SOME TETRACYCLINE DRUGS SUPPRESS MITOGEN-STIMULATED LYMPHOCYTE GROWTH BUT OTHERS DO NOT

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1 Eight tetracycline drugs were tested for inhibitory effect on phytohaemagglutinin (PHA) stimulated growth (measured by [³H]-TdR uptake) of peripheral blood mononuclear cells: at least 26 normal subjects were tested with each drug.

2 Doxycycline reduced DNA synthesis appreciably at concentrations within and just above the therapeutic range of blood levels: it was the most potent inhibitor, but demeclocycline, methacycline and minocycline had similar, if less potent, effects. Tetracycline, oxytetracycline, chlortetracycline and clomocycline did not inhibit DNA synthesis of 3-day cultures even at concentrations five to ten times greater than the therapeutic blood level.

3 Volume spectroscopy measurements showed that none of the eight tetracycline drugs interfered with the recruitment of cells into G₁-phase growth of the first cell cycle after PHA stimulation.

Keywords tetracyclines lymphocyte growth

Introduction

Certain tetracycline drugs (such as doxycycline and minocycline), but not others (such as tetracycline and oxytetracycline), suppress the growth of mitogen-stimulated human lymphocytes when present in the culture fluid at concentrations comparable to peak blood levels sometimes achieved during treatment of infections (Banck & Forsgren, 1979; Thong & Ferranti, 1979). Finch (1980) has argued that it is unlikely that any of the tetracycline drugs will have an appreciable adverse effect on the immune response during the treatment of an acute infection in a previously healthy person since the duration of treatment with the antibiotic is short relative to the time-course of the immunological reaction, but he has emphasized that the immunosuppressive effects of antimicrobial agents may be clinically relevant in long-term chemotherapy for chronic infections.

The tetracyclines are still widely used and remain the treatment of choice for infections caused by *Chlamydia*, *Rickettsia*, *Mycoplasma* and *Brucella* (British National Formulary, 1982). They are also widely prescribed for exacerbations of chronic bronchitis and, at low dosage, in the long-term treatment of acne vulgaris. We have therefore investigated the relative toxicity of the various drugs used in pharmaceutical preparations.

Methods

Table 1 lists the tetracycline drugs that were studied: fresh serial dilutions of the drugs in tissue culture medium were prepared for each experiment.

Mononuclear cells were isolated from heparinised blood donated by healthy volunteers (mainly male laboratory workers, aged 20–55 years) by density gradient centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and washed three times in TC199 (Gibco Bio-cult Ltd, Paisley, Scotland), buffered at pH 7.3 with Hepes and supplemented with L-glutamine (200 mM), penicillin (200 i.u./ml) and streptomycin (100 µg/ml). The purity of lymphocytes in the cell suspension was not measured at this stage. The mononuclear cell suspensions were cultured in round-bottomed microtitre plates (Sterilin): each well contained 2×10^5 cells in 100 µl TC medium, 20 µl autologous serum, 50 µl TC medium with or without drug (for test or control) and 25 µl phytohaemagglutinin (PHA) solution (HA15, Wellcome Reagents Ltd, Beckenham, England) used at optimal stimulating concentrations of 2.5 µl HA15/ml TC199. The plates were sealed with adhesive tape (Flow Laboratories, Irvine, Scotland) and incubated at 37°C. The cells were harvested after culture for 21–24 h for volume spectroscopy experiments and at 72 h for liquid scintillation. The exact duration of

Table 1 Tetracycline drugs studied

Name	Source	Main pharmacological features			Chemical structure	
		Serum half-life* (h)	Peak blood concentration in clinical tests* (µg/ml)	Solubility Octanol/aqueous buffer (pH 7.5)*	Groups attached at position 6	Other differences from parent tetracycline molecule
Tetracycline	Lederle Laboratories	6-10	3	0.036	-CH ₃ : -OH	-
Oxytetracycline	Pfizer Ltd	6-9	3	0.025	-CH ₃ : -OH	-OH at C ₅
Chlortetracycline	Lederle Laboratories	6-9	3	0.13	-CH ₃ : -OH	-Cl at C ₇
Clomoxycine	Pharmax Ltd	6-9	2	—	-CH ₃ : -OH	-Cl at C ₇ and -CH ₂ OH amide side chain at C ₂
Doxycycline	Pfizer Ltd	10-20	3	0.60	-CH ₃ : -H	-OH at C ₅
Demeclocycline	Lederle Laboratories	10-17	2	0.050	-H : -OH	-Cl at C ₇
Methacycline	Pfizer Ltd	7-11	2	0.43	= CH ₂	-OH at C ₅
Minocycline	Lederle Laboratories	17-20	3	—	-H : -H	-N(CH ₃) ₂ at C ₇

These drugs are listed in British National Formulary (1982). The only other tetracycline analogue listed is Lymecycline which was not tested since a specimen of the pure drug could not be obtained: however, Lymecycline is simply tetracycline substituted with lysine at the C₂ position.

* Data from Doluisio & Dittert (1969), Sande & Mandell (1980), Wade (1982).

* Data from Colaizzi & Klink (1969).

culture of the cells in each well was noted at harvesting when the lymphocytes were dislodged and mono-dispersed by repeated Pasteur pipette mixing using a technique which gives good recovery and avoids bias (Potts *et al.*, 1981): many of the monocytes in the inoculating cell suspension had remained adherent to the walls of the plastic culture plate. The extent of cellular contamination in the lymphocytes recovered from the control cultures, determined by volume spectroscopy (Potts *et al.*, 1980), ranged from 3.2 to 11.1% in the experiments: this measurement of contamination did not bear any relation to the observed *in vitro* response to tetracycline analogues.

Measurement of pre-S-phase cell cycle kinetics from volume spectroscopy measurements

The methods have been described in detail previously (Brown *et al.*, 1979; Gibbs *et al.*, 1979). Briefly the cell suspension was measured in an electronic particle counter (Model Fn, Coulter Electronics, Luton, England) and the output accumulated in a multi-channel analyser (Channelyzer C1000, Coulter). The growth during the first G₁-phase of the cell cycle was calculated from a comparison of the size distribution profiles of each stimulated culture with that of the corresponding unstimulated control culture. The computer program estimates the best values for the proportion of cells recruited into growth and for the two parameters describing the volume growth (basal and incremental growth rates) by choosing those values which minimise the size of the chi-squared statistic derived from comparison of the computer-generated profile and that observed experimentally. Computer simulation experiments have shown that the percentage of growing cells deduced from the mathematical model is not appreciably perturbed by the presence of non-responsive cells (such as monocytes and neutrophil granulocytes) provided this contamination is less than 15% (Gibbs *et al.*, 1982).

Measurement of mitogen-stimulated growth by liquid scintillation

After culture for 68 h, 0.5 µCi [³H]-TdR (Radiochemical Centre, Amersham, England) was added to each culture well: the cells were harvested 4 h later with a Skatron harvester (Flow Laboratories, Irvine, Scotland) on filter paper discs. The rate of DNA synthesis was measured by liquid scintillation counting of the air-dried discs in NE233 (Nuclear Enterprises, Edinburgh, Scotland). All experiments were performed in triplicate. Potency studies on the four active drugs were undertaken on peripheral blood mononuclear cells from three normal subjects. In each of these experiments, all the drugs were tested at seven dose levels (from 0.23 to 15.0 µg/ml in doubling dilutions) on a batch of cells harvested from a 40-60

ml sample of venous blood. The analysis of these dose-response tests presented the usual difficulties associated with a curvi-linear response (Finney, 1978), consequently (a) the logarithm of the recorded counts/min was analysed to minimise the effect of greater variability in the samples with higher counts and (b) quadratic curves of the type

$$\log(\text{count}) = a + b \text{ dose} + c (\text{dose})^2$$

were fitted to take into account the non-linearity of the observed log-dose response curves. Calculations were performed with the GLIM statistical package (Baker & Nelder, 1978).

Results

Eight drugs, currently in clinical use, were compared in pairs for their capacity to reduce DNA synthesis of PHA-stimulated peripheral blood mononuclear cells grown for 3 days in the presence of doubling dilutions of the drug (1.85–30.0 $\mu\text{g/ml}$). Each drug was tested on cells from at least 26 normal male subjects. Figure 1 shows the result of a typical experiment. Four of the drugs (demeclocycline, doxycycline, methacycline and minocycline) were inhibitory to cells of all subjects at, and above, 3.75 $\mu\text{g/ml}$ and the shapes of the log-dose response curves were similar in the 26 subjects tested. The other four drugs (chlortetracycline, clomocycline, oxytetracycline and tetracycline) did not influence the growth of cells from any subject over a wide range of concentrations extending into, and well beyond, those encountered in the blood during treatment with recommended doses.

The possibility of interaction between tetracycline analogues and the antibiotics on the tissue culture medium was investigated in separate experiments.

Figure 1 Comparison between effects of doxycycline and tetracycline on PHA-stimulated growth of peripheral blood mononuclear cells from a healthy young adult male: the cultures were grown for 3 days and pulsed with [^3H]-TdR for the last 4 h of culture. --- tetracycline: — doxycycline.

Peripheral blood mononuclear cells were cultured for 3 days in the presence of dilutions of tetracycline and doxycycline (0.94–15.0 $\mu\text{g/ml}$) in tissue culture medium without other antibiotics, with penicillin or streptomycin or with both in the concentration present in the standard tissue culture medium: tetracycline did not affect DNA synthesis at 3 days in any culture conditions and the doxycycline-induced depression of DNA synthesis was unaffected by the presence or absence of the other antibiotics. The influence of suboptimal, optimal and supraoptimal mitogen stimulation on tetracycline action was investigated with checkerboard titrations of tetracycline and doxycycline (0.94–15.0 $\mu\text{g/ml}$) against PHA (0.14–2.28 $\mu\text{g/ml}$). Tetracycline did not inhibit DNA synthesis in any of these experiments: doxycycline had a generally similar depressive effect with all stimulating PHA concentrations and its potency was not enhanced with suboptimal mitogen stimulation.

The structural formula of the parent molecule (tetracycline) is shown in Figure 2 and the modifications incorporated in the other molecules are summarised in Table 1. It seems possible that potency as an inhibitor is associated with substitution at position 6 and that the other changes are irrelevant.

The relative potency of the four active drugs was studied in the concentration range 0.23–15.0 $\mu\text{g/ml}$. The fitted dose-response curves for one subject are shown in Figure 3. Generally similar results were found with cells from the other two subjects. The analysis of variance (Table 2) shows that (a) the dose-response curves are significantly non-linear, (b) there are statistically significant minor differences between the responses of the three subjects for any particular drug and (c) there are statistically significant differences between the dose-response curves of any particular subject to each of the drugs. The latter difference is accounted for largely by the fact that doxycycline is significantly more inhibitory than the other three drugs which give essentially similar and intersecting curves (Figure 3).

The effect of the eight tetracycline drugs on cell volume growth during the first 24 h of culture was

Figure 2 Chemical structure of tetracycline.

Table 2 Analysis of variance of DNA synthesis rate (measured as counts/min after pulsed labelling with [^3H]-TdR of PHA-stimulated lymphocytes from three normal subjects with various concentration (0.23–15.0 $\mu\text{g/ml}$) of drugs (demeclocycline, doxycycline, methacycline and minocycline). Regression fitted to curve $\log(\text{count}) = a + b \text{ dose} + c (\text{dose})^2$.

Source of variation	Variance ratio	Degrees of freedom	P value
Nature of overall dose-response curve			
linear term	12610	1 and 232	< 0.001
quadratic term	479	1 and 232	< 0.001
Differences between curves for different drugs			
linear term	133	3 and 232	< 0.001
quadratic term	14.7	3 and 232	< 0.001
Differences between curves for different subjects			
linear term	250	2 and 232	< 0.001
quadratic term	26.4	2 and 232	< 0.001

studied by volume spectroscopy. Peripheral blood mononuclear cells (> 85% lymphocytes) from four healthy young adult males were stimulated with optimal concentration of PHA in the presence of a range of concentrations of the drug (1.85–30.0 $\mu\text{g/ml}$) or in control medium. None of the drugs (even at 30 $\mu\text{g/ml}$) modified the appearance of the size distribution profile of either the unstimulated or the PHA stimulated cells. Analysis of the profiles by computer confirmed that neither the numbers of cells responding to stimulation, or their averaged rate of volume growth in the pre-S-phase period was modified by any of the drugs.

Figure 3 'Best-fit' dose-response curves for four inhibitory tetracyclines generated during analysis of variance. Doxycycline is significantly more inhibitory than the other three drugs, which give essentially similar intersecting curves.

Discussion

This study has indicated that some tetracycline analogue drugs reduce mitogen-induced lymphocyte proliferation whereas others have very little effect. The tetracycline drugs, as with other pharmaceuticals, are subject to stringent International Regulations with regard to purity and the contaminants in tetracycline, oxytetracycline and chlortetracycline are not different from those found in doxycycline and demeclocycline (Hermansson & Andersson, 1982). It is therefore highly unlikely that our findings could be explained by selective contamination of the 'toxic' drugs by a highly potent lymphocyte growth inhibiting trace substances. There is no obvious pharmacological explanation for the separation of the inhibitor drugs from the non-inhibitors (Table 1). It is interesting to note, however, that inhibitor tetracyclines differ from non-inhibitors chemically by the nature of the substituted group at the C_6 position: by contrast the antibiotic functions of these molecules are determined by the substitution of the opposite side of the molecule.

Relatively little is known about the effect of tetracycline analogue drugs on mammalian cellular metabolism, but these drugs appear to be capable of inhibiting protein synthesis at pharmacological concentrations by interfering with the binding of aminoacyl-s-RNA to the RNA ribosome complex (Beard *et al.*, 1969). It is possible that in certain tissues (such as liver) this effect is limited by failure of the drug to penetrate cells (Franklin, 1963), but this is unlikely to be important with human leucocytes since Brown & Percival (1978) have shown that their cell membranes are fairly permeable to these drugs. Banck & Forsgren (1979) have argued that tetracyclines act on mammalian cells by interference with protein synthesis.

Potts *et al.* (1983) have recently shown that doxycycline causes an almost insignificant reduction in early RNA synthesis, without interference with

volume growth: the rate of recruitment of cells into the first S-phase is reduced and the cells increase in number at a slower rate than normal. By the third day, the growth fraction is reduced and there is increased cell debris, but the surviving cells appear relatively healthy on light microscopy. On-going EM studies indicate that many of the cells surviving in cultures containing doxycycline have cytoplasmic abnormalities including 'myelin' bodies and residual bodies indicating increased membrane turnover (Hassan, Hopwood, Potts & Beck, in preparation). These defects cannot be attributed solely to interference with protein synthesis. The functional defect was not identified but in view of the results reported in the present paper with various tetracycline analogues, it is likely that the lymphocyte growth inhibiting effect has a different biochemical basis from that of the bacteriostatic effects. Moreover it is a direct effect on the proliferating cells and not an indirect effect on a controlled mechanism, such as the interleukin systems.

When normal subjects take the usual recommended dose of doxycycline orally (100 mg daily), the plasma levels are in the range 3–5 µg/ml (Green *et al.*, 1976). Clearly, even higher blood levels may be found in other clinical situations such as excessive dosage (e.g. doxycycline in daily doses of 200 mg or more) or failure of clearance of demeclocycline, methacycline and minocycline in patients with renal failure (doxycycline does not accumulate in renal impairment).

We have found that the DNA synthesis rate of stimulated lymphocytes may be reduced by as much as 50% with concentrations of doxycycline that correspond to therapeutic blood levels (and by 20–40% with demeclocycline, methacycline and minocycline). There are theoretical reasons to believe that growth retardation of this extent could interfere with the immune response *in vivo* (Beck & Browning, 1983) and the present study followed our observation of opportunistic infections and poor responses to lymphocyte stimulation tests in a patient inadvertently given twice the usual dose of doxycycline for several weeks. There is still no conclusive evidence that some of the tetracyclines are immunosuppressive in man, nevertheless it would be prudent to use tetracycline, oxytetracycline, chlortetracycline or clomocycline for long-term treatment, or regular intermittent treatment of chronic infections. It is perhaps fortuitous that the only two drugs that are available for injection, tetracycline and oxytetracycline (British National Formulary, 1982) and therefore liable to give rise to very high initial concentrations, have been found to be inactive in depressing lymphocyte growth *in vitro*.

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