Thermochemotherapy: Synergism Between Hyperthermia (42–43°) and Adriamycin (or Bleomycin) in Mammalian Cell Inactivation

(cancer chemotherapy/cell membranes)

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ABSTRACT The sensitivity of cells exposed in vitro to the antibiotics bleomycin or Adriamycin is only mildly increased at 41° over that seen at 37°. However, at 43° a marked synergism between the effects of hyperthermia and drug is observed. This synergism can also be demonstrated to occur in solid tumors in vivo. Cells after bleomycin exposure at 37° repair potentially lethal damage, and 43° inhibits this repair. This inhibition may in part account also for the observed sensitization of the cells to bleomycin, but not to Adriamycin, since for the latter no repair can be demonstrated. However, fluorescence measurements show that at 43° much more Adriamycin is able to enter the cells than at 37°. The possible implications of the results for cancer treatment are discussed.

Bleomycin (1) and Adriamycin (2) are two antibiotics currently used in cancer chemotherapy. Both agents show some degree of effectiveness against a variety of solid tumors. However, as with all other currently used anti-tumor drugs, normal tissue toxicity limits dosages to levels far below those required to sterilize most malignant lesions. Attempts to improve the efficacy of treatment by simultaneously or sequentially administering several drugs (with differing modes of cytotoxic action and nonoverlapping normal tissue toxicities) have been only moderately successful. Hence, any treatment or combination of treatments that would increase tumor cell toxicity without a concomitant increase in damage to the treatment-limiting normal tissue would be highly desirable. In this preliminary communication, we show that above 42° the cell-killing ability of these two antibiotics is markedly increased when compared to 37° and that this difference holds both in vitro and in a solid tumor system in vivo. Local heating of tumor volumes to 43° appears technically feasible. Since treatment-limiting tissue appears to be lung for bleomycin (3) and heart for Adriamycin (4), the data presented here may point the way towards greatly improved chemotherapeutic management of isolated lesions not located near those organs.

MATERIALS AND METHODS

Cells. Chinese hamster cells (HA1), free of mycoplasma, are grown in monolayers as described (5, 6). Exponentially growing cells are obtained by seeding 10⁶ cells into 60 mm plastic petri dishes. The cultures are used for experiments at a cell density of 10⁴–10⁵ cells per cm². “Unfed” cultures are seeded similarly but used on day 8; cell density is about 5 × 10⁶ cells per cm². Finally, plateau-phase cultures are obtained by changing medium daily beginning with day 5. The cultures stabilize at a density of 10 to 12 × 10⁶ cells per cm². Drug treatments (7) are carried out in cultures of the indicated cell density. Heat treatment, either with or without drug, is carried out under conditions of controlled gas flow (CO₂ plus air) in an incubator controlled to ±0.1° (8). In all experiments, cell survival is measured via Puck’s cloning technique.

Tumors. The EMT-6 mammary sarcoma is a tumor whose cells are capable of growth in vitro or in vivo (9). Tumors taken directly from mice can be converted to single cell suspensions and plated on plastic dishes to give rise to colonies with a plating efficiency usually exceeding 30%. Conversely, if 5 × 10⁶ cells grown in tissue culture are injected intradermally in the thighs of Balb/c mice, 10–12 days later 90–100% of the animals develop tumors (about 200 mg/tumor). Thus it is possible to treat the tumor in situ and then to assay the effect of any treatments rapidly and precisely in vitro. Comparison of the fraction of clonogenic cells from treated and untreated tumors permits quantitative evaluation of the treatments’ effectiveness. For heating experiments, animals with tumors in both hind legs are anesthetized (Diabutol, about 50 mg/kg), and one of the legs is put into a hot water bath (43.5°). Thermistor measurements show that within 10–15 min the tumor stabilizes at a temperature of 42.8–43.1°. The other leg is kept out of the water and used as “37°” control (actual temperature is 36.8 ± 0.3°). Bleomycin is injected intraperitoneally; Adriamycin intravenously (Hahn, Rockwell, and Gordon, in preparation).

Fluorescence Measurements. Cells in monolayers are exposed to Adriamycin for 1 hr, trypsinized, and then resuspended in a balanced salt solution at a concentration of about 10⁶ cells per ml. They are then passed through a flow microfluorometer (Cytofluorograph; Biophysics Inc.), an instrument that measures the amount of laser-excited fluorescence of the individual cells. The resultant data are sorted and stored on a multichannel analyzer.

RESULTS

Survival of HA1 Cells Exposed to Bleomycin. The survival characteristics of “unfed” cells exposed to graded doses of bleomycin are shown in Fig. 1. These cultures were treated, then trypsinized immediately and plated for the survival assay. The 37° curve is quite similar to that published by others (10, 11). There is only minor sensitization at 41°, con-
posed peratures. Exposure drug); (no 420, results in increase remarkable basis of Q, 370; Q, 43°. Error bars: ± 1 SEM. consistent with earlier findings (8). However, at 43° there is a remarkable increase in cytotoxicity; the combined treatment results in cell killing far in excess of that predicted on the basis of independent action of hyperthermia and bleomycin.

For example, exposure to 30 μg/ml of bleomycin at 37° permits 40% of the cell population to maintain their reproductive integrity. A 1 hr exposure to 43° in the absence of bleomycin reduces survival by approximately 50% (8). Hence, the predicted survival for combined exposure (in the absence of synergistic interaction) should be about 20%. In fact, the measured survival is 4 × 10^{-4}, lower by a factor of 500. Somewhat similar sensitization to bleomycin at 43° was observed for density-inhibited cultures as well as for exponentially growing cells, although these latter cultures are more resistant to bleomycin (7) and to hyperthermic shock (8).

The clonogenicity of cells exposed to bleomycin is also influenced by their ability to repair potentially lethal lesions (7, 10, 12). This repair results in higher survival, if the time between drug exposure and stimulation for cell proliferation (e.g., by trypsinization and plating or tumor excision in vivo) is delayed. If HA1 cells are placed at 43° for about 1.5 hr after

fig. 1. Survival of HA1 cells ("unfed" cultures) exposed to graded doses of bleomycin at different temperatures (exposure time: 1 hr). Surviving fraction ratio (S.F.R., ordinate) is the ratio of the fraction of cells surviving drug plus hyperthermia to the fraction surviving hyperthermia alone. The survival of cells exposed to 41° for 1 hr is about 50%; at 43° it is about 50%.

fig. 2. Survival of exponentially growing EMT-6 cells exposed to a fixed dose (0.5 μg/ml) of adriamycin at different temperatures. Exposure time is variable (abscissa). O, 41°, control (no drug); ©, 42°, control; +, 37°, adriamycin; O, 41°, adriamycin; O, 42°, adriamycin.

fig. 3. Survival of EMT-6 cells from tumor-bearing mice treated with adriamycin (1 hr). •, "37°" controls (see text); O, cells from tumors heated to 43°.

fig. 4. Fluorescence of HA1 cells exposed to adriamycin (30 μg/ml; 1 hr). •, Integrated fluorescence histograms from cells exposed to adriamycin at 37°; O, similar data from cells exposed at 43°. *Abacida*: channel number; increasing numbers correspond to increasing fluorescence/cell and hence to increasing intracellular adriamycin concentration. *Ordinate*: cumulative percentage of cells. Shaded area represents experiment-to-experiment variation of 37° curve at low channel numbers.
bleomycin exposure, they lose their ability to effect this type of repair (Braun and Hahn, in preparation).

Response of Cells to Adriamycin. Fig. 2 shows the results of experiments in which exponentially growing EMT-6 cells were exposed to a fixed dose of adriamycin for variable lengths of time at 37, 41, and 42°C and plated immediately to assay survival. The 37°C results are again consistent with those published. At 41°C there is also only modest sensitization (if any). By contrast, at 42°C a pronounced synergism is observed. Qualitatively similar sensitization at 43°C occurs for plateau-phase cultures. Repair after adriamycin is not observed (Hahn, unpublished); hence repair inhibition experiments, analogous to those performed after bleomycin exposure, were not performed.

Survival of EMT-6 Solid Tumors Exposed to Adriamycin In Vivo. A comparison of the survival of EMT-6 cells from tumor-bearing mice that were injected with graded doses of adriamycin is shown in Fig. 3. The closed symbols reflect the clonogenicity of cells from control tumors, i.e., those kept at body temperature during the 1.8 hr interval after adriamycin injection. These survival values are, therefore, a measure of the cytotoxicity of adriamycin against the EMT-6 tumor. Apparently even after massive drug doses, cell killing does not exceed 80%. Immersion of tumor-bearing legs into the hot water bath for the 1.8 hr period in the absence of adriamycin is also ineffective: about 40% of the cells survive. However, massive cell killing results if adriamycin and hyperthermia are combined, as is indicated by the open symbols of Fig. 3. At 30 mg/kg, the combined effect has reduced survival to about 1×10⁻⁴. Qualitatively similar results are obtained if bleomycin replaced adriamycin.

Intracellular Adriamycin Concentrations. In order to obtain some idea of why there should be such a synergism between adriamycin and 43°C hyperthermia, we compared the intracellular fluorescence of adriamycin (13) after 37 and 43°C exposures. Cells were exposed in vitro to the drug (30 μg/ml) as described, then immediately trypsinized, washed, and re-suspended in buffer (Hanks' balanced salt solution) at a concentration of approximately 10⁵ cells per ml. They were then passed through the cytofluorograph. Results of integrated fluorescence histograms are shown in Fig. 4. There is a marked shift to the higher channel numbers for the 43°C curves, showing that cells exposed at the higher temperature contained a higher average amount of adriamycin/cell, since fluorescence is proportional to adriamycin concentration. Furthermore, the very slow rise of this curve shows that very few if any of the 43°C cells contained no drug, while a substantial number of the 37°C cells may have contained an insufficient amount of adriamycin for the instrument to detect (shaded part of 37°C curve).

Discussion

The data presented here show a striking synergism in the cytotoxicity of 42-43°C hyperthermia combined with either bleomycin or adriamycin. It seems unlikely that such results can be explained on the basis of increased activation energies, as reported by Johnson and Pavletic (14) for the alkylating agent thio-tepa, particularly in view of the very minor sensitization at 41°C. In the case of bleomycin it is probable that at least part of the cells' sensitization at 43°C is related to inhibition of a repair mechanism. The importance of such repair in determining the clonogenicity of bleomycin-treated cells, both in vivo (7) and in vitro (12), has already been documented. For example, in an experiment involving chemotherapy of the EMT-6 tumor in vivo, cell survival 2 hr after a dose of 50 mg/kg was approximately 10⁻⁴. However, if the tumor was permitted to remain in situ for 24 hr (presumably so the non-cycling cells could complete repair), survival rose to 0.5; i.e., about 99% of the cells could deal with their potentially lethal lesions. Clearly, inhibition of repair, even if only during the time of bleomycin exposure, could appreciably modify survival. No such mechanism can be evoked to explain sensitization to adriamycin, since experiments both in vivo and in vitro show no evidence for repair (Hahn and Gordon, unpublished). However, the data of the fluorescence experiments show clearly that adriamycin gets into the HA1 cells much more easily at 43°C than at 37°C. A variety of other experiments (not detailed here) indicate that the plasma membrane may change properties during exposure to 42-43°C. In this connection the reported phase transition that some lecithins undergo in this temperature range (15) might be relevant, as the membranes contain about 30% lecithins. Thus, it is not unlikely that the permeability of the cells' membrane is sharply modified as the temperature is raised from 41 to 43°C. Alternatively, active drug exclusion mechanisms, such as those postulated by Terasima et al. (11), might be inhibited.

Whatever the mechanisms involved, the synergism of the two modalities could prove very useful in the chemotherapy of solid tumors; of particular interest is the apparent threshold in sensitization occurring above 41°C. Heating normal tissue to 41°C would only enhance cell killing slightly, but if the tumor volume could selectively be raised to 43°C, the increase in therapeutic ratio should be considerable.

There are two additional features that would make hyperthermia an attractive adjunct to chemotherapy: tumor cells, because of their poor nutritional state, may be much more sensitive to elevated temperatures even in the absence of drugs (8), and malignant cells may be inherently more sensitive to hyperthermia than their normal counterparts (16, 17). While there are difficult technical problems associated with heating specific tumor-containing tissue volumes (without excessively elevating normal tissue temperature), these are probably solvable. Certainly design of equipment to do such heating, utilizing either electromagnetic or ultrasound radiation, is well within present technology.

A final consideration might be that the data could point toward the development of a new class of chemotherapeutic agents. If there are really marked changes in membrane properties between 41 and 43°C, one could conceive of agents which at normal body temperatures would not enter the cell at all (or at least not in cytotoxic quantities) but which could penetrate the modified membrane during or after heating to 43°C. This would permit the "thermochemotherapy" of localized lesions with drugs not showing any systemic side effects.

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