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Inhibition of mitochondrial protein synthesis influences the glucocorticoid sensitivity of lymphoid cells

Coby Van den Bogert, Bert H.J. Dontje, Trudi E. Melis, Cobi van der Veen and Albert M. Kroon

Laboratory of Physiological Chemistry, State University Groningen, Bloemsingel 10, Groningen (The Netherlands)

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Inhibition of mitochondrial protein synthesis impairs the formation of the 13 polypeptides encoded on the mitochondrial genome. These polypeptides are part of enzyme complexes involved in oxidative phosphorylation. Prolonged inhibition of mitochondrial protein synthesis thus reduces the oxidative phosphorylation capacity which ultimately results in impairment of energy-requiring processes. Via a different mechanism glucocorticoid hormones also decrease the oxidative phosphorylation capacity of, e.g., lymphoid cells. The present study shows that inhibition of mitochondrial protein synthesis influences glucocorticoid-induced responses of lymphoid cells in two opposing manners. (a) It is enhanced after induction in cells with a reduced oxidative phosphorylation capacity resulting from preceding inhibition of mitochondrial protein synthesis. This can be explained by the synergistic effects of glucocorticoids and prolonged inhibition of mitochondrial protein synthesis on energy-producing processes. (b) It is counteracted when mitochondrial protein synthesis is impaired during induction of the response. The latter observation suggests that mitochondrial protein synthesis is involved in the generation of glucocorticoid-induced effects on lymphoid cells.

Introduction

Mitochondria contain mitochondrial specific DNA which contains in mammalian cells 13 polypeptide genes. These genes all code for subunits of enzymes of the oxidative phosphorylation system [1]. The mitochondrial genome is expressed by mitochondrial-specific transcription and translation systems. These processes can be inhibited by compounds which do not directly interfere with nuclear gene expression. Mitochondrial protein synthesis is inhibited by, e.g., low concentrations of tetracyclines, whereas cytoplasmic protein synthesis remains unaffected [2]. Continuous inhibition of mitochondrial protein synthesis reduces the concentration of the polypeptides of mitochondrial-genetic origin, which, in turn, reduces the capacity for oxidative phosphorylation. Once the capacity to produce ATP becomes limiting, energy-demanding processes proceed more slowly and subsequently are impaired. We have demonstrated previously that cell proliferation is inhibited as a consequence of prolonged impairment of mitochondrial protein synthesis in several in vivo and in vitro systems [3–6].

We also investigated the effect of tetracycline treatment on the proliferation of malignant

Correspondence (present address): C. Van den Bogert, Laboratory of Biochemistry, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

lymphoid cells in the rat [7,8]. In this tumor model, cytostatic and cytotoxic effects of tetracycline treatment were found consistently. However, the treatment appeared to be more effective when it was started in the later stages of tumor progression. Furthermore, we noticed changes in the pattern of nuclear proteins of the tumor cells which we could ascribe to glucocorticoid-induced effects (Van den Bogert, C., Bakker, H.M., Kuzela, S., Melis, T.E. and Kroon, A.M., unpublished data). The course of these changes was also dependent on the stage of tumor progression at which tetracycline treatment was started. Glucocorticoid hormones are known to have inhibitory effects on leukemic cell growth [9], the hormones affect oxidative ATP generation among other processes. Furthermore, tumor growth in murine model systems is frequently reported to induce the production of stress hormones such as the corticosteroids [10]. Interference of tetracycline treatment with effects of endogenous corticosteroids might thus explain the relationship between the effectivity of tetracycline treatment and the stage of tumor progression in which tetracycline administration is started.

To investigate this, we studied the influence of inhibition of mitochondrial protein synthesis on glucocorticoid action in normal and leukemic lymphocytes of the rat in vivo, as well as in human leukemic cells in vitro. The results of our study show that, depending on the duration of inhibition of mitochondrial protein synthesis, glucocorticoid-induced effects are either antagonized or enhanced.

Materials and Methods

Experimental animals. Male PVG/c rats (CPB, Nijmegen, The Netherlands) weighing about 220 g were used for the studies on the Roser leukemia. Male Wistar rats (CDL, Grongingen, The Netherlands) weighing about 200 g were used in studies on healthy animals.

Cell systems. The Roser leukemia was serially transplanted by intravenous (i.v.) inoculation of 10^4 tumor cells derived form the peripheral blood of tumor-carrying rats. The characteristics of this tumor, which is strictly syngenic with the rat strain PVG/c, have been described before [7,8]. In short, the pathophysiology of the tumor bears much resemblance to human acute leukemia. The pattern of tumor spread is complex, but reproducible in various series of experiments. After i.v. injection of tumor cells, tumor growth is first observed in the bone marrow, the peripheral blood and the spleen. Subsequently, leukemic cells are found in various lymphoid organs, while disappearing from the blood in this stage. After inoculation of 10^4 tumor cells, the rats die at about 38 days after inoculation, because of the disease which in its terminal stage also affects several non-lymphoid vital organs.

The human leukemia cell line Molt-4 was kindly provided by the Division of Hematology, Department of Internal Medicine, University Hospital, Nijmegen, The Netherlands. The cells were cultured in RPMI 1640 medium (Boehringer) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 IU penicillin/ml, 100 μg streptomycin/ml and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air.

Tetracycline and glucocorticoid treatment. For in vitro studies, dexamethasone acetate (Sigma) and doxycycline (Sigma) were added in final concentrations of 1 μM and 10 μg/ml, respectively, using stock solutions prepared in ethanol. The final concentration of ethanol never exceeded 0.1%, control cultures received the same amount of ethanol.

For in vivo studies, dexamethasone phosphate (Decadron, Merck Sharp & Dohme, Haarlem, The Netherlands) or doxycycline (Vibramycin, Pfizer, Rotterdam, The Netherlands) diluted with 0.15 M NaCl, were 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 [11]. The doxycycline dosage was chosen in such a way that doxycycline serum levels remained between 5 and 10 μg/ml. To this end, 20 mg doxycycline/kg per day was administered during the first week of treatment, 30 mg doxycycline/kg per day during the second, and 40 mg doxycycline/kg per day in the weeks thereafter. Dexamethasone was administered in the dosages indicated in the results. Control animals were infused with 0.15 M NaCl only. In some experiments, adrenalectomized rats were used. Adrenalectomy was performed under diethyl ether anesthesia 10 days or more before

the installation of the continuous infusion device. In the period before infusion, adrenalectomized rats were given 0.15 M NaCl solution to drink. The continuous infusion device was installed 7–10 days before tumor implantation or the start of dexamethasone or doxycycline treatment.

Registration of the effects of doxycycline and glucocorticoids. Tumor carrying animals were inspected three times a week. Their weight was recorded, and about 50 µl of blood was taken from the tail vein. The blood sample was used to register the number of nucleated cells with the aid of an electronic cell counter. At the end of each experiment, the wet weights of the thymuses were determined.

The body weight of normal Wistar rats was recorded every other day. At the end of the experimental period, the wet weight of the thymus was registered.

Effects on the in vitro growth of Molt-4 cells were followed by counting the number of cells in the cultures at regular intervals. The number of viable cells was determined by Trypan blue exclusion. The values given are the mean of four independent determinations of comparable samples. For analytical purposes, the cells were collected by centrifugation, washed twice with phosphate-buffered saline (15 mM phosphate/0.15 M NaCl, pH 7.4) and stored at −20 °C.

Analytical assays. Cytochrome *c* oxidase activity was measured spectrophotometrically according to a modification of the method of Cooperstein et al. [12]. The activity was calculated per min and expressed as the first-order reaction rate constant, *K*. The ATP content of the cells was assessed in a neutralized perchloric acid extract by reverse-phase HPLC using a µBondapak C8 column and 0.1 M KH_2PO_4 (pH 4.6) as eluent (cf. Ref. 13).

Results

Effects of doxycycline and glucocorticoids on the growth of the Roser leukemia

As outlined in the introduction, interference of the effects of tetracyclines and endogenous glucocorticoids might explain the relationship between the effectivity of tetracycline treatment and the stage of tumor progression in which tetracycline administration is started. This implies that Roser leukemic cells are sensitive to glucocorticoid treatment and that in vivo tumor growth is modulated by endogenous glucocorticoids. To test this essential part of our hypothesis two approaches were used.

First, the effect on tumor growth of continuous i.v. infusion of dexamethasone was investigated. Fig. 1 shows the results of different doses on the development of the tumor in the peripheral blood of control animals; the wet weights of the thymuses illustrate the effect of dexamethasone treatment on the solid form of the tumor. Dexamethasone treatment clearly reduces tumor growth in a dose-dependent way. The differential sensitivity to dexamethasone between malignant and normal

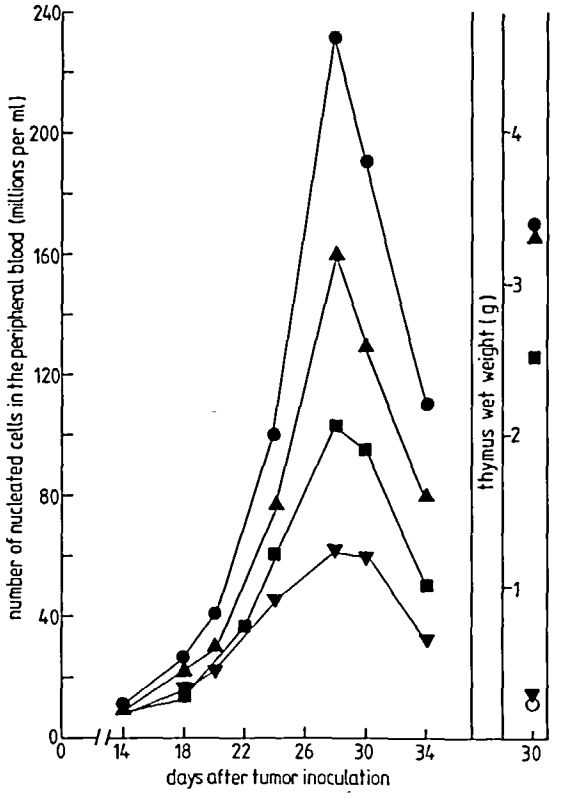

Fig. 1. Effect of dexamethasone treatment on the development of the Roser leukemia. Dexamethasone treatment was started at day 0 of tumor inoculation. ●, 0 µg dexamethasone/kg per day; ▲, 12.5 µg dexamethasone/kg per day; ■, 25 µg dexamethasone/kg per day; ▼, 50 µg dexamethasone/kg per day; ○, untreated controls, no tumor. The mean value found in 2 (○, ●, ▲, ■) or 6 (▼) animals is given. The S.E. ranged from 0.5 to 2.1% of the mean values.

tissues is, however, not large. The lowest dexamethasone dosage which still shows inhibition of tumor growth also affects normal tissues as is illustrated by, e.g., the effects on body weight (Fig. 2). Doxycycline treatment, started simultaneously with dexamethasone administration, appears, however, to reduce the dexamethasone-related loss of body weight with about 50% (12.5–25 μg dexamethasone/kg per day) to 30% (50 μg dexamethasone/kg per day).

The data of Table I demonstrate that doxycycline also interferes with the inhibitory effects of dexamethasone on tumor growth. Dexamethasone treatment reduces the number of nucleated cells in the peripheral blood to about 50% in any stage of tumor development in control rats. In rats treated with doxycycline on day 0 of inoculation (DC_0), however, a significant effect of dexamethasone on the tumor load was not observed at day 18 after inoculation. Between day 18 and day 30 the effect of dexamethasone increased gradually, but remained less than that in the controls. The effect of dexamethasone was, in contrast, far more pronounced in DC_0 rats than in control rats at days 30 and 35. In this period, the cytostatic effect of doxycycline treatment was fully achieved [7,8].

The course of the dexamethasone-related antitumor effects in the presence of doxycycline suggest that doxycycline antagonizes dexamethasone-induced responses as long as doxycycline treatment has not yet resulted in cytostasis. The data also suggest, however, that dexamethasone-induced responses are enhanced considerably in cells of which the proliferation has been arrested by prolonged doxycycline treatment.

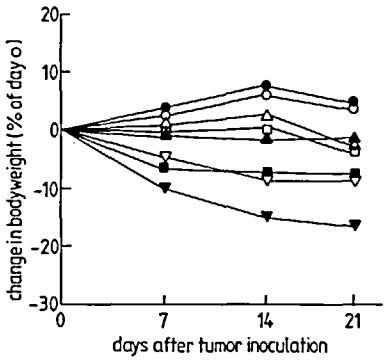

Fig. 2. Effect of dexamethasone on the body weight of Roser-carrying rats. Dexamethasone treatment was started at day 0 of tumor inoculation. ○, ●, 0 μg dexamethasone/kg per day; △, ▲, 12.5 μg dexamethasone/kg per day; □, ■: 25 μg dexamethasone/kg per day; ▽, ▼: 50 μg dexamethasone/kg per day. Open symbols: +doxycycline, treatment started at day 0 after tumor inoculation. Closed symbols: without doxycycline. The mean value found in 24 (●, ▲, ■), 18 (○, △, □) or 6 (▽, ▼) animals is given. The S.E. ranged between 2 and 5% of the mean value.

TABLE I

EFFECT OF DEXAMETHASONE (25 μg/kg PER DAY) ON THE DEVELOPMENT OF THE ROSER LEUKEMIA IN THE PRESENCE OF DOXYCYCLINE

Subscripts denote the day tumor inoculation at which treatment was begun. S.E. was maximally 2.8%.

Days after tumor inoculation	Number of nucleated cells in the peripheral blood (10^6/ml)			
	treatment		treatment	
	none (control)	plus DEX_0 ($n = 24$)	DC_0 ($n = 18$)	DC_0 plus DEX_0 ($n = 18$)
18	27 [c]	13 [c] (48) [a]	20 [d]	18 [d] (90) [a]
24	101	60 (59)	68	51 (74)
28	232	108 (47)	123	80 (65)
30	194	95 (49)	90	15 (17)
35	110	50 (46)	57	10 (18)

[a] Percentage of the control value (control and DC_0, respectively).

Second, the growth pattern of the Roser leukemia in adrenalectomized rats was studied. This approach may indicate whether or not tumor development is influenced by endogenous glucocorticoids. Unfortunately, over 50% of the adrenalectomized tumor-carrying control rats died within 5 weeks after tumor inoculation. This high mortality was observed neither in adrenalectomized control rats without a tumor nor in adrenalectomized tumor-carrying rats treated with doxycycline. Conclusions about the influence of endogenous glucocorticoids on tumor development in control rats can therefore not be drawn. Studies on the effects of adrenalectomy and dexamethasone administration on the course of the wet weight of the thymus in tumor-bearing doxycycline-treated rats, lead, however, to an interesting observation. In the presence of doxycycline, tumor growth is arrested in this organ at about day 28 after inoculation, both in DC_0 and DC_{14} rats. Thereafter, the size of the thymus (but also of

TABLE II

EFFECT OF ADRENALECTOMY OR DEXAMETHASONE (25 μg/kg PER DAY) TREATMENT ON THE WET WEIGHT OF THE THYMUS OF ROSER LEUKEMIA-CARRYING RATS

Subscripts denote the day of tumor inoculation at which treatment was begun. ADM, adrenalectomized. S.E. was maximally 4.8%.

Days after tumor inoculation	Thymus wet weight (g)								
	treatment			treatment			treatment		
	none ($n = 24$)	plus ADM ($n = 12$)	plus DX_0 ($n = 18$)	DC_{14} ($n = 24$)	DC_{14} plus ADM ($n = 12$)	DC_{14} plus DEX_0 ($n = 18$)	DC_0 ($n = 24$)	DC_0 plus ADM ($n = 12$)	DC_0 plus DEX_0 ($n = 18$)
30	3.38	3.82	2.52	1.84	1.54	1.48	0.88	0.86	0.80
35	4.32	– [a]	3.15	0.19	0.79	0.22	0.52	0.58	0.18

[a] No animals survived.

other tumor-infiltrated organs) declines at a rate which depends on the stage of tumor progression in which doxycycline treatment is started [7]. As shown in Table II, the rate of weight reduction is considerably faster in DC_{14} than in DC_0 rats. Treatment with dexamethasone abolishes this difference: under these conditions the decline in tumor mass is comparable to that found in DC_{14} rats not treated with dexamethasone. Adrenalectomy, on the other hand, results for both DC_{14} and DC_0 rats in a rate of size reduction comparable to that found in normal DC_0 rats. These data strongly suggest that the action of endogenous corticosteroids is responsible for the fast decline in tumor load once tumor growth has been arrested in DC_{14} rats, and that such an effect is absent or less in DC_0 rats. The data confirm, moreover, that the anti-tumor effect of either exogenous or endogenous corticosteroids is enhanced considerably

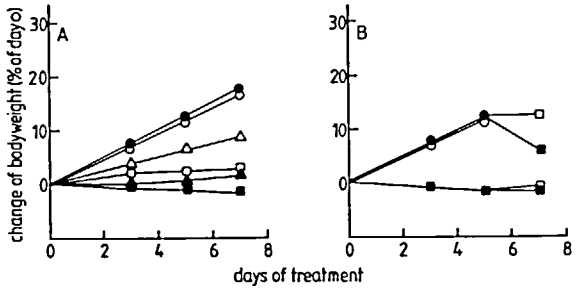

Fig. 3. Effect of doxycycline and dexamethasone on the body weight of wistar rats. (A) ●, untreated controls ($n = 20$); ○, doxycycline-treated ($n = 32$); ▲, 12.5 μg dexamethasone/kg per day ($n = 9$); △, 12.5 μg dexamethasone/kg per day + doxycycline ($n = 9$); ■, 25 μg dexamethasone/kg per day ($n = 9$); □, 25 μg dexamethasone/kg per day + doxycycline ($n = 9$). Doxycycline and dexamethasone treatments were started at day 0. (B) ●———□, controls, dexamethasone (25 μg/kg per day) treatment started at day 5 ($n = 6$); ○———□, doxycycline treatment started at day 0, dexamethasone (25 μg/kg per day) treatment started at day 5; ■———■: controls, dexamethasone (25 μg/kg per day) treatment started at day 0 ($n = 6$); ■———□: dexamethasone (25 μg/kg per day) treatment started at day 0, doxycycline treatment started at day 5 ($n = 6$). The initial weights ranged between 200 and 220 g, the S.E. was maximally 5% of the percentage change.

TABLE III

WET WEIGHTS OF THYMUSES OF WISTAR RATS TREATED WITH DOXYCYCLINE AND/OR DEXAMETHASONE DURING 7 DAYS

Subscripts denote day of experimental period at which treatment was begun.

Treatment	Thymus wet weight (g ± S.E.)	No. of animals
None	0.52 ± 0.03	(20)
DC_0	0.54 ± 0.05	(32)
$+DEX_0$ (12.5 μg DEX/kg per day)	0.25 ± 0.02	(9)
$DC_0 + DEX_0$ (12.5 μg DEX/kg per day)	0.41 ± 0.03	(9)
$+DEX_0$ (25 μg DEX/kg per day)	0.21 ± 0.01	(9)
$DC_0 + DEX_0$ (12.5 μg DEX/kg per day)	0.33 ± 0.02	(9)
$DC_5 + DEX_0$ (12.5 μg DEX/kg per day)	0.24 ± 0.01	(6)
$+DEX_5$ (25 μg DEX/kg per day)	0.36 ± 0.02	(6)
$DC_0 + DEX_5$ (12.5 μg DEX/kg per day)	0.45 ± 0.02	(6)

TABLE IV

EFFECTS OF DEXAMETHASONE AND DOXYCYCLINE ON THE GROWTH OF MOLT-4 CELLS

Subscripts denote the day of culture at which treatment was begun. Values are mean percentages found in three different experiments, individual values ranged maximally 5% from this mean. The percent increase is given as a percentage of the value of the previous day.

Day	% increase in cell number per day						
Treatment:	DC_0	DEX_0	DEX_0DC_0	DEX_0DC_1	DEX_0DC_2	DC_0DEX_1	DC_0/DEX_2
1	79 (< 5) [a]	94 (< 5)	80 (< 5)	94 (< 5)	94 (< 5)	79 (< 5)	79 (< 5)
2	28 (< 5)	41 (< 5)	3 (15)	27 (7)	41 (< 5)	19 (< 5)	32 (< 5)
3	3 (< 5)	41 (< 5)	1 (40)	1 (35)	13 (37)	−46 (51)	−17 (42)

[a] % of dead cells.

when tumor growth has been arrested by prolonged doxycycline treatment.

Effects of doxycycline and glucocorticoids on normal rats

To investigate whether or not interference of DC- and glucocorticoid-induced effects is limited to tumor cells or tumor-carrying animals, experiments on healthy Wistar rats were performed. Body and thymus wet weights were used as measures for dexamethasone-induced effects. Fig. 3 shows that doxycycline counteracts the dexamethasone-related reduction of body weight also in normal rats. Table III demonstrates that comparable effects of doxycycline are found for dexamethasone-related changes in wet weight of a glucocorticoid-sensitive organ, the thymus. Doxycycline treatment decreases the dexamethasone-induced reduction of body- or thymus weight with 50% at a dexamethasone dosage of 12.5 μg/kg per day, and with 20–30% at a dexamethasone dosage of 25 μg/kg per day. The antagonizing effect of doxycycline is found whenever doxycycline is present if dexamethasone treatment is started. Doxycycline treatment does not have cytostatic effects in normal rats within an experimental period of 7 days [11]. This explains why only the antagonizing effect of the doxycycline/dexamethasone combination is seen. The effect of dexamethasone treatment started at day 5 is, e.g., still counteracted by DC_0 treatment.

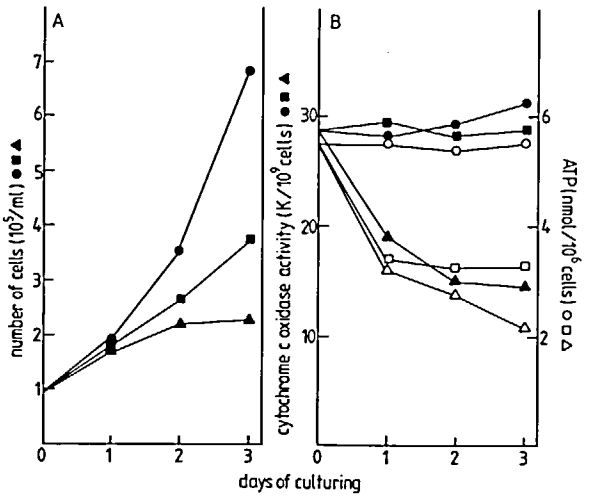

Fig. 4. Effect of doxycycline or dexamethasone on Molt-4 cells. Dexamethasone (1 μM, □, ■) or doxycycline (10 μg/ml, △, ▲) were added at day 0 of culturing. ○, ●: controls. The mean values from four different experiments are shown. A, effects on proliferation; B, effects on mitochondrial function.

Effects of doxycycline and glucocorticoids on leukemic cells in vitro

To analyze the effects of doxycycline and dexamethasone further, the investigations were extended to leukemic cells in tissue culture. To this purpose the human leukemic line Molt-4 was chosen for two reasons. First, it has not been possible so far to maintain the Roser leukemia in culture and second, glucocorticoids are not cytotoxic for Molt-4 cells [14]. The latter allows experiments in which the effect of dexamethasone and doxycycline can be studied for a number of days.

The effects of either doxycycline or dexamethasone on the growth pattern of Molt-4 cells are shown in Fig. 4A. As outlined in the introduction,

doxycycline treatment leads to proliferation arrest secondary to inhibition of mitochondrial protein synthesis. Inhibition of mitochondrial protein synthesis impairs the formation of a number of enzymes involved in oxidative phosphorylation, as is illustrated for cytochrome *c* oxidase in Fig. 4B, and halves the oxidative phosphorylation capacity per cell doubling. In Molt-4 cells, this dilution leads after one cell doubling to a shortage of ATP to such an extent that cytostasis is the result [6]. The decline of the ATP content of Molt-4 cells grown in the presence of doxycycline is also shown in Fig. 4B. The growth-arrested cells remain, however, viable for at least five days of treatment.

Dexamethasone treatment does not lead to cytostasis, and influences neither the cytochrome *c* oxidase activity nor the viability of the cells (Fig. 4, Table IV). It does, however, reduce the ATP content of the cells and decreases their growth rate. The ATP content decreases during the first day after dexamethasone addition and remains thereafter constantly above the level which accompanies cytostasis in doxycycline-treated cells (Fig. 4).

The presence of both doxycycline and dexamethasone affects the cells significantly more than either doxycycline or dexamethasone alone. The presence of both compounds leads to cytotoxicity and results in a severe reduction of cell viability (Table IV). The extent to which the effects of doxycycline and dexamethasone potentiate each other depends on the way in which they are combined. The synergistic effect is the most pronounced if dexamethasone is added at a moment when doxycycline treatment has led to cytostasis (Table IV, DC_0DEX_1; DC_0/DEX_2). A synergistic effect, however, is less pronounced or absent if dexamethasone and doxycycline are added simultaneously (Table IV, DC_0DEX_0).

Discussion

Our study demonstrates that the effects on lymphoid cells of doxycycline and glucocorticoids interfere with each other, leading to either antagonizing or synergistic results. Synergism is found whenever dexamethasone exerts its effect on cells of which the growth has been arrested by preceding doxycycline treatment. The synergism can be explained by the inhibitory effect of both doxycycline and dexamethasone on ATP generation. Doxycycline treatment leads to a shortage of ATP-producing capacity as the secundary result of inhibition of de novo enzyme synthesis. The onset of this effect depends on the reserve of mitochondrial oxidative phosphorylation capacity and the turnover rate of mitochondrial proteins. Glucocorticoids affect, amongst other processes, also oxidative ATP generation. This effect is usually found within a few hours after glucocorticoid addition [15], and may even be the main cause of the catabolic effects exerted by these hormones on lymphoid cells [15,16].

Our results show that the growth rate of Molt-4 cells is decreased and their ATP content is reduced by dexamethasone treatment. The proliferation of the cells is not blocked, indicating that the ATP level remains high enough to allow this process. The ATP level is, however, likely to drop considerably when dexamethasone is added to cells of which the ATP content is already lowered to a critical level by prolonged inhibition of mitochondrial protein synthesis. This results in cell death, as is shown in Table IV (DC_0DEX_1, DC_0DEX_2). When mitochondrial protein synthesis is inhibited in cells in which a dexamethasone response has already been induced, the cells likewise will reach a situation sooner in which the shortage of ATP leads to cytotoxicity (Table IV, DEX_0DC_1, DEX_0DC_2). The synergism between doxycycline and dexamethasone via inhibitory actions on oxidative ATP production offers a solid explanation for the results obtained in the studies obtained on the Roser leukemia. When doxycycline treatment has resulted in cytostasis, and the leukemic cells thus have a lack of ATP-generating capacity, the cytotoxic effect of glucocorticoids is enhanced (Tables I and II).

The synergism between (prolonged) doxycycline treatment and dexamethasone makes investigation of the possibility of combined modality treatments of lymphoid tumors with doxycycline and glucocorticoids worthwhile. If both treatments are scheduled in an optimal sequence, their effects may potentiate each other. This is all the more interesting, since most human leukemic cells (as illustrated by Molt-4 cells, Table IV [14]) are rather insensitive to glucocorticoids, which implies

that prolonged treatment with high concentrations are needed to achieve cytotoxic effects. This is especially true for leukemias with large growth fractions [17,18]. Such leukemias have a low percentage of early G_1 or G_0 cells, whereas lymphoid cells are probably the most sensitive to steroid mediated lytic kill in early G_1 and G_0 cells [19]. Doxycycline treatment is, however, most effective in rapidly dividing cell populations and leads, moreover, to proliferation arrest in early G_1 cells [5].

Another aspect of our study which is of fundamental importance is that it strongly indicates that mitochondrial protein synthesis is involved in the glucocorticoid-induced response. Doxycycline treatment clearly reduces the dexamethasone-induced effects on body weight and normal or malignant lymphoid cells when doxycycline treatment has not yet resulted in cytostasis. Several other authors [20–22] have reported that the activity of mitochondria becomes enhanced relatively early during the induction of steroid-mediated responses in, e.g., liver. This may be the (indirect) result of an anabolic effect of the hormones on such tissues, but may also point at involvement of, e.g., mitochondrial protein synthesis for the generation of the hormone-induced response [22]. Furthermore, other authors have shown that inhibition of mitochondrial protein synthesis with chloramphenicol reduces or even blocks hormone-dependent induction of nuclearly encoded proteins in tadpoles [23] and steroid hormone-dependent follicular maturation and oocyte maturation in mice [24]. These data, together with those of our present study, lead to the conclusion that the role of mitochondrial protein synthesis in the generation of steroid-induced responses deserves further investigation.

The results of our study also allow a conclusion concerning the hypothesis proposed in the introduction. We tried to find an explanation for the observation that tetracycline treatment seemed to be more effective in its anti-tumor effect when treatment was started in a later stage of tumor development [7,8]. From our results it follows that the Roser leukemia is sensitive to exogenous glucocorticoids. The dexamethasone effect is antagonized initially by doxycycline and potentiated later on, at the time cytostasis is achieved by doxycycline treatment. If tumor growth is also modulated by endogenous glucocorticoids, tumor growth in normal control rats should be less than that in adrenalectomized control rats. If, on the other hand, doxycycline counteracts the anti-tumor effects of endogenous glucocorticoids, such differences are not expected for DC_0 rats and are expected to be less for, e.g., DC_{14} rats. The additional effect of endogenous glucocorticoids on tumor growth in control rats might thus mask the degree of doxycycline-related anti-tumor action. This might explain the rather small anti-tumor effect of doxycycline when its administration is started during the early stages of tumor development and its effect is compared with tumor growth in normal control rats. To test this, data on tumor growth in adrenalectomized doxycycline-treated as well as in adrenalectomized control rats are required. However, as mentioned in the results, adrenalectomy leads to high mortality in tumor-bearing control rats. This high mortality is not seen in doxycycline-treated rats, which might be explained by the reduced tumor development in presence of doxycycline. Conclusions which would allow a clear explanation for the difference in effectiveness of doxycycline treatment in relation to the stage of tumor progression in which treatment is started can, therefore, not be drawn. However, this holds only for the stages of tumor development in which doxycycline does not yet exert a completely tumor growth-arresting effect. In the latter stage, the differences in the effects of exogenous and endogenous glucocorticoids between DC_0- and DC_{14}-treated rats offer another clue to explain the effectiveness of doxycycline treatment in relation to the stage of tumor progression in which treatment is started. Table II shows that exogenous glucocorticoids exert a synergistic effect once tumor growth is arrested by doxycycline in DC_0- as well as in DC_{14}-treated rats, whereas a synergistic effect of endogenous glucocorticoids is limited to DC_{14}-treated rats. This is most likely due to inhibiting effects of prolonged doxycycline treatment on endogenous glucocorticoid production, either directly or indirectly, or even both.

A direct mechanism might involve impairment of mitochondrial function in the cells of the adrenocortex, since the rate-limiting step in steroid synthesis is located within the mitochondria of

steroidogenic cells [25]. Prolonged doxycycline treatment may indirectly counteract corticosteroid production via its anti-tumor effect: as the tumor load remains lower, the induction of stress hormones will be less [10]. When doxycycline treatment is started later in the tumor progression, the synergistic effect of doxycycline and glucocorticoids will, thus, be more pronounced.

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