METRONIDAZOLE (FLAGYL): CHARACTERIZATION AS A CYTOTOXIC DRUG SPECIFIC FOR HYPOXIC TUMOUR CELLS

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Summary.—The cytocidal properties of metronidazole against hypoxic mammalian cells are described. This chemotherapeutic action has been shown to be dependent on drug concentration and duration of exposure. The x-ray TCD50 for a murine anaplastic carcinoma was reduced from 6081 rad to 4643 rad when animals were given metronidazole orally for 36 h before radiation treatment. The effect is attributed to the direct killing of hypoxic tumour cells by a mechanism analogous to that proposed for the action of the drug on anaerobic micro-organisms. It is concluded that further work with metronidazole as a cytotoxicant specific for hypoxic cells is warranted, particularly in view of the reported lack of toxicity associated with the preliminary clinical use of the drug as a radiosensitizer in man.

Metronidazole (Flagyl, May and Baker Ltd) has been shown to be an effective radiosensitizer of several murine tumour systems in vivo (Begg, Sheldon and Foster, 1974; Stone and Withers, 1974; Rauth and Kaufman, 1975). Preliminary clinical trials using drug doses of about 200 mg/kg are now being undertaken (Urtasun et al., 1974, 1975; Deutsch et al., 1975).

The drug was originally screened as a radiosensitizer of hypoxic cells because it is an organic nitro-compound possessing extremely favourable pharmacological and toxicological properties, which had been emphasized several years earlier as important prerequisites for sensitizing activity in vivo (Emmerson and Howard-Flanders, 1965). In addition the drug is well known for its potent cytocidal action on anaerobic but not aerobic micro-organisms (McFadzean, 1971).

Subsequent experiments have indicated that the drug does have an analogous chemotherapeutic effect on hypoxic tumour cells. The rates of growth of tumours of mice given metronidazole following radiation treatment have been found to be lower, and the rates of cell loss higher, than those of animals given radiation alone (Begg et al., 1974; Inch and McCredie, 1975). The drug had also been found to kill non-cycling, mammalian cells grown in vitro as spheroids (Sutherland, 1974). It was thus apparent that prolonged continuous treatment with metronidazole before and during the first part of fractionated radiotherapy might provide an additional method of attacking the problem of radioresistant hypoxic tumour cells (Foster and Willson, 1976).

We now report experiments on mouse tumours in which this possibility of using metronidazole as a chemotherapeutic agent has been further evaluated.

MATERIALS AND METHODS

(a) In vitro incubation of Ehrlich ascites carcinoma cells.—Sterile procedures were used throughout. Approximately $3 \times 10^6$ ascites tumour cells were injected into the peritoneal cavity of CBA/CA mice. The cells were harvested 7 days later and placed in a tube containing 5 ml of phosphate-
buffered saline (PBS, pH 7.3, containing 100 u of heparin). The cells were washed with lysing buffer (7-4 g NH₄Cl, 2-06 g Tris HCl in 1 l brought to pH 7.2 with conc. HCl) to remove erythrocytes and centrifuged at 700 g for 5 min to produce a pellet. The pellet was gently resuspended in 5 ml PBS and the tumour cell concentration determined using a Coulter particle counter (model D). Residual erythrocytes present in samples removed for counting were lysed with "Zapoglobin" (Coulter Electronics Ltd). After further centrifugation the cells were resuspended in tissue culture medium (TC199, Wellcome Reagents Ltd, containing 200 u of benzyl penicillin and 100 u streptomycin per ml), to a concentration of 4 x 10⁷/ml. This suspension was buffered to pH 7-4 using HEPES (Flow Labs. Ltd). Aliquots (5 ml) of this suspension were placed into amber bottles with aluminium foil caps and gently shaken in a water bath at 37°C for 20 min. A further 5 ml of tissue culture medium was added to 7 of these bottles and designated controls. To the remainder, 5 ml of tissue culture medium containing metronidazole was added to give drug concentrations of 5 mM and 10 mM (10 mM = 1710 μg/ml). All the bottles were stoppered with plastic caps through which two syringe needles were inserted to allow equilibration of the cell suspensions with the appropriate gas. Pure N₂ or air was gently bubbled through the suspensions for 30 min. The needles were then removed and the caps of the bottles containing cells equilibrated with N₂ were sealed. The caps of those bottles containing cells that had been equilibrated with air were replaced with loose-fitting aluminium foil. All the bottles containing the cells were then incubated for 0, 2, 4 or 6 h at 37°C whilst being gently shaken to ensure that the cells remained in suspension, and that they continued to be in equilibrium with the appropriate gas. After the required intervals 0-2 ml aliquots of the cell suspension (4 x 10⁸ cells) were injected into groups of 5 or 10 CBA/CA mice from the inbred Brunel colony. The times of death of the mice were recorded.

(b) Tumour control probability determination.—Syngeneic female CBA/Ht mice were used in batches of 100. A fast-growing anaplastic carcinoma, designated NT, was transplanted subcutaneously by trocar on to the ventral surface of the thorax whilst the animals were under Penthrane-induced anaesthesia.

The resulting tumours were measured twice a week with Vernier calipers and those mice with tumours of approximately 5 mm diameter were selected for use. Each selected batch was divided into two: one half was set aside and 2 days later these mice were assigned to one of 6 radiation dose groups. The other half was given an oral dose of metronidazole (0-3 mg/g body wt.) every 6 h for 36 h (6 administrations) and irradiated 6-8 h after the last drug dose. All the mice were irradiated as described previously (Begg et al., 1974) with 240 kV x-rays (HVL 1-3 mm Cu) at a dose rate of 240 rad/min whilst breathing air at room temperature. The mice were then kept for 130 days. Those mice developing tumours greater than 8 mm in diameter were scored as recurrences. The x-ray dose required to cure 50% of the mice (TCD₅₀) and the s.e. mean of this value was calculated from the percentage cures using a computer program (Peters and Porter, private communication). The program assumes that single cell survival kinetics apply so that the probability of tumour control is given by exp (-SN) where N is the initial number of clonogenic cells and S is the recurring (i.e. surviving) fraction which is an exponentially decreasing function of dose.

Another batch of tumour-bearing mice was dosed with metronidazole as described above. At hourly intervals, for up to 8 h, groups of 3 or 4 mice were exsanguinated by heart puncture whilst under ether-induced anaesthesia. The concentration of metronidazole in the serum from these blood specimens was measured by polarography (Kane, 1961).

RESULTS

(a) In vitro incubation of Ehrlich ascites tumour cells

The results are shown in the table. Incubation of the cells in metronidazole under aerated conditions resulted in little or no increase in survival time of mice injected with these cells compared with those injected with control cell samples. In contrast a large increase in survival time was seen in mice receiving cells
### Table.—Survival Time of Male CBA/CA Mice Receiving a Standard Inoculum of Ehrlich Ascites Carcinoma Cells After Treatment with Metronidazole Under Anoxic and Aerated Conditions

| Drug exposure time (h) | Drug conc. (mM) | Mean day of death ± s.e. mean | Range of death | Mean day of death ± s.e. mean | Range of death |
|-----------------------|----------------|-------------------------------|----------------|-------------------------------|----------------|
| 0†                    | 0              | 18.7 ± 0.45                  | 17–21          | 18.2 ± 0.49                  | 17–19          |
|                       | 5              | 20.6 ± 0.92                  | 18–23          | 17.4 ± 0.40                  | 17–19          |
|                       | 10             | 18.8 ± 0.47                  | 17–21          |                              |                |
| 2                     | 0              | 21.4 ± 0.60                  | 20–23          | 19.2 ± 0.49                  | 18–20          |
|                       | 5              | 21.0 ± 1.05                  | 17–23          | 22.2 ± 1.07                  | 20–26          |
|                       | 10             | 22.4 ± 0.87                  | 21–24          | 25.4 ± 2.18                  | 22–34          |
| 4                     | 0              | 22.0 ± 0.60                  | 19–24          |                              |                |
|                       | 5              | 25.8 ± 1.64                  | 24–27          |                              |                |
|                       | 10             | >46.8 ± 6.34                 | >22–>60        |                              |                |
| 6                     | 0              | 21.6 ± 1.09                  | 13–24          | 21.8 ± 0.80                  | 19–23          |
|                       | 5              | >60                          | >60            |                              |                |
|                       | 10             | >60                          | >60            | 25.4 ± 2.18                  | 22–34          |

* Mean survival time of 5 animals; the remaining 5 animals within this group lived beyond day 60 and were arbitrarily defined as indefinite survivors.
† Cells removed immediately after gassing.

treated with metronidazole under anoxic conditions. Six hours' exposure to 5 mM metronidazole was required to cause a significant increase in survival time. A marked effect was seen after only 4 h exposure to 10 mM metronidazole.

(b) **Tumour control probability determination**

The results of the tumour irradiation experiment are shown in Fig. 1. Tumours in the metronidazole-treated mice show an increased sensitivity to irradiation. A dose of 4643 rad ± 97 controlled 50% of the tumours in the drug-treated mice compared with a dose of 6081 ±135 for the control group. It can be seen (Fig. 1) that for a dose of 5300 rad less than 20% of the control mice were cured compared with over 80% of the drug-treated mice.

The serum concentration/time curve (Fig. 2) indicates that the serum concentration of metronidazole at the time of irradiation was less than 30 μg/ml. The peak concentration of the drug was in the region of 200 μg/ml and the half-life of metronidazole in the serum was approximately 1½ h. No mice were lost from this study due to drug toxicity. However, 8/64 animals from the drug-treated group were excluded from the experiment due to the development of metastases, mainly in the lungs, and similarly 15/101 from the control group. Also, 9 mice died within 3 weeks of irradiation due to the unavoidable occasional inclusion of part of the intestine in the radiation field in a few cases.

**DISCUSSION AND CONCLUSION**

The results of the incubation of ascites cells with metronidazole in vitro clearly shows that the drug is cytocidal for anoxic cells at concentrations producing little or no effect on aerated cells. It has been reported (Hawes, Howard and Gray, 1964) that incubation of ascites cells under anaerobic conditions with or without mechanical agitation greatly reduces the viability of these cells and increases their radiosensitivity. In the present study no reduction was seen in the survival time of mice injected with cells incubated under anoxic conditions for up to 6 h when compared with the analogous aerated controls. How-
Fig. 1.—The percentage of tumours as a function of x-ray dose for transplanted anaplastic carcino-
mata in CBA/Ht mice.  × tumours given x-rays only.  ○ tumours given x-rays after the mice
received 36 h metronidazole treatment.  Curves drawn by eye.  Horizontal bars ± s.e. mean.

Fig. 2.—The serum concentration of metronidazole as a function of time after the last of
6 injections of 0·3 mg/g body wt. spaced 6 h apart.

However, it is possible that the ascites cells
which were incubated under N₂ were
made unusually sensitive to the cytotoxic
effect of metronidazole.  If this is to
invalidate our conclusion, then a dif-
ference between cells that are naturally
hypoxic in vivo (i.e. hypoxic tumour
cells) and the cells as we have used
them in vitro would have to be demon-
strated.

It is not possible to make a quantita-
tive estimate of the fraction of cells
killed by the drug treatment schedules
used in this experiment.  However, there
can be little doubt that the increased
survival time of the mice receiving the
drug-treated cells is due to a substantial
reduction of the viable fraction of the
standard cell inoculum.  Since these stu-
dies were begun a similar effect of metroni-
dazole against anoxic mammalian cells
has been reported (Mohindra and Rauth,
1976).  Nitrofurazone was found to
be similarly active.  In vitro experiments
also showed that the activity is de-
pendent on cell type, O₂ concentration,
temperature of incubation and concen-
tration of both drugs.  Recently a 2-
nitroimidazole drug has also been found
to be cytoidal for anoxic mammalian
cells (Hall and Roizin-Towle, 1975).  Ex-
posure of anoxic cells to this drug for up
to 24 h resulted in marked cell killing
with drug concentrations as low as 200
μg/ml. Results from studies with fractionated irradiations led to the suggestion that exposure to the drug between doses of radiation prevents the repair of sub-lethal radiation damage.

The increased sensitivity of the solid carcinoma to x-rays reported here is interpreted as being due to the killing of a substantial fraction of the hypoxic cells in the tumour by the metronidazole treatment before irradiation. For significant radiosensitization of hypoxic cells to occur in vitro (Asquith et al., 1974) or in vivo (Rauth and Kaufman, 1975) it has been found that metronidazole concentrations 5 times greater than the value of 30 μg/ml found in the present study are required at the time of irradiation.

Unfortunately, the fast growth rate of murine tumours of the type used in this experiment restricts the time available for pre-irradiation treatment with a drug. In particular, the short half-life of metronidazole in mice (1.5 h) means that either the drug has to be administered very frequently or very high drug doses have to be used to maintain an effective drug concentration over an extended time period. The design of the experiments reported here took these factors into account as far as possible. It is not known how fast hypoxic cells are generated in the tumour used nor the minimum effective concentration of metronidazole under in vivo conditions. Therefore, it is possible that some hypoxic tumour cells survived the treatment schedule used. For these reasons further experiments have been started where metronidazole has been administered for a time after irradiation as well as before and in which a range of drug doses has been used. The serum half-life of metronidazole in man is 8–10 h and the growth rate of most solid human neoplasms is much slower than their murine counterparts. Thus any future clinical application will be more straightforward than might be suggested by the present experiments. The drug regimes reported are directly applicable only to mice.

It is probable that many organic nitro-aromatic compounds have activity against anoxic mammalian cells. On reduction, whether by radiation or biochemically, they can form a toxic species (Willson, Cramp and Ings, 1974; Willson and Searle, 1975; Foster and Willson 1976). In the case of metronidazole this species is either inactivated by O₂ or only produced in its absence; hence the drug’s lack of activity on aerobic microorganisms (Ings, McFadzean and Ormerod, 1974), or on normal well-oxygenated cells as reported herein. Thus, as with radiosensitizers, drugs which show the greatest efficiency on hypoxic cells in vitro (i.e. having relatively high one-electron electrode potentials), are more likely to produce unacceptable side-effects in vivo.

In conclusion we suggest that the use of metronidazole as a cytotoxin specific for hypoxic cells in solid tumours should be more fully explored. The lack of unwanted side-effects recently reported following its use as a radiosensitizer, in man at high doses, is encouraging (Urtasun et al., 1975). In addition, the use of the drug as a chemotherapeutic agent before the commencement of radiotherapy would obviate the necessity of altering well established fractionation schedules to accommodate tolerable drug regimes.

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