Virchows Archiv A Pathol Anat (1991) 419:223–230

Virchows Archiv A
Pathological Anatomy and Histopathology

Effect of α-difluoromethylornithine on the polyamine levels and proliferation in two transplantable tumours

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Received April 30, 1991 / Accepted May 30, 1991

Summary. The effect of inhibition of polyamine biosynthesis by α-difluoromethylornithine (DFMO) on the growth of two murine transplantable tumours was studied. Female CBA mice were implanted with either the sarcoma F (SaF) or an anaplastic mammary carcinoma (CaNT), and 3% DFMO in the drinking water was provided once the tumours were established. Over a 10-day period control SaF tumours increased exponentially from 20 mm^3 to over 800 mm^3, whereas DFMO-treated SaF reached only 300 mm^3. CaNT grew more slowly, requiring 22 days to achieve a similar volume increase, and DFMO was as effective in retarding growth as it had been in SaF. DFMO depleted tumour tissues of putrescine and spermidine, but did not reduce spermine levels. Metaphase arrest experiments with vincristine demonstrated that DFMO could substantially reduce the rates of tumour cell production, but there was no indication the DFMO accelerated the rate of cell loss from the tumours. Despite reduced rates of cell production, labelling studies with bromodeoxyuridine failed to detect differences between control and treated tumours: an increase in transit time through the S-phase was suspected. The number of nuclear organizer regions, detected by the argyrophilia of their associated proteins, was less in DFMO-treated tumours, and within a tumour the degree of silver deposition unequivocally reflected the proliferative heterogeneity. Ultrastructural studies revealed no differences between DFMO-treated and untreated tumours.

Key words: Difluoromethylornithine – Polyamines – Cell proliferation – Mitosis – Bromodeoxyuridine

Introduction

Polyamines are ubiquitous polycationic molecules synthesized seemingly by all nucleated cells. There is general agreement that polyamines are essential for growth (Pegg 1988), probably because of their ionic binding to nucleic acids; their basic nature causing strong binding to acidic phosphate groups of nucleic acids. The first step in the synthesis of polyamines is the decarboxylation of the amino acid ornithine by the enzyme ornithine decarboxylase (ODC, 4.1.1.17) to form the diamine putrescine. Putrescine is metabolized further to spermidine and spermine through the consecutive action of two aminopropyl transferases, spermidine synthase and spermine synthase. Marked increases in polyamine biosynthesis and accumulation are characteristically associated with elevated rates of cell and tissue growth caused by hormones and growth factors (Bachrach 1984), and are particularly pronounced when cells are triggered from proliferative quiescence to active proliferation as occurs to hepatocytes after a two-thirds partial hepatectomy (Luk 1986).

The role of polyamines in cell proliferation has become somewhat clearer through the use of specific inhibitors of their biosynthesis (Pegg 1986). Particularly important in this respect is the enzyme-activated irreversible inhibitor of ODC, difluoromethyl ornithine (DFMO, MDL 71.782), synthesized at the Merrell-Dow-Research Institute (Metcalf et al. 1978). In vitro, DFMO retards the growth of many cell types and these observations have lead to many studies of the effects of DFMO on a variety of chemically induced or implanted rodent tumours, and on human tumours xenografted into nude mice. Profound reductions in overall tumour growth rate have been recorded for xenografted human tumours (Tutton and Barkla 1986; Upp et al. 1988), as well as for transplantable rodent tumours (Grossie et al. 1987; Marx et al. 1987) and chemically induced rodent tumours (El-Ela et al. 1989; Manni et al. 1989; Zhang et al. 1988).

The present study was undertaken to define more exactly the kinetic mechanisms by which DFMO retards tumour growth. Two murine transplantable tumours of differing growth rate were studied, and measurements have been made at two time points during the exponen-

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tial phase of growth. Actual and potential doubling times for the tumours were calculated to assess the effects of DFMO on cell loss rates as well as cell production rates. Ultrastructural studies were performed to find if there were any subcellular morphological changes associated with the treatment.

Materials and methods

Animals. Sixteen female CBA mice were implanted with sarcoma F (SaF), a round cell tumour of murine connective tissue, and a further 16 female mice were implanted with carcinoma NT (CaNT) an anaplastic corded mammary carcinoma. Implants were made subcutaneously on the flank, each containing approximately 1×10^6 tumour cells. Mice bearing the faster-growing SaF tumours were killed at either 12 or 16 days post-implant, while mice bearing CaNT were killed at either 26 or 35 days post-implant. These time intervals were chosen so that both tumours had a mean diameter of around 0.5 cm at the first time point and a mean diameter of around 1 cm at the latter time point.

Tumour measurement. Starting on the 6th (SaF) or 13th (CaNT) day after implantation, when they were first palpable, tumours were measured at intervals for up to 16 days (SaF) or 35 days (CaNT) after transplantation. The three principal diameters of each tumour were measured using a Vernier caliper, and the volume of each tumour was calculated using the formula:

$$\text{Volume} = (\text{Product of the 3 diameters}) \times \frac{\pi}{6}$$

DFMO administration. DFMO was a gift from C.D. Houldsworth, Research Support Manager at Merrell Dow Research Institute, Strasbourg Research Center, Strasbourg, France. Six days after implantation of SaF or 13 days after implantation of CaNT, each category of tumour-bearing mouse was randomly divided into two groups of 8; the treatment groups were immediately placed on drinking water containing 3% DFMO.

Estimates of tumour cell proliferation. Three separate techniques were employed to assess the proliferative status of the tumours. First, the rate of entry into mitosis was calculated by performing a metaphase arrest experiment after injection of vincristine sulphate (VCR) (Oncovin; Eli Lilly, Basingstoke, UK). Briefly, at each time point of the study, groups of 6 mice were given a single i.p. injection of VCR and subsequently killed at 30-min intervals over the following 3 h. The metaphase index (%) for each animal was estimated by scanning 2000 consecutively observed tumour cells, and graphs of metaphase index versus duration of VCR treatment were constructed for each experimental group. The regression coefficient for each group was calculated using the method of least squares, the calculated value representing the rate of entry into mitosis or the cell birth rate (Kb) with units of cells born, per cell, per hour. This method allows the potential doubling time (t_{pd}) of the tumour to be calculated from the formula:

$$t_{pd} = \frac{\ln 2}{Kb} \text{ (Wright and Alison 1984)}$$

The S-phase labelling index was measured after injection of the pyrimidine analogue bromodeoxyuridine (BrdUrd; Gratzner 1982). Mice were injected i.p. with BrdUrd (Sigma, Poole, UK) at a dosage of 50 mg/kg body weight 1 h prior to death. Tissue was fixed for 4 h in Carnoy's solution and subsequently transferred to 70% alcohol prior to immunostaining. Dewaxed sections underwent partial DNA denaturation (1 M HCl at 60° C for 5 min) and were exposed to the primary antibody (rat anti-BrdUrd; Sera Lab., Crawley, UK) at a 1:20 dilution overnight at 4° C. Visualization of labelled cells was achieved by a standard peroxidase/DAB method (peroxidase conjugated rabbit anti-rat immunoglobulins, Dako, High Wycombe, UK; diaminobenzidine, Sigma). The labelling index (%) for each tumour was calculated from the number of stained cells in 2000 consecutively observed tumour cells. Tumour cell proliferation was further assessed by enumerating the rRNA gene clusters. Nucleolar organizer regions (NORs), as they are called, are located on the short arms of the appropriate pair of acrocentric chromosomes in the diploid cell (Wolfe 1981). NORs can be demonstrated by means of the argyrophilia of their associated proteins, using a simple silver-staining technique (Ploton et al. 1986), the so-called AgNOR technique which was applied to formalin-fixed, paraffin-embedded tissue sections. Two hundred nuclei from each tumour were assessed for AgNOR number.

Polyamine determination. Analysis was as described by Seiler (1984). Tumour specimens were snap frozen in liquid nitrogen and stored until analysis. Samples were homogenized in 0.1 M sodium phosphate buffer, and an equal volume of 0.4 M perchloric acid was then added. Following sonication, samples were centrifuged at 4000 *g* for 15 min and the supernatant after centrifugation was stored at −20° C until analysis using a high-pressure liquid chromatograph (Model 352, ACS, Macclesfield, UK). Polyamines were derivatized using *o*-phthalaldehyde and quantified using 1,7-dia-

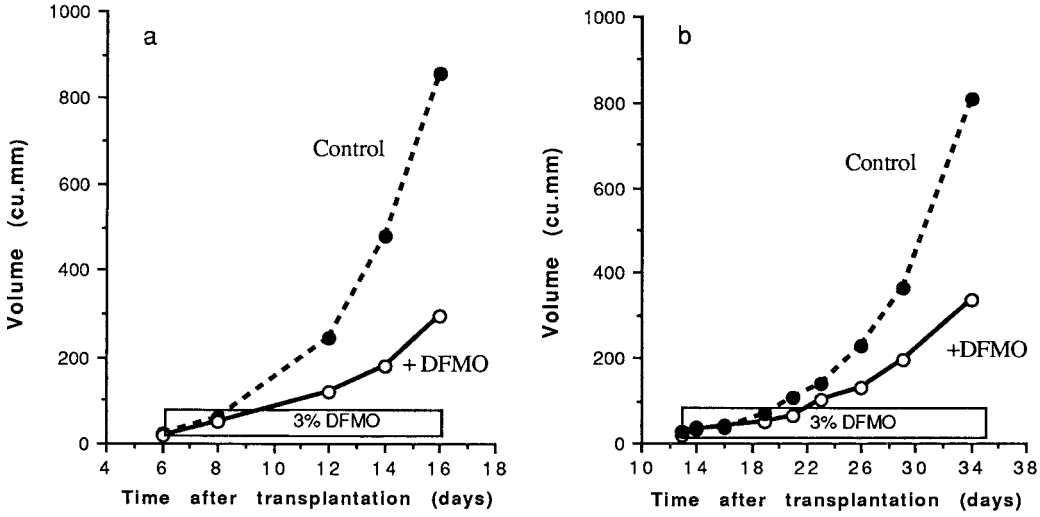

Fig. 1. Growth curves for control and α-difluoromethylornithine (DFMO)-treated sarcoma F (SaF) (**a**) and carcinoma NT (CaNT) (**b**) tumours

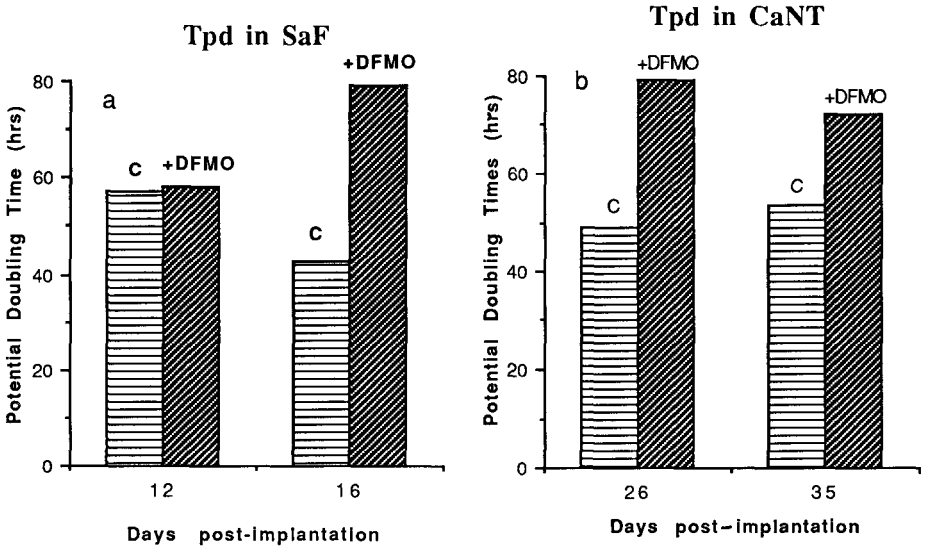

Fig. 2. Potential doubling times for control and DFMO-treated SaF (**a**) and CaNT tumours (**b**) estimated from rates of entry into mitosis after vincristine injection

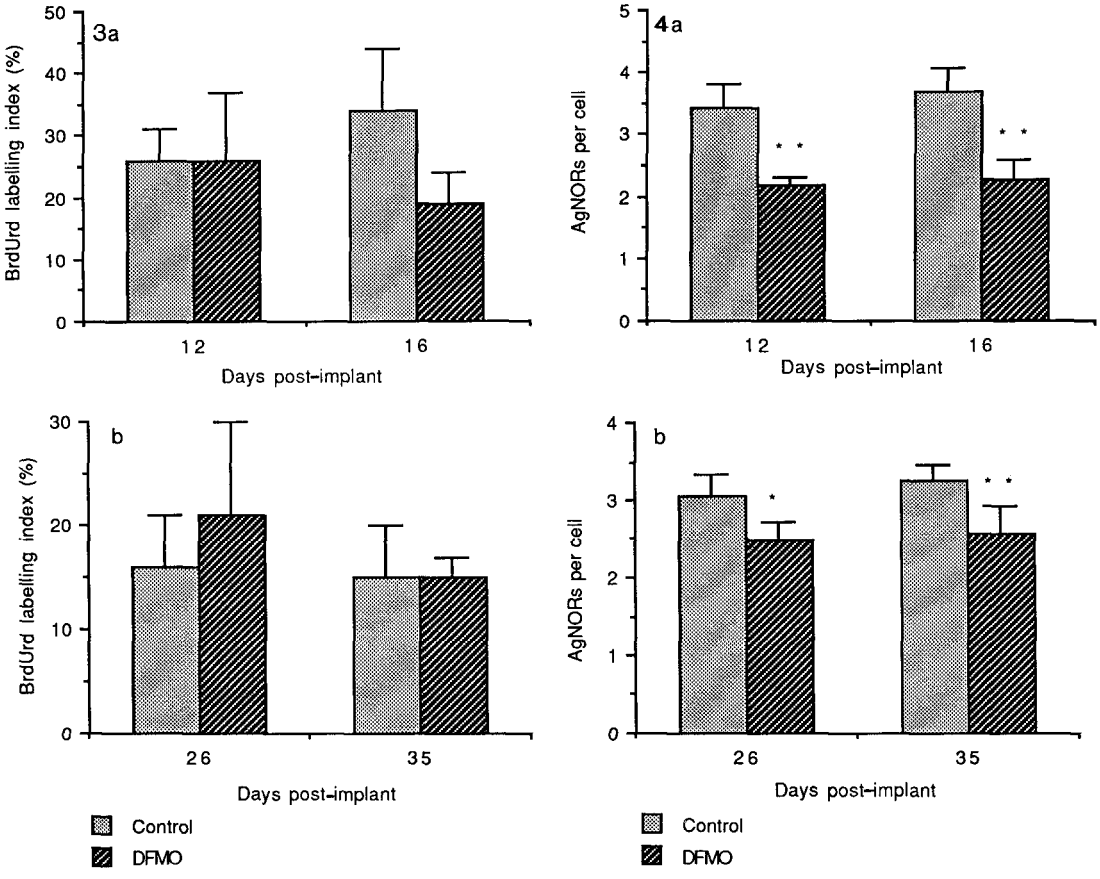

Fig. 3. Bromodeoxyuridine (BrdUrd) labelling indices in control and DFMO-treated SaF (**a**) and CaNT tumours (**b**)

Fig. 4. Silver-stained nucleolar organizer region (AgNOR) numbers in control and DFMO-treated SaF (**a**) and CaNT tumours (**b**); $*P<0.05$, $**P<0.01$

minoheptane (final concentration 10 nmol/ml) as an internal standard; the buffer flow rate was 90 ml/h. Protein content was determined by the Biuret assay (Plummer 1978), and polyamine concentrations were expressed as nmol/mg of protein.

Electron microscopy. Tumour samples, not exceeding 1 mm^3 in volume, were fixed in 2% glutaraldehyde for 2 h. After washing in phosphate buffer, tissues were osmicated and dehydrated in acidified DMP before routine embedding in Taab resin. Sections were cut (1 μm thick) and stained with toluidine blue for observation at light microscope level, followed by ultrathin sections of approximately 100 nm, collected on nickel grids and stained with uranyl acetate and lead citrate, for observation on a Philips CM-10 electron microscope.

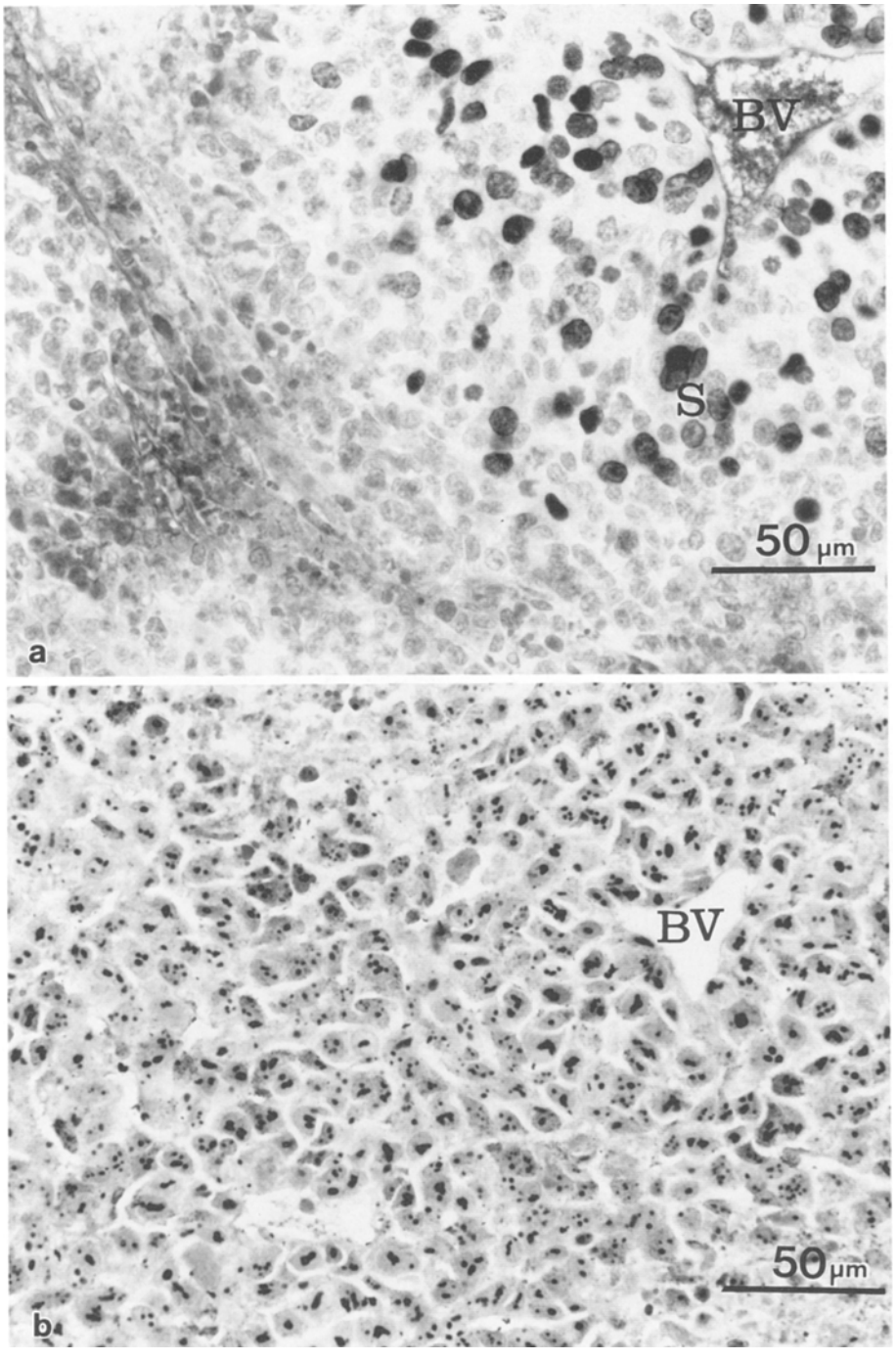

Fig. 5. a BrdUrd labelling in the CaNT tumour; the S-phase cells (*S*) are located near the blood vessel (*BV*). (**b**) AgNOR staining in the same tumour, the cells with the largest silver deposits are also close to the blood vessel (*BV*)

Results

DFMO resulted in a dramatic impairment in growth of both SaF (Fig. 1a) and CaNT (Fig. 1b) and both treated tumours were only one-third the volume of their respective controls at the end of the study. All tumours grew logarithmically over the period of observation and DFMO increased the doubling time (t_d) of SaF from 1.9 to 2.6 days, and likewise lengthened the t_d of CaNT from 4.3 to 5.6 days.

The metaphase arrest experiments allowed the t_{pd} of the various tumours to be calculated, i.e. the time in which the tumours would be expected to double in cell number if no cells were lost. In SaF (Fig. 2a), no difference in t_{pd} was detected at day 12 after implantation, but at day 16 t_{pd} in the DFMO-treated SaF tumours was almost twice as long as that in controls. In CaNT (Fig. 2b), DFMO had lengthened t_{pd} at both times of observation. In terms of the size of the S-phase fraction (the BrdUrd labelling index), DFMO had no statistically significant effect on either tumour at the times studied (Fig. 3). In SaF (Fig. 3a), similar labelling at day 12 but a trend towards reduced activity in the DFMO-treated tumours on day 16 was in accord with the metaphase

arrest data (Fig. 2a). In CaNT, however (Fig. 3b), DFMO-induced increases in t_{pd} were not reflected in reduced BrdUrd labelling indices.

The study of AgNORs per tumour cell nucleus did reveal differences between DFMO-treated and control tumours (Fig. 4). In both tumours DFMO treatment resulted in statistically significant, albeit small, reductions in AgNOR number per tumour nucleus. The notion that the quantity of AgNOR proteins increases with proliferative activity and/or transcriptional activity in general was strongly supported by the finding of much larger silver deposits in areas of intense proliferative activity. CaNT has a definite corded structure of proliferative cells adjacent to blood vessels (Fig. 5a), separated from each other by concentric zones of hypoxic and necrotic cells more distant from the afferent blood supply. This zonation was mimicked by larger silver deposits in the proliferative areas (Fig. 5b), in which many of the cells would be in the process of duplication of the NOR-bearing chromosomes; the larger deposits probably reflecting coalescence of the more numerous NORs.

In the untreated tumours, the relative abundance of the normally synthesized polyamines was as expected with spermidine being present at the highest concentraton (Fig. 6a, b), with lesser amounts of spermine, and putrescine present in only minor quantities. Cadaverine, a bacterial polyamine produced through the action of the enzyme lysine decarboxylase, occurred in measurable quantities in all samples analysed. DFMO treatment had the same effect on both tumours (Fig. 6a, b), depleting tissue levels of putrescine and spermidine to immeasurable amounts, while at the same time the levels of spermine were actually increased.

Extensive ultrastructural studies failed to reveal any consistent differences between control and DFMO-treated tumours, although in some treated samples of both SaF and CaNT there was a suspicion of distension of the rough endoplasmic reticulum. SaF (Fig. 7a) is a round cell tumour; electron microscopy revealed infiltration with host macrophages with little or no intercellular stromal material present. Cell nuclei were large and irregular in shape with a high euchromatin/heterochromatin ratio; nucleoli were prominent and often marginated in common with a proportion of heterochromatin. Although demonstrating a full complement of organelles, the cells of SaF were not rich in either rough or smooth endoplasmic reticulum; no desmosomes, and no other junctional complexes were present, but the cells were generally closely apposed, sometimes with pinocytotic activity on adjacent plasma membranes. CaNT, on the other hand, was more pleomorphic with the plasma membranes typically thrown into folds which interdigitated with those of neighbouring cells. Frequently the cells appeared to lie in nests bounded by small amounts of connective tissue. Once more, nuclei were large with prominent nucleoli and a proportion of marginated heterochromatin. Typical for highly proliferative cells organelles were sparse though fully represented, rough and smooth endoplasmic reticulum were present, comparable with SaF, but in addition cells of CaNT were joined by the occasional desmosome.

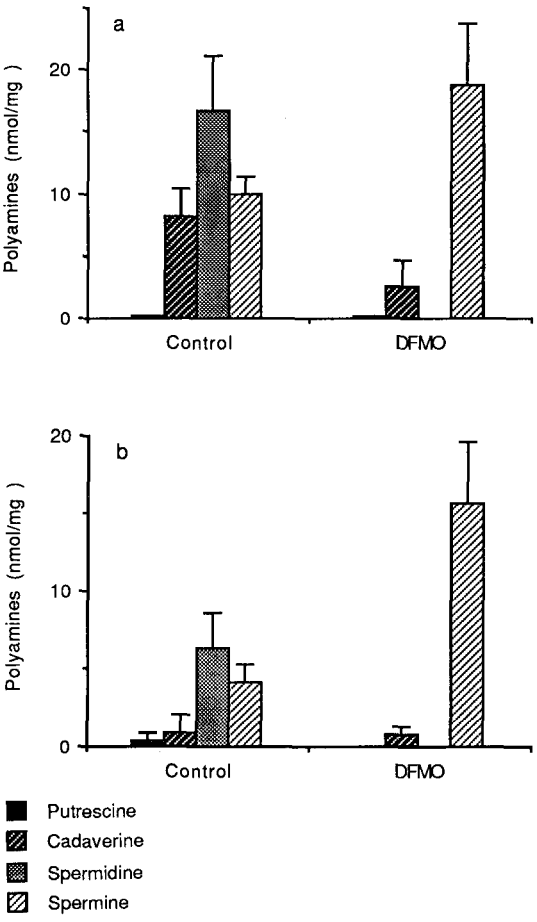

Fig. 6. Polyamine levels in control and DFMO-treated SaF (**a**) and CaNT tumours (**b**)

Discussion

A number of studies have been devoted to the effects of DFMO on tumour growth, but these have largely confined themselves to observations of overall tumour growth rate. As such the data are undoubtedly impressive, with reports ranging from a 100% or more increase in the tumour doubling time (Marx et al. 1987; Tutton and Barkla 1986; Upp et al. 1988) to an almost cessation of tumour growth (Manni et al. 1989; Zhang et al. 1988). The present study was undertaken to explore the underlying kinetic mechanisms through which DFMO retarded tumour growth, examining DNA synthesis, mitotic activity and AgNORs in two tumours, one a mammary carcinoma and the other a round cell sarcoma.

The SaF sarcoma was the faster-growing tumour, t_d was 1.9 days, achieving 5.26 doublings in the 10-day period of observation, enlarging from around 20 mm^3 to over 800 mm^3. The presence of a 3% concentration of DFMO in the drinking water lengthened the t_d to 2.6 days; growth remained exponential, but only 4.0 doublings in the 10-day period resulted in the tumours averaging a volume of just under 300 mm^3. Largely similar results were obtained over a 22-day period of study of the slower-growing CaNT, with the DFMO-treated tumours achieving only 3.9 doublings against 5.1 dou-

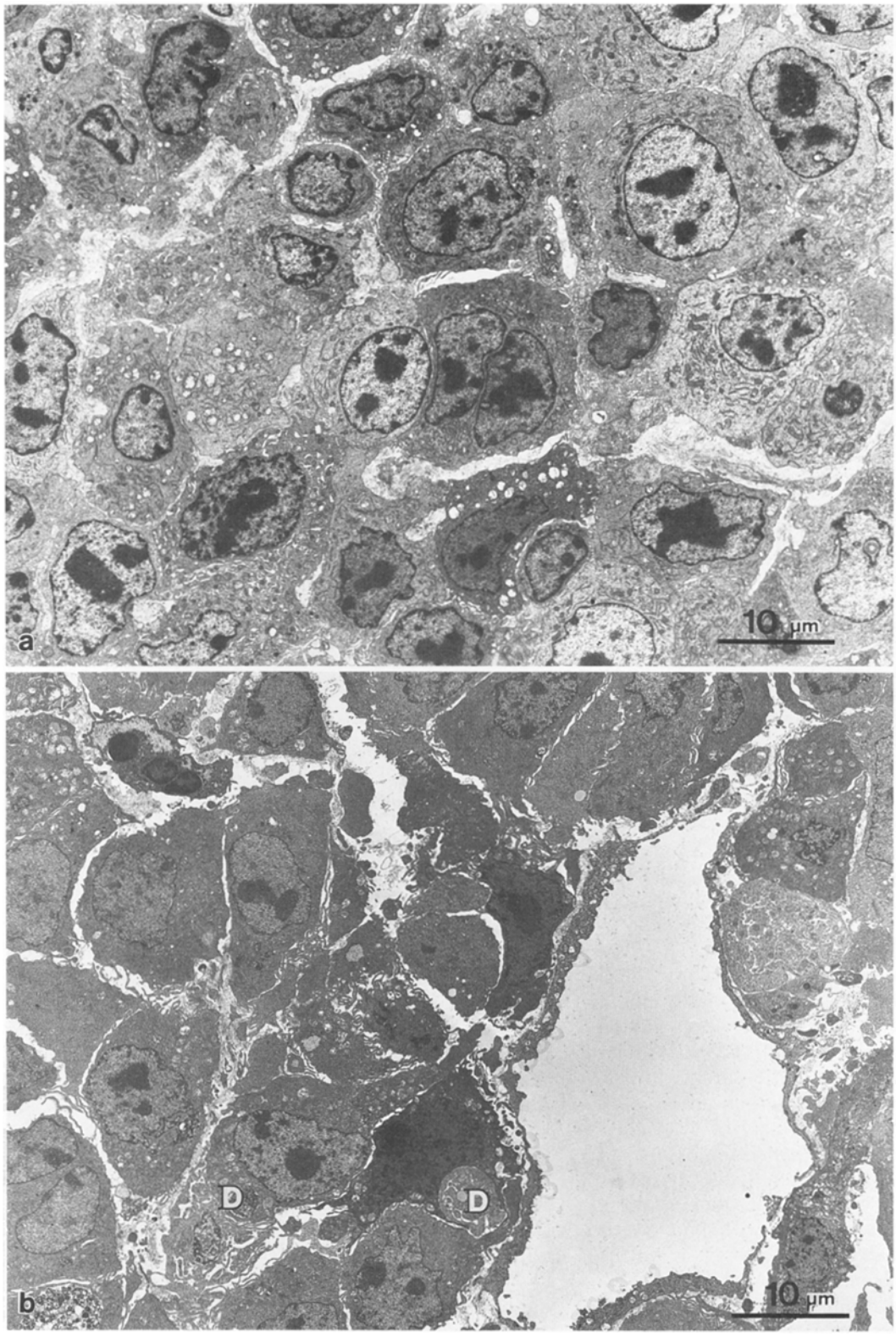

Fig. 7. Electron micrographs of control SaF (**a**) and control CaNT tumours (**b**). Note phagocytosed cell debris (*D*), probably apoptotic bodies, in tumour cells and macrophages in the CaNT tumour. ×2400 (reduced to 75%)

blings in the control tumours. DFMO had broadly similar effects on the tissue levels of polyamines in the two tumours. DFMO effectively depleted the tumours of putrescine and spermidine, but had no effect on spermine. In fact the tumour concentrations of spermine were almost doubled in the DFMO-treated mice; a reduced rate of spermine breakdown could be responsible, or alternatively the uptake of preformed polyamines from the plasma could have been stimulated by ODC inhibition. Indeed, Ehrlich ascites cells cultured for 2 days in

the presence of DFMO show a strikingly enhanced uptake of polyamines from the medium (Alhonen-Hongisto et al. 1980). In many studies, DFMO has been remarkably ineffective in reducing tumour levels of spermine with levels actually rising in a DFMO dose-dependent fashion in a transplantable fibrosarcoma (Grossie et al. 1987), or remaining unaltered in a chemically induced breast cancer (Manni et al. 1989) and in human gastric cancer xenografts (Upp et al. 1988). Clearly, in the present study the absence of putrescine and spermidine slows down the growth rate of two very rapidly growing tumours, but it can not be emphasized too strongly that even the DFMO-treated tumours are still rapidly growing, in the context of human cell proliferation (Steel 1977). It is also worth noting that cadaverine, a bacterial polyamine, was invariably present in the tumour tissues. Cadaverine is synthesized by the bacterial flora of the gut and its uptake, like that of the other polyamines, can be enhanced by polyamine deprivation (Sarhan et al. 1989), though in our study it was present in all tumours. A specific function of cadaverine has not been described and unfortunately most studies ignore its presence.

Tumour cell proliferation was assessed by three independent techniques. Metaphase arrest experiments produce a firm kinetic parameter, the rate of entry into mitosis, which is independent of the duration of the mitotic phase cf. DNA labelling. DFMO caused a substantial reduction in the rat of entry into mitosis and hence an extension of t_{pd} at three of the four time points studied, the exception being SaF at day 12 post-implantation, but this may have been due to the relatively short period (6 day) of exposure to DFMO compared to the other tumour groups. We can, of course, proceed to measure the cell loss factor ϕ, which is the rate of cell loss to cell birth (Steel 1977) and can be calculated using the relationship

$$\phi = 1\text{-}t_{pd}/t_d$$

where t_{pd} is the potential doubling time derived from the metaphase arrest experiment and t_d is the actual doubling time derived from the growth curve. Thus, in untreated CaNT at day 26,

$$\phi = 1\text{-}49/103 = 0.53$$

i.e. for every 100 cells born some 53 are lost from the population. A similar calculation makes $\phi = 0.41$ in the DFMO-treated CaNT at this time. Therefore it seems unlikely that DFMO is accelerating the rate of cell loss from the tumour; in fact, the converse could be true. However, there are several caveats. First, as DFMO reduces the cell production rate we are dealing with a smaller tumour, and since it is widely acknowledged that tumours tend to have higher cell loss rates as they enlarge (Steel 1977), the higher cell loss factor in the untreated tumours could be due simply to their larger physical size. Perhaps more germane to the present discussion is the fact that estimates of t_d are based on growth curve measurements, be they tumour weights, or as in this case, volume estimations. It is to suspend disbelief to assume that such measurements truly represent the increase in viable tumour cell number for these tumours; like all tumours they contain inflammatory cells, apoptotic cells, cystic spaces and areas of necrosis; indeed the discrepancy between cell loss factors estimated according to gross tumour weights and net tumour weights after the removal of necrotic material has already been documented (Sarraf and Browen 1986). Therefore any estimates of ϕ. must be viewed with considerable caution. Perhaps surprisingly, DFMO had little effect on BrdUrd labelling in the two tumours, and broadly similar results were reported by Zhang et al. (1988), who found even a small increase in the thymidine labelling index in DFMO-treated rat colonic tumours. However, the labelling index depends not only on the rate at which cells enter DNA synthesis but also on the duration of the synthetic phase. Thus, the absence of any reduction in labelling caused by DFMO could simply be due to DFMO causing a prolongation of the synthetic phase. Indeed, there is a good deal of evidence to support this hypothesis; both Ehrlich ascites tumour cells and CHO-A7 cells cultured under conditions of polyamine deprivation markedly increase the duration of DNA synthesis as judged by flow cytometric analysis (Anehus et al. 1984; Heby et al. 1978).

There has been considerable debate regarding the relationship of AgNOR counts to proliferative status (Quinn and Wright 1990). The AgNOR count depends on the stage of the cell cycle (Anastassova-Kristeva 1977), the number of NOR-bearing chromosomes (Wolfe 1981) and even the degree of dispersal of the NOR-associated proteins (Underwood and Giri 1988). The fact that chromosomes fuse during interphase making individual NORs difficult to discern simply compounds the problem. Nevertheless, the slower growth rates caused by DFMO treatment were consistently associated with a lower AgNOR count. The belief that AgNORs are related in some way to cell proliferation and/or transcriptional activity was unequivocally supported by the finding of much larger silver deposits in relatively oxic, highly proliferative areas of CaNT; these large deposits clearly represent coalescence of AgNORs that cannot individually be discerned. Thus, the amount of silver deposited may be a better qualitative assessment of the amount of NOR-associated proteins than the number of individual silver deposits.

That treatment with DFMO resulted in no ultrastructural changes in either tumour type was of some interest. In some samples there was a suspicion of distension of the rough endoplasmic reticulum, but this was inconsistent and would need further study with higher doses and/or longer treatment time to establish whether the inhibition of polyamine synthesis manifested itself morphologically in these tumours.

In conclusion, DFMO has proved to be retardant of growth in two rapidly growing transplantable murine tumours. Despite the absence of putrescine and spermidine the tumours still grew much faster than human neoplasms. Metaphase arrest experiments indicated that DFMO reduced the rates of tumour cell production and BrdUrd labelling studies strongly suggested that transit through the DNA synthetic phase was prolonged. De-

spite the pitfalls in comparing t_d with t_{pd}, the data did not suggest that DFMO promoted a greater rate of cell loss from the tumours. Ultrastructurally the treatment produced no change in the tumours under these conditions.

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