Arrest of the Proliferation of Renal and Prostate Carcinomas of Human Origin by Inhibition of Mitochondrial Protein Synthesis

Coby van den Bogert, Bert H. J. Dontje, Marijke Holtrop, Trudi E. Melis, Johannes C. Romijn, Jan W. van Dongen, and Albert M. Kroon

ABSTRACT

The results described in this paper demonstrate that proliferation arrest by low concentrations of tetracyclines, which has previously been shown in experiments with animal tumor systems, can also be achieved in tumor systems of human origin. Tetracyclines specifically inhibit mitochondrial protein synthesis. Prolonged and continuous impairment of protein synthesis inside the mitochondria leads to reduction of the cellular concentration of the polypeptide products which are coded and synthesized within mitochondria. These products are part of the oxidative phosphorylative system of the cell. Long-term tetracycline treatment leads to a decrease of oxidative ATP-generating capacity as monitored by cytochrome c oxidase activity. This may cause severe energetic or metabolic disturbances which explain the proliferation arrest observed. Proliferation arrest, provided that mitochondrial protein synthesis is blocked effectively, is found in vitro as well as in vivo. It is shown that the effect of doxycycline is not limited to cytostasis; prolonged doxycycline treatment is clearly cytotoxic for the tumor cells.

INTRODUCTION

Mitochondria contain a small DNA which is transcribed and translated inside the mitochondria (1). The translation machinery of mitochondria can be selectively blocked by antibiotics such as the tetracyclines (2). Interference with mitochondrial protein synthesis impairs the expression of the polypeptide products of the mitochondrial genome which are all part of enzyme complexes involved in oxidative phosphorylation. Continuous inhibition of mitochondrial protein synthesis will have energetic and metabolic consequences for the strictly aerobic mammalian cells which may lead for instance to proliferation arrest, because their oxidative phosphorylation capacity is reduced at the turnover of the cytoplasm or at cell division. It can thus be expected that rapidly proliferating cells are affected by continuous impairment of mitochondrial protein synthesis sooner than resting cells. This holds even more if proliferating cells possess a low reserve capacity for oxidative phosphorylation of their own already, as is the case in many tumor cells (3). As we have argued before (4, 5), the mitochondrial genetic system can, therefore, be considered as a target for chemotherapy in malignant tumor growth and the tetracyclines as rather selective cytostatics. The postulated cytostatic action of the tetracyclines is confirmed by the results obtained in various animal tumor systems. We have shown that the reduction of the oxidative phosphorylation capacity of tumors by treatment with the tetracyclines leads to in vivo proliferation arrest of an ascitically growing hepatoma (6), of a solid growing Leydig cell tumor (7), of a chemically induced mammary gland tumor (4), and of a T-type leukemia (8). We have shown further that prolonged tetracycline treatment does not, at least not within the period of time required to achieve antitumor effects, affect normal, nondividing tissues (9, 10). The effect of the tetracyclines on proliferating normal cells has been studied as well. The experiments showed that, due to permeability barriers for the tetracyclines, RBC and B-lymphocyte formation is not impaired (11). The proliferation of at least one subset of T-lymphocytes (11) and of monocytes,3 on the other hand, is inhibited. The same holds for the intestinal epithelium, but the latter has only moderate functional consequences even after 3–4 weeks of continuous treatment (9). The low toxicity and the pronounced antitumor effect of prolonged, continuous tetracycline treatment suggested that these antibiotics may be of value in human antitumor therapy. To lay a firmer basis for the possible application in humans, we initiated a study on the effect of DC4 treatment on the in vitro and in vivo growth of renal and prostate tumors of human origin. The results of this study are reported in this paper.

MATERIALS AND METHODS

Animals and Reagents. Female athymic (nude) rats weighing about 200 g were obtained from the CPB, TNO, Zeist, The Netherlands, and used to study the effect of DC treatment on the growth of s.c. transplanted tumors. Before and during the experiments the nude rats were housed in a separate stable under laminar flow conditions. Cages, bedding, food, and drinking water were sterilized before use. DC in its standard commercial preparation form for i.v. injection (Vibramycin) was kindly provided by Pfizer B.V., Rotterdam, The Netherlands, and administered to the rats by means of continuous i.v. infusion. In tissue culture studies we used DC obtained from Sigma. The proteinase Nagarse was obtained from Nagase Biochemical, Ltd. All other chemicals were of analytical grade.

In Vitro Tumor Systems. The permanent cell line PC-3, derived from a prostatic carcinoma (12), was cultured in Eagle’s medium (obtained from Gibco Europe, The Netherlands), supplemented with nonessential amino acids (Gibco) and 10% fetal calf serum (obtained from Commonwealth Serum Labs, Australia).

For the in vitro studies with renal tumor lines, tumor tissues obtained from nude mice were minced and digested with collagenase (400 units/ml) for 4 h. The resulting cell suspensions were, after extensive washing, plated in Petri dishes. The cultures were maintained in Eagle’s medium plus 10% fetal calf serum. The experiments with NC-65 were carried out with cells that were passaged four times; with the other renal tumor lines primary cultures were used.

DC was added to the cultures at the concentrations indicated after dissolving in absolute ethanol; the final concentration of ethanol never exceeded 0.1%. Control dishes received the same amount of ethanol. At fixed points after addition of DC, samples were taken for several analytical determinations and stored at −20°C until analytical measurements were made.


3 The abbreviation used is: DC, doxycycline.

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established more recently, were also maintained in nude mice. One of these lines, RCC-14, was obtained by the courtesy of Dr. U. Otto (Hamburg, Federal Republic of Germany). Some of the characteristics of these tumor lines were described before (14).

NC-65 was successfully implanted s.c. in nude rats. Usually two fragments of 80 mg (wet weight) of tumor tissue were transplanted in a symmetrical way, perpendicular to the backbone, one near the left and one near the right shoulder blade of the animals. Three days after tumor implantation DC treatment was started. Starting 1 week after tumor transplantation, the rats were inspected every 2 days. They were brought under light ether narcosis and tumor growth was followed by measuring the length, width, and height of the tumor. The tumor volume was calculated as if the tumor were spherical, using the measured sizes to obtain the mean radius of this sphere (7).

Administration of DC to Rats. Vibramycin was diluted with 0.15 M NaCl and administered at a rate of 0.1–0.2 ml/h by means of continuous i.v. infusion via the jugular vein as described previously (15). The dosage used was chosen in such a way that DC serum levels remained at about 5 µg/ml. This can be achieved by administering 20 mg/kg/day during the first week of treatment, 30 mg/kg/day during the second week, and 40 mg/kg/day in the weeks thereafter (9). Control animals were similarly infused with 0.15 M NaCl or Vibramycin vehicular solution. Three days before the implantation of the tumors the continuous infusion system was installed under laminar flow conditions, using sterilized operation equipment and sterilized continuous infusion devices.

Preparation of Tumor Mitochondria. Mitochondria from tumors growing on nude rats were isolated by differential centrifugation from a 10% tumor homogenate. The homogenate was obtained by cutting the tumor into small fragments in 0.25 M sucrose and incubating it for 20 min with Nagarse (0.8 mg/g tumor) at 0°C. During the incubation period the tumor fragments were roughly homogenized with the aid of a loosely fitting Teflon-glass homogenizer. After the incubation period the final 10% homogenate was made with a tightly fitting Teflon-glass homogenizer, in 0.25 M sucrose. The nuclear fraction was sedimented at 600 × g for 5 min, and subsequently mitochondria were sedimented from the supernatant at 18,000 × g for 10 min. The mitochondrial pellet was resuspended and centrifuged again for 10 min at 18,000 × g. The fluffy layer was discarded and the final mitochondrial pellet was suspended in 0.25 M sucrose. Samples were taken from the homogenate, the postnuclear supernatant, and the final mitochondrial pellet and the total volumes of these fractions were registered.

Analytical Assays. Cytochrome c oxidase was assayed spectrophotometrically at 20°C (16). Prior to incubation, the enzyme was activated in 1.5% digitonin at 0°C during 15 min. The cytochrome c oxidase activity in the several fractions of the tumors grown in vivo was measured between 2 and 3 h after the addition of Nagarse to the homogenate. The activity was calculated per min and expressed as the first-order reaction rate constant. Triplicate cultures were used for control cultures to which DC was added. Each point represents the mean value found per culture. The SE was maximally 4.5%.

RESULTS

In Vitro Studies

Preliminary studies revealed that mitochondrial protein synthesis in in vitro cultured PC-3 and NC-65 tumor cells could be inhibited specifically by the addition of DC to the culture medium in a final concentration of 10 µg/ml. In analogy to our previous work it can be expected, therefore, that DC exerts an inhibiting effect on cell proliferation if it is continuously present during a few cell cycles. In Fig. 1 it can be seen that NC-65 as well as PC-3 cells indeed stop growing after, respectively, 9 and 5 days of culturing in the continuous presence of 10 µg DC/ml. The doubling time of the PC-3 cells (about 2 days) is shorter than that of the NC-65 cells (about 4 days). From this it follows that both tumor lines can perform about 2 cell cycles in the presence of DC before cell proliferation becomes arrested.

In order to investigate to what level the mitochondrial capacity had to be reduced by continuous inhibition of mitochondrial protein synthesis before proliferation stopped, we made a detailed study of the effect of DC on the in vitro growth of PC-3 cells. The results of these studies are given in Fig. 2, which shows the total amounts of protein, DNA, and cytochrome c oxidase activity (as a parameter of the mitochondrial capacity) determined at various days of culturing of control and DC-treated PC-3 cells. The cytochrome c oxidase activity per culture remains nearly constant in the presence of 10 µg DC/ml, whereas it increases in the control cultures. The amount of
protein and DNA found in the DC cultures does not differ, however, from the protein and DNA content of the controls during the first 5 days of culturing. Because cytochrome c oxidase is partly dependent on mitochondrial gene expression, it follows that DC blocks mitochondrial protein synthesis specifically and almost immediately. At about the fifth day of culturing in the presence of 10 \( \mu \text{g} \) DC/ml cell proliferation stops; the DNA and protein contents of the cultures no longer increase. Comparison of the specific cytochrome c oxidase activity, which can be calculated from Fig. 2, on either a protein or a DNA basis, in control and DC cultures at the fifth day of culturing learns that reduction of this activity to about 35% leads to proliferation arrest of the PC-3 cells. The interpretation of the data on the extent of the decrease of the cytochrome c oxidase activity required to achieve proliferation arrest becomes more complex, however, if the specific cytochrome c oxidase activity of the control cultures at various days of culturing is taken into account (Table 1).

The specific cytochrome c oxidase activity as measured in control PC-3 cells is obviously not constant; it increases as culturing lasts longer. This increase of the specific cytochrome c oxidase activity will be impaired if mitochondrial protein synthesis is blocked by DC. From this it follows that calculations on the degree of inhibition of the specific cytochrome c oxidase activity required to impair proliferation should be made with the use of the control value at the moment of DC addition to the cultures as the 100% specific activity. The data in Table 1 show, therefore, that DNA synthesis becomes impaired by effective inhibition of mitochondrial protein synthesis if the specific cytochrome c oxidase activity is reduced to about 75% and that protein synthesis is blocked if the cytochrome c oxidase activity has been reduced to about 50%. It can be concluded that PC-3 cells are easily arrested in their \textit{in vitro} proliferation by DC. About one cell cycle during which mitochondrial protein synthesis is effectively blocked is sufficient to stop proliferation. Besides, it appears that the decrease of the oxidative ATP-generating capacity leads first to inhibition of DNA synthesis and later on to impairment of protein synthesis.

Table 2 shows the effect of DC on cultures of various renal tumor lines of human origin. In these experiments, cytochrome c oxidase activities, and DNA and protein contents were determined only at the end of the culture period. It cannot be concluded to what extent the specific cytochrome c oxidase activities of these lines must be reduced to arrest cell proliferation because the kinetics has not been studied as for PC-3 cells. The tested lines are, however, all sensitive to DC because the cytochrome c oxidase activity decreases in all tumor cell types studied. The data given in Table 2 and Fig. 1A suggest that the initial growth kinetics of the several renal tumor lines in the presence as well as in the absence of DC are mutually well comparable. It can be concluded that culturing for 5 days in the presence of DC is too short a time to achieve a cytostatic effect in the renal tumor cell lines, because after this period no significant differences between the DNA and protein content of the control and the DC-treated cultures are found. It can be seen, however, that the specific cytochrome c oxidase activity is lowered during this period of inhibition of mitochondrial protein synthesis. At 14 days of culturing in the presence of DC, when the specific cytochrome c oxidase activity has been reduced further, the renal tumor cell lines tested reveal without exception the cytostatic effect because after this period of DC treatment the total number of cells per culture, measured either as the DNA or as the protein amount per culture, is significantly lower in the DC-treated cultures. This implies that inhibition of mitochondrial protein synthesis precedes proliferation arrest also in renal tumor cell lines.

**In Vivo Studies**

Tumor Growth in Control and DC-treated Rats. Table 3 shows the frequency and course of NC-65 tumor growth patterns in control and in DC-treated rats. In control rats tumor growth usually became measurable at 10–12 days after tumor implantation, the tumors had doubled their size twice between days 16 and 20, when their volume was 2–3 cm\(^3\), and grew at about the same rate until they reached a volume of at least 8 cm\(^3\). This volume was usually reached between days 20 and 24 after tumor implantation. Thereafter, some tumors continued to grow, whereas other tumors remained at a size of about 8–10 cm\(^3\). The growth pattern described above is indicated as progressive in Table 3. Tumor regression, defined in our experiments as initial tumor growth to a volume of at least 1 cm\(^3\) and the complete disappearance of the tumor thereafter, was rarely found in control animals. In the DC-treated rats growth patterns comparable to those in control rats were found; the frequency at which these patterns occurred differed significantly, however, from the frequencies found in control rats (Table 3).

It should be stressed that the distribution of tumors which showed proliferation arrest in the presence of DC was not at random; per series of simultaneous experiments usually either all tumors regressed or none at all. Fig. 3 shows some representative curves of NC-65 tumor growth as found in the majority of the control and DC-treated rats, respectively. The kinetics of tumor growth in presence of DC is in line with the prediction made on the basis of the results shown in Figs. 1 to 3. The tumors first increase in volume in the presence of DC and only after some time is their growth arrested. Subsequently, the tumors disappear completely, even if DC treatment is stopped. The initial tumor growth in the presence of DC is most probably required to reduce the oxidative ATP-generating capacity by inhibition of mitochondrial protein synthesis to the extent that proliferation becomes impossible. If so, then it can be expected that the tumor size maximally reached in the presence of DC depends on the size of the tumor fragments used for implantation. Fig. 4 shows the results of an experiment in which 2 tumor fragments of different wet weights were implanted on the same animal. In the case of DC treatment, both tumors grow initially. The tumor arising from implantation with a fragment of about 100 mg reaches a volume of 3 cm\(^3\), whereas the one originating from a fragment of about 20 mg reaches a size of only 0.5 cm\(^3\) before growth is arrested. These experiments support, therefore,
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Table 2  Specific cytochrome c oxidase activities and protein and DNA content of various renal tumor cell cultures, grown in the presence or absence of DC

DC was added at day 0 of the culturing period in a final concentration of 10 μg/ml culture medium. Triplicate cultures were used to measure the DNA, protein, and cytochrome c oxidase activity. Each value represents the mean of the values found per culture. The SE was maximally 5.2%.

<table>
<thead>
<tr>
<th>Period of culturing (days)</th>
<th>Tumor cell line</th>
<th>Cytochrome c oxidase activity*/mg protein</th>
<th>Cytochrome c oxidase activity/mg DNA</th>
<th>μg protein/culture</th>
<th>μg DNA/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>NC-65</td>
<td>17.9 (55)</td>
<td>174.6</td>
<td>438 (110)</td>
<td>50 (114)</td>
</tr>
<tr>
<td></td>
<td>NC-65 + DC</td>
<td>9.9 (55)</td>
<td>93.6 (54)</td>
<td>483 (110)</td>
<td>50 (114)</td>
</tr>
<tr>
<td>5</td>
<td>RC-21</td>
<td>15.2 (73)</td>
<td>205.6</td>
<td>266 (90)</td>
<td>22 (79)</td>
</tr>
<tr>
<td></td>
<td>RC-21 + DC</td>
<td>11.0 (73)</td>
<td>172.5 (84)</td>
<td>330 (90)</td>
<td>22 (79)</td>
</tr>
<tr>
<td>14</td>
<td>RC-2</td>
<td>30.5 (29)</td>
<td>420.0</td>
<td>1213 (87)</td>
<td>69 (79)</td>
</tr>
<tr>
<td></td>
<td>RC-2 + DC</td>
<td>8.9 (29)</td>
<td>107.2 (26)</td>
<td>841 (69)</td>
<td>69 (79)</td>
</tr>
<tr>
<td>14</td>
<td>RCC-14</td>
<td>63.8 (16)</td>
<td>483.5</td>
<td>61 (9)</td>
<td>9 (67)</td>
</tr>
<tr>
<td></td>
<td>RCC-14 + DC</td>
<td>9.9 (16)</td>
<td>44.9 (9)</td>
<td>36 (59)</td>
<td>6 (67)</td>
</tr>
<tr>
<td>14</td>
<td>RC-43</td>
<td>50.2 (26)</td>
<td>658.7</td>
<td>492 (39)</td>
<td>39 (69)</td>
</tr>
<tr>
<td></td>
<td>RC-43 + DC</td>
<td>11.2 (26)</td>
<td>102.4 (16)</td>
<td>246 (50)</td>
<td>27 (69)</td>
</tr>
<tr>
<td>14</td>
<td>RC-8</td>
<td>32.1 (33)</td>
<td>599.6</td>
<td>769 (42)</td>
<td>42 (57)</td>
</tr>
<tr>
<td></td>
<td>RC-8 + DC</td>
<td>10.5 (33)</td>
<td>166.5 (28)</td>
<td>372 (48)</td>
<td>24 (57)</td>
</tr>
<tr>
<td>14</td>
<td>RC-51</td>
<td>16.5 (29)</td>
<td>327.1</td>
<td>867 (45)</td>
<td>45 (53)</td>
</tr>
<tr>
<td></td>
<td>RC-51 + DC</td>
<td>4.8 (29)</td>
<td>85.1 (26)</td>
<td>432 (50)</td>
<td>24 (53)</td>
</tr>
</tbody>
</table>

* Cytochrome c oxidase activity is expressed as the first-order rate constant.
* Numbers in parentheses, percentage of the value found in the controls.

Table 3  Frequency and course of NC-65 tumor growth during 35 days after tumor transplantation in control and continuously DC-treated nude rats

The growth patterns of NC-65 tumors was studied in 15 control and 15 DC-treated rats carrying 2 tumor transplants and in 14 control and 12 DC-treated rats carrying 1 tumor transplant. Rats were treated with DC such that the serum level remained at about 5 μg/ml during the total experimental period. DC treatment was started at day 3 and stopped at day 38 after tumor transplantation.

| Characteristics of NC-65 Tumor Mitochondria of Control and DC-treated Rats. To judge if inhibition of mitochondrial protein synthesis precedes the in vivo proliferation arrest of NC-65 tumors, information about, e.g., the cytochrome c oxidase activity in untreated and DC-treated tumors and their mitochondria is essential. Extended studies revealed, however, that the control or 100% cytochrome c oxidase activity, expressed as either per mg protein or per mg DNA in tumor homogenates the fact that the growth-arresting effect of DC was not always found, an extensive study was made on the properties of tumors and their mitochondria after various periods of treatment with DC.

Fig. 3. Effect of DC on the in vivo growth of NC-65 tumors. The growth patterns of a number of typical experiments (2 control and 2 DC-treated rats, each implanted with 1 tumor fragment) are shown. DC was given in such a way that DC serum levels remained at about 5 μg/ml. DC treatment was started at day 3 and stopped at day 38 after tumor transplantation. •, tumor growth in controls; ○, tumor growth in DC-treated rats; ◯, growth pattern after stopping DC infusion; □, implanted with a tumor fragment of about 100 mg (wet weight); △, implanted with a tumor fragment of about 20 mg (wet weight).
or per mg protein in the isolated mitochondria, varied considerably. The SE ranged between 10 and 20% for all these parameters in 15 control tumors tested. Spectral studies of mitochondria indicated that the amount of cytochromes $c + c_1$ (cytochromes composed of cytoplasmically made subunits exclusively) and the amount of cytochrome $aa_3$ (the spectral equivalent of cytochrome $c$ oxidase) also showed large variations. The ratio between these cytochromes appeared, however, to be quite constant. For these reasons it was decided to use the ratio of the amount of cytochrome $aa_3$ to the amount of cytochromes $c + c_1$ in the isolated mitochondria as a measure for the extent of inhibition of mitochondrial protein synthesis in the NC-65 tumors of DC-treated animals. Table 4 shows these ratios in the mitochondria of NC-65 tumors of control rats and rats treated for various periods with DC. Depending on whether the treated rats belonged to a series of experiments in which the tumor growth-inhibiting effect of DC was seen or not, the DC-treated animals were divided in 2 categories in this table. It can be seen in Table 4 that in the tumors of rats of the former category, proliferation arrest is preceded by inhibition of mitochondrial protein synthesis. It can also be deduced from Table 4 that the amount of cytochrome $aa_3$ (and, therefore, the cytochrome $c$ oxidase activity) must be reduced to about 30% before proliferation stops. This value is in good agreement with the value which was extrapolated from our in vitro experiments with NC-65 cells (Fig. 1, reduction to about 25%). It can also be seen in Table 4 that mitochondrial protein synthesis in the tumors, belonging to the series of experiments in which growth arrest was rarely found, is less effectively blocked than in the tumors of the series in which growth arrest was usually seen. It can, therefore, without doubt be stated that prolonged DC treatment leads also in vivo to proliferation arrest of NC-65 tumor growth, provided that mitochondrial protein synthesis is inhibited to an extent that the amount of mitochondrialy made polypeptide products is reduced to about 30%.

The spectral data of the mitochondrial fractions isolated from regressive DC-treated tumors deserve, as will be clear from Fig. 5, A to C, some special comments. Fig. 5A shows the spectrum of control tumor mitochondria. Fig. 5B shows mitochondria derived from a tumor arrested in its growth by DC, and Fig. 5C shows the mitochondrial fraction from a tumor regressing in the presence of DC. In the latter tumors, spectral analysis reveals that the ratio of cytochrome $aa_3$ to cytochromes $c + c_1$ increases again, even to ratios higher than found in controls. Because of the large variety in the amounts of cytochromes $aa_3$ and cytochromes $c + c_1$ per mg mitochondrial protein, it cannot be decided whether this increase is due to a decreasing amount of cytochromes $c + c_1$ or to an increasing amount of cytochrome $aa_3$. Fig. 5C shows very clearly that the ratio of cytochrome $b$ to cytochromes $c + c_1$ is greatly enhanced. This could mean that the amount of cytochromes $c + c_1$ is largely reduced in the regressing tumors. It seems, however, more likely that the regressing DC-treated tumors are infiltrated with phagocytic cells. These cells are equipped with peroxisomes, which may contaminate the tumor mitochondria. Peroxisomes contain large amounts of cytochrome $b_5$ perform a variety of oxygen-consuming activities, and possess, in contrast to mitochondria, no oligomycin-sensitive ATPase activity. Because the latter two characteristics were also found in the mitochondrial fractions of the regressing tumors, contamination is a reasonable explanation.

Distribution of DC over NC-65 Tumor Cells. Table 5 shows the DC concentration, expressed per mg protein in the homogenates and in the mitochondria, of the tumors in the rats described in Table 4. Comparison of the data in Tables 4 and 5 leads to two conclusions. First, the difference between the series of experiments in which tumor growth arrest was usually found and those in which this result was an exception is clearly based on differences in the DC concentrations in the respective mitochondria. The DC concentration in the homogenates of tumors of different series, however, is well comparable. From

### Table 4: Ratio of cytochrome $aa_3$ to cytochromes $c + c_1$ in the mitochondrial fractions of NC-65 tumors of control and DC-treated rats

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>Group 1*</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive, controls</td>
<td>0.38 (8)*</td>
<td>0.35 (7)</td>
</tr>
<tr>
<td>Progressive, DC-treated for 6 days</td>
<td>0.25 (2), 69%</td>
<td>0.16 (3), 67</td>
</tr>
<tr>
<td>14–16 days</td>
<td>0.13 (4), 36</td>
<td>0.24 (3), 44</td>
</tr>
<tr>
<td>21–24 days</td>
<td>0.14 (2), 39</td>
<td>0.16 (4), 44</td>
</tr>
<tr>
<td>24–35 days</td>
<td>0.18 (2), 50</td>
<td>0.22 (4), 61</td>
</tr>
<tr>
<td>Arrested, DC-treated for 16–20 days</td>
<td>0.10 (3), 29</td>
<td></td>
</tr>
</tbody>
</table>

* Group 1, values found in sets of experiments in which DC-caused tumor growth arrest was usually seen; Group 2, values found in sets of experiments in which DC-caused tumor growth arrest was generally absent.

### Table 5: DC concentration in homogenates and mitochondria of NC-65 tumors of rats treated for various periods

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive, DC-treated for 14–16 days</td>
<td>0.72 (3)*</td>
<td>0.70 (3)</td>
</tr>
<tr>
<td>21–24 days</td>
<td>0.78 (2)</td>
<td>0.71 (4)</td>
</tr>
<tr>
<td>24–35 days</td>
<td>0.72 (1)</td>
<td>0.69 (4)</td>
</tr>
</tbody>
</table>

* Group 1, DC concentrations found in sets of experiments in which DC-caused tumor growth arrest was usually seen; Group 2, DC concentrations found in sets of experiments in which DC-caused growth arrest was generally absent.

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This it follows that the intracellular distribution of DC varies per set of experiments. In Table 6 the intracellular distribution of cytochrome c oxidase and DC in 4 series of experiments is given. Because cytochrome c oxidase is located exclusively in the mitochondria, the distribution of this enzyme is a reliable measure for the distribution of the mitochondria over the tumor cell fractions. It can be concluded from the data in Table 6 that the distribution of the mitochondria does not significantly differ in the various experiments. The affinity of the tumor mitochondria for DC shows, however, significant variations, depending on the series of experiments the recovery of DC versus the recovery of cytochrome c oxidase is either 0.4 or 0.7. Because the experiments were done with one batch of nude rats and with tumor fragments derived from one tumor-carrying rat or mouse per series, it seems probable that heterogeneity with respect to the affinity of the mitochondria for DC is inducible in the NC-65 tumor.

The second conclusion which can be drawn from the comparison of the data in Tables 4 and 5 is that it cannot be excluded that DC becomes less effective in inhibiting mitochondrial protein synthesis if treatment lasts longer. Within a series of experiments the mitochondrial DC content does not vary significantly (Table 5), whereas the inhibiting effect is gradually lost after 20 days of treatment (Table 4).

**DISCUSSION**

The results of this study demonstrate that in principle mitochondrial protein synthesis can be used as a target for chemotherapy in the case of tumors of human origin. Effective inhibition of mitochondrial protein synthesis by DC in tissue culture leads to the arrest of proliferation of the human tumor lines tested. The sequence of events found in the experiments on the relationship between inhibition of mitochondrial protein synthesis and growth arrest is fully in line with our previous results (6, 10). Also in tumor lines of human origin, specific inhibition of mitochondrial protein synthesis is the primary effect of DC treatment, whereas growth arrest can be achieved as a secondary result.

Proliferation arrest of these tumor lines was found whenever the specific cytochrome c oxidase activity had been reduced by DC treatment to about 30% of the control value. This implies that these tumor cells of human origin do not differ largely in their reserve capacity for oxidative phosphorylation from the animal tumor systems, inasmuch as we have shown before that the specific cytochrome c oxidase activity had to be reduced to 40% in rat Leydig cell tumor mitochondria (7) and to 25% in methylnitrosourea-induced rat mammary gland tumors (4) to achieve this arrest. The *in vivo* results of the present study thus indicate that the tetracyclines may be of value as cytostatic agents in humans.

The decisive criterion for this possible application is of course whether the cytostatic action of the tetracyclines is also found in *in vivo* studies. For this reason we made a detailed study in a nude rat model system on the effect of DC treatment on the growth of the NC-65 tumor, a renal adenocarcinoma of human origin. These experiments lead to a number of important conclusions. First of all, the NC-65 tumors stop growing *in vivo* if the specific cytochrome c oxidase activity (determined spectrally as cytochrome aa₃ and expressed as the ratio of cytochrome aa₃ to cytochromes c + c₁) has been reduced by inhibition of mitochondrial protein synthesis to about 30%. Also the growth arrest *in vivo* is preceded by a gradual decrease of the specific cytochrome c oxidase activity. There are, therefore, no basic differences between the *in vitro* and *in vivo* effects of effective prolonged inhibition of mitochondrial protein synthesis by DC on NC-65 tumor growth. It appeared, however, that the DC concentration inside the tumor mitochondria, at rat serum levels of 5 µg DC/ml, was not always sufficient to impair mitochondrial protein synthesis effectively. In the present study it was found that the NC-65 tumors showed heterogeneity as far as the intracellular distribution of DC was concerned. Therefore, it seems likely that the absence of growth arrest seen in the tumors with a relatively low mitochondrial affinity for DC is due to an inadequate inhibition of mitochondrial protein synthesis. It is very probable that similar differences in the intracellular DC distribution as well as differences in the tissue to serum ratio exist between various kinds of tumors. Therefore, it should be realized that the growth of some tumor types may be influenced at low DC serum levels whereas for other kinds of tumors higher DC serum levels are required. If clinical trials to the effect of tetracycline treatment on malignant tumor growth in humans are considered, the affinity for the tetracyclines of tumor types and their mitochondria should certainly be taken into account.

Another important conclusion is that effective DC treatment causes not only proliferation arrest of the NC-65 tumor cells but also complete tumor regression. In previous studies the cytostatic effect of the tetracyclines on solid tumor growth appeared to be reversible (7). In those studies the tetracyclines were, however, administered for maximally 3 weeks. Experiments in which the effect of DC treatment for 2–3 weeks on the growth of the NC-65 tumor were studied (not shown) resulted also in a reversible cytostatic effect. After 2–3 weeks of treatment either the originally proliferating cells are, therefore, able to restore their ATP-generating capacity, or resting tumor cells still have the possibility to reenter the cell cycle. Recruitment from the resting compartment will, because of the continuous turnover of the cytoplasm which reduces the amount of oxidative energy-generating capacity also in resting tumor cells, only be possible for a limited period during prolonged DC treatment. Therefore, it seems likely that a period of 5 weeks of DC administration to NC-65-carrying rats is long enough to reduce also the oxidative ATP-generating capacity of the cells in the resting tumor compartment to such an extent that, in the case of recruitment, proliferation is inhibited. Complete exhaustion of a potentially cycling compartment leads to tumor regression because under such circumstances the size of the cell loss fraction exceeds the size of the growth fraction.

Whereas treatment for 5 weeks leads to an irreversible effect

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**Table 6 Intracellular distribution of DC and cytochrome c oxidase in NC-65 tumors in 4 sets of experiments on the effect of DC on in vivo NC-65 tumor growth**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sets</th>
<th>Total DC content (%)</th>
<th>Total cytochrome c oxidase activity (%)</th>
<th>Recovery of DC</th>
<th>Recovery of cytochrome c oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1, 2, 3, 4 (21)</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postnuclear</td>
<td>1, 2, 3, 4 (21)</td>
<td>73.3 ± 1.2</td>
<td>73.7 ± 1.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>1, 2, 3, 4 (21)</td>
<td>33.3 ± 1.4</td>
<td>33.3 ± 1.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>1, 3 (10)</td>
<td>13.8 ± 0.6</td>
<td>13.8 ± 0.6</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

* cytochrome c oxidase activity is expressed as the first-order rate constant.
* Numbers in parentheses, number of experiments.
* Mean ± SE of the percentages of the DC or cytochrome c oxidase activity content in the homogenate.
on tumor growth and treatment for about 3 weeks to a reversible cytostatic effect, there were no indications that the side effects for normal tissues are far more serious after 5 weeks than after 3 weeks of treatment. In healthy rats DC treatment at a serum level of 5 μg/ml for 5 weeks leads to some loss of weight, which is probably based on a reduced function of the small intestine (9). This side effect is, however, found no sooner than after about 17 days of continuous treatment and is readily reversible if DC treatment is stopped. Untreated nude rats, carrying the NC-65 tumor, lose weight because of the presence of the tumor. Death, as a result of tumor growth, was, at least within the experimental period studied, not recorded. DC-treated NC-65-carrying rats lose weight to about the same extent as the controls, most likely as the result of the (initial) presence of the tumor and because of DC treatment. Also in this case we never observed any deaths. After DC infusion was stopped the treated nude rats in which tumor regression was found regained weight quickly. An explanation for the apparent absence of serious side effects for most normal tissues after a period of 5 weeks of treatment may be based on the large reserve capacity for oxidative phosphorylation of the majority of the normal tissues (10).

It could be argued that the NC-65 xenograft model system is that fragile that a slight cytostatic effect of DC is amplified by, e.g., host immunity which subsequently would lead to tumor regression. Recent experiments with a leukemic tumor in the rat (8) revealed, however, that prolonged DC treatment leads to tumor regression also in a less artificial tumor system. Because this leukemic tumor is strictly syngeneic, effects of DC which would lead to specific or nonspecific immune stimulation and consequently to tumor regression are unlikely under these conditions. It is moreover improbable that the NC-65 tumor, growing in nude rats, induces host immunity responses or that DC treatment evokes or intensifies a possible host immunity. Control rats, having carried the NC-65 for 40 days as well as DC-treated rats in which complete NC-65 regression was found after 35 days of treatment, do not reject a second NC-65 graft. Because we have evidence that the mitochondrial fractions of regressing tumors are contaminated with peroxisome-like organelles, it seems, however, likely that the NC-65 tumors become infiltrated with, e.g., macrophages or monocytes once their growth is arrested by DC treatment. It cannot therefore be excluded that aspecific host defense mechanisms can more successfully combat tumor cells if these cells have a severely reduced energy-generating capacity or a reduced growth rate. Such conditions will also cause or contribute to tumor regression.

From the results presented, it will be clear that the tetracyclines may have therapeutic potential in the treatment of human tumor growth. It can be argued that the NC-65 model represents a rapidly growing tumor system. Such tumors generally can be treated rather successfully. It should be realized, however, that the success of tetracycline treatment in anticancer therapy does not depend only on the size of the proliferating compartment of a tumor, because mitochondrial protein synthesis will also be impaired in the resting compartment. It should, moreover, be noticed that the NC-65 tumor belongs to a tumor class (renal tumors) which is highly resistant to currently used cytostatic agents. This high resistance is found not only clinically (21) but also in studies on the results of treatment with almost any cytostatic agent known in NC-65-carrying nude mice (22).

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REFERENCES

Arrest of the Proliferation of Renal and Prostate Carcinomas of Human Origin by Inhibition of Mitochondrial Protein Synthesis

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