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>5</td>
<td>3229</td>
<td>82.6</td>
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<td></td>
<td>DM</td>
<td>10</td>
<td>4269 (3566-4691)</td>
<td>109</td>
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<tr>
<td></td>
<td>+2 DM</td>
<td>5</td>
<td>2851 (2738-3417)</td>
<td>73</td>
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<tr>
<td></td>
<td>+2 DM</td>
<td>10</td>
<td>4448 (3625-5157)</td>
<td>113.9</td>
</tr>
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</table>

* Mean ± S.E.

### Table 4

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Drug dose (mg/kg i.v.)</th>
<th>Size of tumor inoculum</th>
<th>Mean survival time (days)</th>
<th>D/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10⁶</td>
<td>&gt;90</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>15 10⁶</td>
<td>13.0 ± 1.5⁰</td>
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<td>DM</td>
<td>15 10⁶</td>
<td>8.2 ± 1.2⁰</td>
<td>10/10</td>
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<tr>
<td>AM</td>
<td>7.5 10⁶</td>
<td>13.5 ± 1.5⁰</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>7.5 10⁶</td>
<td>8.5 ± 1.1⁰</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10⁶</td>
<td>&gt;90</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>15 10⁶</td>
<td>&gt;90</td>
<td>0/10</td>
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</tr>
<tr>
<td>DM</td>
<td>15 10⁶</td>
<td>&gt;90</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E.

### Notes

- *p < 0.05 versus controls.
- *p < 0.01 versus controls.
- *p < 0.01 versus AM.
leukemia cell numbers in the peritoneal cavity also revealed that in this experimental condition substantial differences existed between AM and DM. In fact, in DM-pretreated mice the leukemia cells underwent a progressive expansion beginning on Day 4, reaching a peak of approximately $190 \times 10^6$ cells on Day 8 and abruptly declining thereafter so that neoplastic cells were no longer detectable by Day 10 (Chart 3). In contrast, tumor growth was abortive in the AM-pretreated mice and only $20 \times 10^6$ cells were counted on Day 8. Results similar to those presented were obtained also when the interval between drug treatment and tumor transplantation was 48 hr. In addition, the administration of AM and DM (15 mg/kg) 24 hr before L1210 inoculation to compatible DBA/2 or to X-irradiated (400 R) C3H mice did not result in any significant prolongation in survival over that of untreated control animals. Since the L1210 leukemia is sensitive to both these antibiotics, these findings permit exclusion of the possibility that residual circulating drug present at the time of tumor transplantation might have influenced the results.

Effect on Hematopoietic CFU. Chart 4 shows the dose-response curves obtained when AM or DM were administered to bone marrow cell donors 24 hr before sacrifice. Under these conditions, bone marrow stem cells showed the same sensitivity to both agents, the dose that was effective for 50% of this cell population being 6.1 and 6.3 mg/kg for AM and DM, respectively. By way of comparison, the calculated doses effective for 50% of the cells for primary anti-SRBC PFC were 2 and 2.8 mg/kg and the acute doses lethal to 50% of the donors in this strain were 31 and 30.5 mg/kg for AM and DM, respectively. In other experiments (Chart 5), it was observed that the number of CFU was lowest 24 hr after drug treatment; values were unchanged on Day 4 and thereafter a tendency towards a return to normal values was detected, with levels of approximately 50% of controls reached on Day 7 and a total recovery found 11 days after an AM or DM dose of 15 mg/kg.

With respect to DNA synthesis in the bone marrow, a very rapid decline in $[^{3}H]$TdR uptake was observed after injection of AM, 15 mg/kg, with a value of approximately 50% of controls 4 hr after treatment and a nadir (41% of control level) at the 14th hr (Chart 6). An increase in isotope uptake was then observed, control values being reached 2 days after injection followed by an overshoot in DNA synthesis to a level of 465% of control on the 4th day. Radioactivity per million bone marrow cells then tended to return to normal, although by the 6th day the counts were still above the baseline. The pattern observed after an equal dose of DM was very similar, the greatest inhibition in $[^{3}H]$TdR uptake (35% of control) also being detectable at the 14th hr. At later times the kinetics of recovery in DNA synthesis was somewhat faster than that seen with AM and the highest value in isotope uptake in the overshoot period was also somewhat lower (370%).

**DISCUSSION**

Our results show that AM and DM have a complex differential capacity to interfere with immune responsiveness; in
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Fact, AM was found to exhibit a greater capacity than its analog DM in depressing a primary response to SRBC and to S-III, whereas DM was significantly more potent in reducing a secondary response to foreign erythrocytes and in depressing allograft reactivity. In contrast, a similar quantitative and qualitative activity was displayed by these agents in affecting hematopoietic stem cells and DNA synthesis in the bone marrow. In showing that these antibiotics possess a strong immunodepressive activity, our findings are in general agreement with other reports (9, 10, 21, 38) using various experimental stimuli; however, past studies were mostly confined to investigations of drug alone and/or on single types of immune reactivities; thus a direct quantitative comparison of the effects of AM and DM on a series of immune parameters was not previously available. On the basis of these data a judgment on the relative immunosuppressive potency of the 2 analogs would in principle appear difficult, since such a rating would depend on the type of immune response considered.

However, in drawing conclusions concerning the possible importance of a differential impairment of host antitumoral defenses in giving AM a better antineoplastic activity over DM, it seems justified to attribute greater importance to the finding that AM was significantly less depressive of tumor allograft resistance.

In another report, evidence linking this finding to a less severe reduction by AM of cellular cytotoxicity and serum potentiating activity will be presented (26). It is generally believed that the mechanisms mediating the response to syngeneic neoplasms are operative also for allogeneic tumors (11). Although other mechanism(s) such as differences in pharmacokinetics, metabolism, and/or tissue distribution may all be of relevance, the relatively lower impairment of antitumoral immune reactivity given by AM can thus reasonably be considered as a factor of great importance in explaining the higher antineoplastic effectiveness of this agent as originally suggested by Schwartz and Grindey (34). Also supporting this view is the finding that AM was more effective than DM in retarding the growth of an antigenic Moloney virus-induced tumor, whereas no differences with DM were detectable with a nonimmunogenic variant (19). The mechanism(s) underlying the capacity of AM and DM to affect differentially the various immune reactivities investigated remain at this time a matter of speculation. Since DM displayed a greater activity on allograft rejection, which is considered a T-cell dependent reactivity (11), whereas it did not impair the responsiveness to a T-independent stimulus such as S-III (4), the hypothesis could thus be advanced that DM acts with a relative preference on T-lymphocytes. This view could also account for the greater reduction by this drug of secondary (indirect) PFC in contrast to the action of AM, which was more depressive on the primary response to SRBC, an antigen requiring T-B cell cooperation; it has in fact been shown (8, 16) that IgG antibody formation to this stimulus is more T-dependent than is IgM production. An interpretation of these results on pharmacokinetic grounds could also be considered, based on the faster accumulation of DM in the spleen (34), whereas AM reaches lower peak levels but persists for significantly longer times. On the other hand, as discussed in detail elsewhere (24), after a single DM dose the decrease in spleen cellularity is significantly faster than that seen with AM, possibly a reflection of the faster and higher accumulation of DM in this organ, whereas AM persists for longer times. On this basis, the higher activity of DM on allograft resistance and on secondary PFC could easily be explained; however, to account for the greater depression of primary PFC by AM, it may be argued that the longer persisting drug levels resulted in an additional, greater killing of replicating PFC progenitors. It has been shown in fact that these agents are cytoxically more effective on proliferating than on nondividing cells (23). In this framework, however, the lack of impairment of the S-III response by DM would require that the greater cell destruction caused by this drug also involve T-suppressor lymphocytes, which are known to operate in the S-III system (3). In the absence of more data, these considerations are still hypothetical.

In view of the differences observed in the activity of AM and DM on the immune system, it was of interest to examine whether similar differences also applied to the hematopoietic system, a critical and drug-limiting cell population in treatment with these drugs (28). The effect of both these antibiotics on normal hematopoietic stem cells has been investigated previously by Razek et al. (30) in AKR mice; they reported that AM was approximately 2.5 to 3 times less inhibitory than was DM. In contrast our findings showed no significant differences between the 2 drugs in the degree of maximal CFU reduction or in the kinetics of the effect as was also evidenced by the similar pattern of the changes in DNA synthesis in the bone marrow after injection of either drug. The reasons for this discrepancy in results are not readily apparent and may reside in the somewhat different experimental conditions used.

REFERENCES


M. G. Donelli and A. Martini, manuscript in preparation.


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