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Effects of oxytetracycline on *in vivo* proliferation and differentiation of erythroid and lymphoid cells in the rat

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SUMMARY

Previous studies revealed that inhibition of mitochondrial protein synthesis by tetracyclines during a number of cell divisions results in proliferation arrest, *in vitro* as well as in *in vivo*. The tetracyclines may, therefore, be considered as cytostatics. In this context it is of interest to know the effect of the tetracyclines on rapidly dividing normal cell types, such as erythroid and lymphoid cells. To investigate the influence of the tetracyclines on the proliferation of these cells, we studied immunological responses to sheep red blood cells and recovery from severe anaemia in rats in the presence or absence of oxytetracycline (OTC). Under the experimental conditions used, effects of OTC on the immune responses were only found for events directly or indirectly related to T cell proliferation. The results presented thus strongly suggest that OTC inhibits the division of T lymphoid cells only. The proliferation of other haemopoietic cells is not influenced, most likely because these cells are not permeable to OTC.

INTRODUCTION

Concentrations of oxytetracycline (OTC) and other tetracyclines used in anti-bacterial chemotherapy inhibit not only prokaryotic but also mitochondrial protein synthesis. Concentrations of at least one order of magnitude higher also impair cytoplasmic protein synthesis (Gijzel, Strating & Kroon, 1972; De Jonge, 1973). Inhibition of mitochondrial protein synthesis leads to dilution of partly mitochondrially made enzymes at every turnover of the cytoplasm or division of the cells involved. As the enzymes concerned are all part of the system for oxidative phosphorylation of the mammalian cell, prolonged inhibition of mitochondrial protein synthesis may therefore have severe energetic or metabolic consequences.

In previous studies we have shown that OTC continuously administered to rats in low doses inhibits mitochondrial protein synthesis in all tissues investigated (Van den Bogert & Kroon, 1981b) and that reduction of the ATP generating capacity below a critical level leads to proliferation arrest of rapidly dividing Zajdela tumour cells *in vivo* (Van den Bogert *et al.*, 1981).

If OTC also permeates the cells of the white and red cell series, interference by this antibiotic—if present during a number of cell cycles—with erythropoiesis and lymphopoiesis is also to be expected.

Many reports deal with immunomodulating effects of the tetracyclines (see Finch, 1980) and chloramphenicol (Weisberger, Daniel & Hoffman, 1964; Cruchaud & Coons, 1964), another mitochondriotropic antibiotic. Whereas the proliferation of red blood cells is severely depressed by low levels of chloramphenicol and its analogues (Nijhof & Kroon, 1974), the tetracyclines are not known to influence erythropoiesis. The study was undertaken to gain insight into the nature and

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cause of the immunomodulating effects and into the lack of interference with red blood cell formation. We report here the results of a detailed study on the influence of OTC on the course of immune responses and on recovery from severe anaemia in rats.

MATERIALS AND METHODS

Animals and reagents. Male Wistar rats, weighing about 200 g were used in all experiments, except in the investigations concerning the effect of OTC on secondary immune responses. In the latter case adult rats of 300 g were prefered, having a more or less stable body weight over the whole experimental period.

Oxytetracycline was supplied by Gist-Brocades N.V., Delft, The Netherlands. [7-^{3}H-(*N*)] tetracycline (^{3}H-TC), 0·94 Ci/mmol was purchased from New England Nuclear, Dreiech, West Germany and used as obtained. All other chemicals were of an analytical grade. Sheep red blood cells (SRBC) were stored in Alsever's solution and washed three times with 0·15 M NaCl directly prior to use.

OTC administration. OTC was given by continuous intravenous infusion at a rate of 0·15 ml/hr as described previously (Van den Bogert & Kroon, 1981b). Dosages are indicated in the various experiments. Control animals were treated in the same way, except that no OTC was present in the infusion solution.

OTC determination. The fluorometric determination (Van den Bogert & Kroon, 1981a) of OTC concentrations in serum, tissues or cells was carried out within 24 hr after the samples had been obtained.

Distribution of OTC and ^{3}H-TC among blood components. The amount of OTC in blood cells and serum was measured after 7 days of continuous infusion with OTC. After separation of serum and total blood cells, the latter were washed three times with 0·15 M NaCl or directly used to determine the OTC content.

The distribution of ^{3}H-TC was investigated *in vitro*, after incubation of blood with ^{3}H-TC (10 μg ^{3}H-TC per ml blood) for 1 hr at 37°C. After a two-fold dilution with Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) the blood was separated in several fractions on a Ficoll gradient. The number of cells per fraction was determined using a cell counter. The amount of radioactivity per fraction was measured in a liquid scintillation counter, using various scintillators on xylene basis. In other experiments, samples containing mainly lymphocytes or red blood cells were pooled, and then washed with 0·15 M NaCl. Cell number and radioactivity counts were registered as described above.

Recovery from severe anaemia. Anaemia was induced in rats by blood withdrawal from the jugular vein—via the infusion system—on the first and second day of the infusion period, leading to haematocrit values of about 20–25%. Recovery from this anaemia was followed for 7 days during continuous infusion of OTC in dosages of 10, 20 and 30 mg/kg/day, which are low enough to inhibit only mitochondrial protein synthesis (Van den Bogert & Kroon, 1981b). For serial determinations of the haematocrit, blood was taken from the tail veins.

Delayed-type hypersensitivity (DTH). Animals were primed by injecting 1×10^9 SRBC subcutaneously on the back on day 0, and challenged on day 5 with 1×10^8 SRBC, injected subcutaneously in the right hind footpad. Twenty-four hours after challenge the increase in footpad thickness was measured with a caliper.

Primary humoral response. Animals were primed by injection via the infusion system of 1×10^9 SRBC into the jugular vein. Serial determinations of antibody titres were made on blood obtained from the tail vein.

Dilutions of the heat-inactivated sera were made in microtitre plates, and equal volumes of 1% v/v neuraminidase treated SRBC were added. After 1 hr incubation at 37°C, haemagglutinatinin titres, both mercapto-ethanol sensitive (IgM) and mercapto-ethanol resistant (IgG), were measured.

Secondary humoral response. Some of the rats used in the study for effects on the primary immune response were again connected to the infusion system and given another dose of 1×10^9 SRBC to induce a secondary immune response 5 weeks after primary immunization. Antibody titres were measured as described above.

RESULTS

Distribution of OTC and ^{3}H-TC among blood components

The data obtained in the distribution studies are given in Table 1. Total blood cells contain considerably less OTC on a wet weight basis than serum or any other tissue examined (Van den Bogert & Kroon, 1981b) even if no precautions are taken to avoid contamination of the cells with serum. ^{3}H-TC added to blood samples is unequally distributed among the blood components as well. The non-homogeneous distribution seems to be due to the very low amount of ^{3}H-TC in the erythrocytes, which form the main part of the blood cells. As can be seen in Table 1, the specific binding or uptake by the lymphocytes is about 2000 times higher than binding or uptake by the erythrocytes on a per cell basis. The ratio of the concentration in the lymphocytes to that in the serum is about 3, a value comparable to that found for other tissues (Van den Bogert & Kroon, 1981b). The estimation of the ^{3}H-TC content of the various blood components on a wet weight basis indicates that, at serum levels of 5–15 μg tetrcycline/ml as normally used in our experiments and reached in anti-bacterial therapy, the tetracycline content is too low to have any effect on erythrocytes and high enough to cause inhibition of mitochondrial protein synthesis in the lymphocytes (Van den Bogert & Kroon, 1981b).

Recovery from severe anaemia in presence of OTC

Fig. 1 shows the course of the recovery at various OTC serum levels. Similar experiments with the chloramphenicol analogue thiamphenicol lead to persistence of the anaemia (Nijhof & Kroon, 1974). In the case of OTC, however, haematocrit values at successive days after induction of anaemia are comparable to control values in spite of the continuous presence of the drug.

The lack of inhibition of red cell proliferation is also apparent from the gain in spleen weight, occurring as well in control as in OTC treated anaemic rats. The absence of inhibition of red cell formation is, therefore, not restricted to the bone marrow, where the well known chelation of the tetracyclines may protect the bone marrow cells from adverse effects.

Table 1. Distribution of OTC and ^{3}H-TC in blood cells and serum

Amount per	OTC	^{3}H-TC
ml blood	10 μg	10 μg
ml serum	18 μg	19 μg
g unwashed blood cells	1·9 μg	1 μg
g washed blood cells	0·42 μg	0·31 μg
g washed erythrocytes	n.d.	0·24 μg
g washed lymphocytes	n.d.	60 μg
10^6 washed erythrocytes*	n.d.	14 pg
10^6 washed lymphocytes*	n.d.	30 ng

Distribution of OTC among blood cells and serum was measured after 7 days of continuous infusion; ^{3}H-TC distribution after 1 hr incubation at 37°C of ^{3}H-TC with total blood. Each value represents the mean value of triplicate measurements on at least four separate samples. The s.d. is about 10%; n.d. = not done.

* These values are calculated, assuming a haematocrit of 50%, the presence of 9×10^9 erythrocytes and of 6×10^6 lymphocytes per ml blood and a volume per lymphocyte 10 times greater than that per erythrocyte.

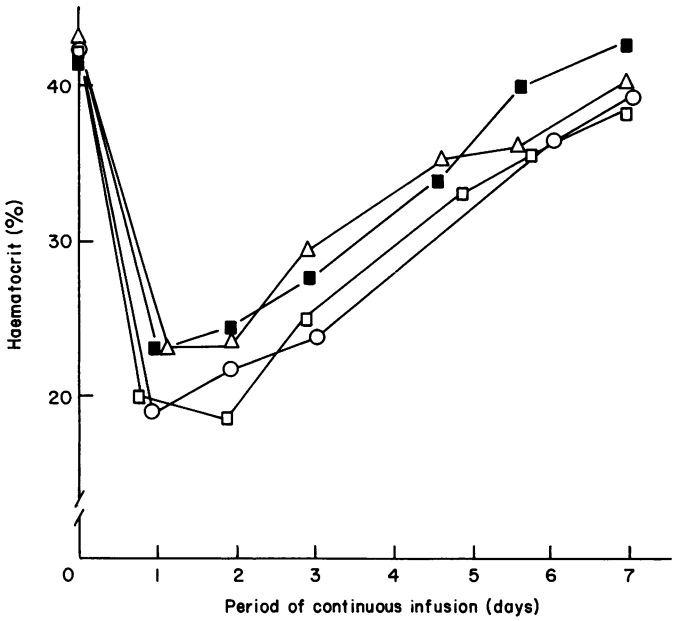

Fig. 1. Recovery of severe anaemia in absence or presence of OTC. Rats were made anaemic by withdrawal of 2 × 2 ml blood (on day 0 and day 1 of the continuous infusion period). 0, 10, 20 or 30 mg OTC/kg/day was administered, leading to serum levels of 0 (■), 4·1 (○), 9·2 (△) and 14·7 (□) μg OTC/ml as indicated by the symbols. Each point represents the mean value found in three animals. Individual values per point ranged maximally between 96% and 104% of the mean value.

Effect of OTC on cell-mediated immunity

The DTH response to SRBC was used to establish a possible effect of OTC on cell-mediated immunity. The first set of experiments showed that continuous OTC administration of 20 mg/kg/day started just before the moment of priming, severely depressed the DTH reaction. Several points of attack by OTC are conceivable such as, in order after priming, inhibition of proper function of antigen presenting cells, interference during the interaction of these cells and helper T cells, proliferation or differentiation arrest of T cells or impairment during the challenge reaction. To discriminate between these possibilities, OTC was given at various periods of the DTH reaction. The results are given in Table 2. This table shows that OTC treatment during the first 18 hr after priming does not affect the DTH response. The challenge of the response in the presence of OTC is also not influenced. If OTC is administered, however, between 18 and 72 hr after priming the response is depressed to the same extent as it is in case of the continuous presence of OTC ((−6)−120 hr). Comparable results are obtained if OTC is given from 18–48 hr or 48–72 hr after priming. The depression of the DTH response found during the continuous presence of OTC is therefore best explained by assuming that OTC interferes with the formation of T cells. The results imply that during the second as well as during the third day after priming the number of T cell divisions is large enough to reduce the ATP generating capacity to such a degree that it results in proliferation inhibition.

Effect of OTC on humoral immunity, effect on the primary response

The first series of experiments on the effect of OTC on humoral immunity revealed that the constant administration of OTC (20 mg/kg/day) during the development of the primary response to SRBC leads to depressed antibody titres. The administration of OTC only during the first 18 hr after priming, however, did not lead to a reduction of the amount of antibodies. Thus, for the humoral response, the induction phase seems not to be influenced by OTC. Because of the T cell dependency of the humoral response to SRBC in mammals (Greaves, Owen & Raff, 1974) the depression of the

Table 2. Effect of OTC on DTH response to SRBC in rats

Period of OTC treatment (hours related to priming at zero time)	DTH response (% increase ±s.e. in footpad thickness, 24 hr after challenge at 120 hr after priming)
none	53±4
(−6)–18	52±5
114–144	57±6
72–144	51±5
(−6)–18 and 72–120	52±5
(−6)–144	13±3
(−6)–120	14±1
24–120	13±1
48–120	12±1
18–72	18*
18–48	25*
48–72	17*

OTC was continuously administered (20 mg/kg/day) at various time intervals before and after priming with SRBC. The arithmetic mean ±s.e. of at least five animals is given;

* These values represent the results found in one experiment.

humoral response observed can be explained either by proliferation arrest of B cells or by reduced T cell formation in presence of OTC. To investigate the nature of the effect of OTC in more detail, the period of OTC administration was varied. The results are shown in Table 3 and indicate that OTC depresses IgM antibody titres only when it is administered during the first 48 hr after induction of the response. B cell proliferation and differentiation most likely do not occur during the first 2 days after priming (Van Ewijk *et al.*, 1977). We assume, therefore, that B cell formation and antibody secretion as such are not affected by OTC.

To investigate a possible influence of OTC on the kinetics of antibody formation, haemagglutinin titres were determined on various days after immunization. Fig. 2 shows the course of the IgM

Table 3. Effect of OTC on IgM response to SRBC in rats

Period of OTC treatment (hours related to priming at zero time)	IgM response (^{2}log IgM titre ±s.e. 120 hr after priming)
none	4·0±0·5
(−6)–18	4·1±0·5
72–120	4·0±0·5
(−6)–18 and 72–120	3·9±0·5
48–120	4·2±0·5
42–120	3·2±0·5
32–120	2·7±0·5
24–120	1·5±0·5
(−6)–120	1·2±0·5
18–72	1·5*

For details, see footnotes to Table 2.

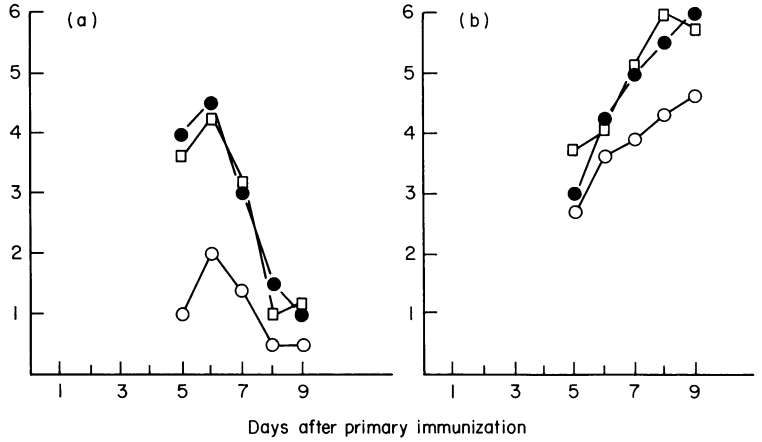

Fig. 2. (a) IgM (b) IgG titre, determined at various days after immunization with 1×10^9 SRBC. ● = control; ○ = OTC treated, administration started 6 hr before priming; □ = OTC treated, administration started 48 hr after priming. Each point represents the mean of values found in nine animals; the s.e. was maximally 0·3.

and IgG titres in control animals, in rats continuously treated with OTC and in rats given OTC from 48 hr after immunization. It can be seen that the kinetics of the humoral responses are not influenced by the constant presence of OTC, in contrast to the amount of antibodies formed.

Effect of OTC on humoral immunity, effect on the secondary response

To study the effect of OTC on memory cell formation, a secondary immune response was induced in rats which were treated in various ways during the primary response. These were immunized a second time with SRBC, 35 days after induction of the primary response.

The course of the IgG titre was subsequently followed, infusing the rats with solutions containing OTC (20 mg/kg/day) or control solutions. The results are given in Fig. 3 and show that OTC does not influence the induction and course of the secondary response as such. It depresses, however, a normal secondary response completely when it has been administered during the first 48 hr of the primary response. It seems likely, therefore, that OTC directly or indirectly blocks memory cell formation when present during this period.

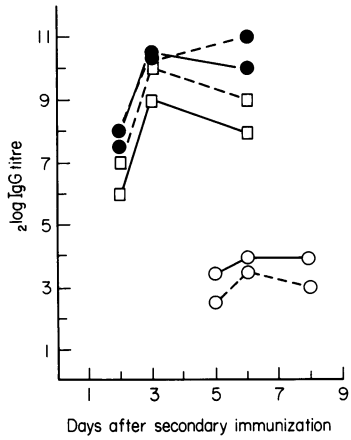

Fig. 3. IgG titre, determined during the secondary immune response by injection of 1×10^9 SRBC on the 35th day after primary immunization. ● = infused with control solution during the primary response; □ = infused with OTC, starting 48 hr after priming of the primary response; ○ = infused constantly with OTC during the primary response; —— infused with control solution during the secondary response; – – – infused constantly with OTC during the secondary response; each value represents the mean of values found in two animals.

DISCUSSION

As pointed out already, inhibition of mitochondrial protein synthesis by OTC can result in proliferation arrest, if it lasts long enough to let a number of cell cycles pass, and if the cells concerned are permeable to OTC. The tetracyclines in general may, as a consequence, be of use for the medication of malignant growth either alone or in combination with other cytostatics or treatments (Leezenberg, Wesseling & Kroon, 1979). It is, therefore, of interest to study also the effect of OTC on other rapidly proliferating cells, such as cells originating from the bone marrow.

From the results presented it is clear that circulating erythrocytes are not permeable to OTC. In previous studies (Gijzel & Kroon, 1978) on the effect of tetracyclines in high doses on the synthesis of haemoglobin it was noticed that protein synthesis in reticulocytes was only moderately impaired, whereas lysates showed the expected inhibition. It was concluded that the reticulocyte membrane is impermeable to tetracycline chelates. The lack of any effect of OTC recovery from severe anaemia suggests that stem cells and precursor cells of the red cell series are likewise impermeable and that this impermeability prevents impairment of mitochondrial protein synthesis by low concentrations of OTC. The OTC concentration in circulating lymphocytes, however, suggests that these are accessible to the tetracyclines. To investigate a possible anti-proliferative effect of OTC on these cells, immune responses were studied. Immunomodulating effects of the tetracyclines in mammals, birds and fishes have been described in a number of publications. The results are confusing: stimulation (Popovic *et al.*, 1973), depression (Nikolaev & Nazarmukhamedova, 1974; Rijkers, Van Oosterom & Van Muiswinkel, 1981) as well as no effect (Thong & Ferrante, 1980) of tetracyclines on *in vivo* humoral responses are reported.

Depression of *in vivo* cellular mediated responses is also found (Thong & Ferrante, 1980; Rijkers *et al.*, 1980). Varied effects of the tetracyclines on polymorphonuclear cells are found, *in vivo* as well as in *in vitro*, like impaired phagocytosis (Forsgren, Schmeling & Quie, 1974) and reduced chemotaxis (Forsgren & Schmeling, 1977; Thong & Ferrante, 1980), while other authors report only minor effects or none. The contradictory results are most likely due to the different conditions used in the various studies. The tetracycline concentrations and the way by which it is administered are important in this respect (Kroon & De Jong, 1979).

At serum concentrations of tetracyclines higher than about 50 μg/ml cytoplasmic protein synthesis can be impaired directly. Also side effects may occur at these high concentrations, e.g. immunomodulation resulting from Ca^{2+} chelation by the tetracyclines (Diamantstein & Odenwald, 1974). At lower concentrations only mitochondrial protein synthesis becomes impaired.

The conditions used in our experiments made it possible to study the effects of OTC at constant low serum levels during successive stages of immune responses. In analysing the results presented, one must keep in mind that with 20 mg/kg/day it takes 6 hr to reach a serum level giving complete inhibition of mitochondrial protein synthesis. The serum levels remain inhibitory for about 8 hr after stopping the infusion (Van den Bogert, unpublished data). OTC has to be present in sufficient amounts (> 5 μg/ml) during a number of cells cycles before its anti-proliferative effect becomes apparent. Comparing our results with the generally accepted course of events during immune response to SRBC, the results indicate that OTC blocks only T cell proliferation.

The depression of the secondary response, found when OTC has been administered during the first 2 days after induction of the primary response, can be explained by inhibition of T memory cell formation or inhibition of T cell-dependent B memory cell formation. On the other hand antibody–antigen complexes seem an important factor in regulation of memory formation and the secondary response as such (Kunkl & Klaus, 1981). Because the antigen to antibody ratio is changed in rats continuously treated with OTC this may also explain the apparent depression of the secondary response by OTC given during the induction of the primary response.

Because of the lack of proliferation inhibition during erythropoiesis we conclude that the impermeability of the erythrocytes and their late precursor cells for OTC *in vivo* is a property retained from the (pluripotent) stem cell stage, rather than a property acquired during differentiation. We found also no inhibition of mitochondrial protein synthesis and thus of the proliferation of tumour cells derived from a pre-B cell line (Rozing, *et al.*, 1982) by OTC under circumstances where the growth of Zajdela tumour cells (Van den Bogert *et al.*, 1981) is strongly

reduced. The proliferation of B cells seems also not affected by OTC. The permeability barrier seems, however, to be lost during the differentiation to T cells. Impairment of T lymphocyte colony formation by inhibition of mitochondrial protein synthesis has been reported (Fao, Zafar & Catovsky, 1980). If our results can also be extrapolated to humans, then the clinical implication of this study is that OTC may cause neither bone marrow depression nor interference with humoral or cellular immunity in the case of responses that have been induced before its administration. It should be stressed once more that this reasoning holds for concentrations of OTC that specifically inhibit only mitochondrial protein synthesis.

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