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The Effect of Dead Cells on the Activity of Actinomycin D against Mouse Sarcoma 180 Ascites[1]

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SUMMARY

It has been demonstrated that cells killed by heat or irradiation have four times greater affinity for actinomycin D (AMD) than do viable tumor cells. By using a double labeling technique, we were able to show that, with increasing amounts of AMD bound in cells, the incorporation of RNA precursors is proportionally decreased.

However, in the presence of nonviable cells or of native DNA, the AMD-induced inhibition of [^{3}H]uridine incorporation is markedly reduced. This reduction does not occur if DNase is added to the system.

The accumulation of dead cells in the tumor vicinity during the natural course of tumor growth or therapy must be taken into consideration in planning therapeutic regimens. We suggest that, in combined chemo- and radiotherapy, increased effectiveness of AMD may be obtained by its use prior to irradiation, thereby assuring its direct access to the tumor cells. The addition of DNase could eliminate or greatly diminish the dead cell competition for the drug.

INTRODUCTION

This study was designed to evaluate the significance of dead cells in a tumor environment, and, specifically, of their influence on the effectiveness of AMD[3] in cancer chemotherapy.

The accumulation of dead cells in tumor-bearing animals may result from destruction of normal cells by the invading tumor, from cell death within the tumor itself, or from lethal effects of radio- or chemotherapy.

The molecular mechanism involved in the ability of AMD to affect the growth of neoplastic cells is related to the property of this compound to bind to double-stranded DNA (3, 6), thereby blocking RNA synthesis (4, 5, 8) and resulting in the arrest of cell division. In comparing the abilities of isolated DNA and DNA in intact cells to bind AMD, Bolund (2) found the efficacy of isolated DNA to be 20 times greater. Other authors have observed that dead cells absorb more AMD than do their living counterparts (2, 10). This observation, which we were able to confirm in our laboratory, led us to a hypothesis that nonviable cells may be competing with the DNA in viable cells for the uptake of AMD. The gradual accumulation of dead cells in the tumor environment would thus suggest an additional explanation of the progressively diminishing effect of AMD observed in the course of therapy.

Since the arrest of cell growth by AMD is expressed by the inhibition of RNA synthesis, we have used this parameter as a gauge of AMD activity. Comparative studies were made on AMD acting directly on live tumor cells, on cells suspended in an environment of killed cells, and on cells in the presence of DNA in different physical states.

MATERIALS AND METHODS

Mouse Sarcoma 180 ascites cells were maintained by weekly transfers into the peritoneal cavity of Charles River mice. Seven days after inoculation, the mice were killed and the cells were suspended in 20 volumes of cold 0.14 M NaCl solution and centrifuged. The sedimented cells were resuspended in 20 volumes of cold Eagle's medium containing 20% fetal bovine serum (Microbiological Associates, Bethesda, Md.).

To estimate the binding of AMD by living and dead cells, a series of samples consisting of 2×10^7 cells suspended in 2 ml of Eagle's medium were incubated for different periods of time at 37° with a constant amount (725,000 cpm/20 μl) of [^{3}H]AMD (specific activity, 6.5 Ci/mmole). (The labeled compounds were purchased from Schwarz/Mann, Orangeburg, N. Y.). The samples were washed with 10 ml of medium with unlabeled AMD (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 μg/ml and the cells were then centrifuged. This procedure was repeated 4 times. After the final washing, the sedimented cells were suspended in 2 ml 0.14 M NaCl solution, 0.2 ml of which was placed in scintillation vials, mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.), and counted in a Packard Tri-Carb scintillation counter.

The binding of radioactive AMD to the cells and the inhibition of the incorporation of labeled RNA precursors were determined by a double-labeling technique. Samples containing 2×10^7 cells in 2 ml of Eagle's medium were preincubated for 10 min at 37° with [^{3}H]AMD. Four μCi of [^{14}C]adenine (specific activity, 52 mCi/mmole) in 20 μl of water were then added to the samples, and incubation was continued for 1 hr. A 0.5-ml aliquot of each sample was then assayed for binding capacity of AMD by the technique described above. The RNA was extracted from the remaining cells according to the method of Mendecki *et al.* (7), and

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[3] The abbreviation used is: AMD, actinomycin D.

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the incorporation of [^{14}C]adenine into RNA was estimated.

In all other experiments, [^{3}H]uridine was used (specific activity, 8.0 Ci/mmole) in place of [^{14}C]adenine.

The influence of dead cells on the effectiveness of AMD was assessed in samples containing a suspension of 2 ml of living cells (2×10^7) and 4 ml of dead cells (4×10^7). AMD, 0.25 μg, was then added for each ml cell suspension, and a 10-min preincubation was carried out, followed by the addition of radioactive RNA precursor (6.25 μCi [^{3}H]uridine per ml sample) and by incubation at 37° for 1 hr. The RNA was extracted, and the radioactivity was determined.

Dead cells were prepared by exposing Sarcoma 180 cells to 15,000 rads from a ^{60}Co source or to heat (65° for ten min). The killed cells remained morphologically indistinguishable from viable cells but were metabolically inactive (Table 2, A) and stained easily with trypan blue.

The effect of native, denatured, or degraded DNA on the inhibition of RNA synthesis by AMD in living cells was determined by adding 0.2 ml (2 mg/ml) of a DNA solution (Type I; Sigma) to 2 ml of the cell suspension. After 5 min at 37°, 0.612 μg/ml of cell suspension of nonradioactive AMD was added for 10 min. The cells were then exposed to [^{3}H]uridine (6.25 μCi/ml sample) for 1 hr, and the RNA was extracted.

Denaturation of DNA (2 mg/ml) was accomplished in 0.1 M NaCl solution by heating at 100° for 10 min, followed by rapid cooling. The DNA (2 mg/ml) was degraded by incubation with 50 μl (1 mg/ml) of bovine pancreatic DNase (Sigma) under optimal conditions of pH and ionic strength for 20 min at 37°.

Following the previously described technique, we examined the effect of AMD in the presence of DNase on RNA synthesis in tumor cells. The model utilized consisted again of samples of living cells to which were added either dead cells or native DNA. The samples were submitted to 50 μl of DNase for 20 min at 37° prior to the addition of AMD (0.25 μg/ml cell suspension). Ten min later, [^{3}H]uridine was added and the samples were incubated for 1 hr.

All samples contained an equal number of cells/unit of medium (1×10^7 living or dead cells/ml of medium). The ratio of added radioactive precursors to the volume of medium was kept constant in all sets of samples. Adequate controls were prepared for each experiment.

RNA extraction was carried out according to the procedure previously described (7). The RNA was precipitated in 200-μl aliquots with 10% trichloroacetic acid and 1 drop of yeast RNA as a carrier. The precipitate was filtered through glass filters and washed with 0.1% trichloroacetic acid. The filters were dried, placed in 10 ml of toluene scintillation mixture, and counted in a Packard Tri-Carb counter.

The results presented are the averages of 3 experiments performed with duplicate samples.

RESULTS

Binding of AMD to Living and Dead Cells. Table 1 shows that maximal binding of [^{3}H]AMD to both living and dead cells was achieved within 5 min and remained constant for the 60-min observation period. Four times as much AMD was bound to cells killed by irradiation or by exposure to 65° than to the living cells.

Our results were similar to those obtained by Bolund (2), who found that AMD uptake by dead HeLa cells was approximately 5 times that of living cells, and our results are in substantial agreement with other reports that heat-killed lymphocytes bound 40 times more AMD than did living cells (10).

Effect of AMD on Incorporation of Labeled RNA Precursors. In a double-labeling experiment using [^{3}H]AMD and [^{14}C]adenine, it was demonstrated that, with increasing amounts of [^{3}H]AMD in the incubation medium, the amount of [^{14}C]adenine incorporated into RNA extracted from the cells decreases as the binding of [^{3}H]AMD to the living cells increases (Chart 1). In experiments where cold AMD was used, the inhibitory effect on the incorporation of [^{3}H]uridine into RNA was found to be identical to that with [^{14}C]adenine.

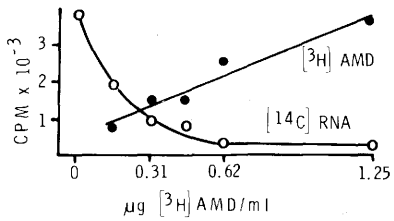

Chart 1. The effect of [^{3}H]AMD binding to living cells on [^{14}C]adenine incorporation into RNA.

The finding (Table 1) that dead cells bind more AMD than do living cells suggested an investigation of the possible effect of dead cells on the uptake of AMD by living cells. This appeared to be of particular interest since, in tumor tissue, *in vivo* living and dead cells exist side by side.

Table 1

[^{3}H]AMD binding to living and dead cells

[^{3}H]AMD incubation with	cpm after		
	5 min	30 min	60 min
Living cells	1206	1018	1350
Dead cells	4201	4351	4182

Our *in vitro* model consisted of a mixture of living and dead cells. Since the 2 groups of cells could not be easily separated from each other, the amount of AMD bound to the living or dead cells could not be separately determined. The AMD uptake by the living cells was therefore indirectly estimated, using as a gauge the incorporation of a radioactive precursor into the RNA.

It was found that 0.5 μg AMD added to 2×10^7 living cells in 2 ml of medium inhibited the uptake of [^{3}H]uridine by 57%. In the presence of dead cells, the same concentration of AMD inhibited the uptake of [^{3}H]uridine by only 15% (Table 2, A).

In order to establish whether these results were due to the preferential binding of AMD by the DNA of the dead cells, a series of experiments was performed, substituting for the dead cells DNA in a native, denatured, or degraded state. An equivalent amount of each form of DNA was added to

Table 2

The effect of dead cells and DNA on the inhibition of RNA synthesis by AMD

Samples of 2×10^7 of living cells and (A) 4×10^7 dead cells, or (B) 0.4 mg native, denatured, or degraded DNA, or (C) dead cells or DNA plus DNase (50 μg) were preincubated for 10 min with AMD (μg/ml sample) and then for 1 hr with [^{3}H]uridine (6.25 μCi/ml sample). The RNA was extracted, and the radioactivity was determined. Appropriate controls were used in each group. The results are the averages of 3 experiments performed with duplicate samples.

	Living cells	Dead cells	DNA	DNase	AMD (μg)	RNA radioactivity cpm	%
A							
1	+					4532	100
2	+				0.25	1949	43
3	+	+				4441	98
4	+	+			0.25	3852	85
5		+				227	5
B							
1	+					4012	100
2	+				0.612	120	3
3	+		Native		0.612	1926	48
4	+		Denatured		0.612	602	15
5	+		Degraded		0.612	201	5
6	+		Native			3972	99
C							
1	+		Native	+		5852	100
2	+		Native		0.25	3336	57
3	+		Native	+	0.25	819	14
4	+	+		+		5911	101
5	+	+			0.25	3745	64
6	+	+		+	0.25	1580	27

the living cells, followed by an equal amount of AMD. After 10 min of preincubation, [^{3}H]uridine was added, and the incubation was continued for 1 hr at 37°. The RNA was then extracted, and its radioactivity was determined in each sample (Table 2, B).

Our results indicate that the incorporation of [^{3}H]uridine in the presence of AMD in a concentration of 1.25 μg/2 ml cell suspension was inhibited by almost 100%. The same concentration of AMD in the presence of 0.2 mg of native DNA inhibited the uridine incorporation by only 52%. In the presence of heat-denatured DNA, the same concentration of AMD still inhibited 85% of the RNA synthesis, while in the presence of degraded DNA, 95% inhibition was achieved (Table 2, B).

These results are consistent with the observation made by other authors (2, 5) that only native DNA binds AMD, while single-stranded or degraded DNA shows minimal binding capacity.

The possibility that DNase may be effectively used to eliminate the competition for AMD by dead cells or their product (DNA) in the tumor environment was explored in a separate series of experiments. Here, DNase was added to a mixture of suspended living sarcoma cells combined with either dead cells or native DNA. Following a 20-min incubation period at 37°, 0.5 μg of AMD was added to the samples. Ten min later, [^{3}H]uridine was added, and incubation was continued for 1 hr. Measurements of [^{3}H]uridine incorporation revealed conclusively that, in the presence of DNase, native DNA as well as dead cells failed to reduce the effect of AMD on RNA synthesis (Table 2, C).

DISCUSSION

Our studies are concerned with the effect of dead cells in the tumor environment on AMD therapy. They provide evidence that dead cells reduce the effectiveness of AMD as expressed by its inhibition of RNA synthesis by malignant cells.

Numerous clinical trials have established that single large doses of antineoplastic agents are more potent than are repeated small doses (1). Our results suggest the possibility that the progressive accumulation of cells killed during the course of treatment or in the process of tumor spread might be partly responsible for the diminishing effectiveness of AMD, as dead cells tend to preferentially absorb the available AMD.

In a series of experiments, we were able to demonstrate that the growth-arresting activity of AMD on cancer cells could be diminished by the addition of an excess of native DNA, while denatured or degraded DNA failed to produce such an effect (Table 2B). Conversely, with the addition of DNase to an actively treated tumor cell population, it was possible to restore the effectiveness of AMD (Table 2, C).

Several investigators have recently demonstrated that, if viable tumor cells are inoculated into mice together with lethally irradiated cells of the same tumor, the resulting tumor develops more rapidly and kills the animal sooner than if inoculated without the dead cells (9).

Other workers (11) have suggested that the dead cells might have a stimulating effect on the growth of tumor cells. On the basis of our experiments, it may be postulated that the introduction of DNase in the chemotherapeutic program may be beneficial, not only by removing the dead cell competition for the drug but also by reducing this stimulating effect.

It must be stressed that our results deal only with a small fragment of a complex problem and that further elaboration will be required in order to reach definite conclusions applicable to chemotherapy in a living organism. However, it is possible to consider some practical applications of our observations. Dead cells present in the vicinity of living cancer cells may be expected to impair the effectiveness of AMD. In planning a therapeutic program that includes the use of combined therapy (irradiation and AMD), the administration of AMD should therefore precede, and not follow, irradiation. In cases where this order must be reversed, the addition of DNase to the regimen will tend to nullify, or at least reduce, the effect of the dead cells, resulting in the destruction of exogenous DNA, and permitting the utilization of AMD by the tumor itself. One other advantage of dead cell removal would be the improved oxygenation of the tumor mass, resulting in its increased radiosensitivity.

The introduction of DNase into the chemotherapeutic regimen would naturally be contingent upon determining its freedom from adverse reactions in the living organism.

The evidence presented, which indicates that dead cells exert an inhibitory effect in cancer chemotherapy, may serve to focus attention on the need for finding an effective method for the removal of necrotic cells and of their products. Moreover, we hope that our studies will add to a better understanding of optimal combinations, dosages, time intervals, and sequences in the administration of chemotherapeutic agents and radiotherapy and that further exploration of the role of adjunct enzyme therapy will prove to be of value in enhancing the effectiveness of cancer chemotherapy.

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