In vitro effects of doxycycline and tetracycline on mitogen stimulated lymphocyte growth

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SUMMARY

Doxycycline and tetracycline were tested for inhibitory effect on PHA stimulated growth (measured by [^{3}H]-TdR uptake) of peripheral blood mononuclear cells from 26 normal subjects. Doxycycline reduced DNA synthesis appreciably at concentrations within and just above the therapeutic range of blood levels: tetracycline did not inhibit DNA synthesis of 3 day cultures even at concentrations five–10 times greater than the therapeutic blood level. Detailed studies on the action of doxycycline by volume spectroscopy and RNA flow cytofluorimetry indicated that this drug did not influence the recruitment of cells into the first G_1-phase. Cell counts, DNA flow cytofluorimetry and autoradiography after pulsed exposure to [^{3}H]-TdR showed a reduction in the numbers of growing cells in 2 and 3 day cultures in the presence of doxycycline compared with those containing tetracycline at the same concentration.

Keywords phytohaemagglutinin lymphocyte stimulation tetracycline doxycycline

INTRODUCTION

Certain tetracycline drugs, such as doxycycline and minocycline, 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 doxycycline and 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, in full dose, for infections caused by Chlamydia, Rickettsia, Mycoplasma and Brucella (Anon, 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 manner in which tetracycline analogues interfere with lymphocyte growth.

MATERIALS AND METHODS

Fresh serial dilutions of the tetracycline drugs in tissue culture medium were prepared for each experiment.

Mononuclear cells were isolated from heparinized 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 (200iu/ml) and streptomycin (100μg/ml). The mononuclear cell suspensions were cultured in round bottomed microtitre plates (Sterilin): each well contained 2×10^5 cells in 100μl TC medium, 20μl autologous serum, 50μl TC medium with or without drug (for test or control) and 25μl phytohaemagglutinin (PHA) solution (HA15, Wellcome Reagents Ltd., Beckenham, England) used at optimal stimulating concentration of 2·5μl 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 21–24h for volume spectroscopy experiments, at 24, 48 and 72h for flow cytofluorimetry, at 72h for liquid scintillation and at the times indicated in the text for autoradiography. The exact duration of culture of the cells in each well was noted at harvesting when the lymphocytes were dislodged and monodispersed by repeated Pasteur pipette mixing using a technique which gives good recovery and avoids bias (Potts *et al.*, 1981). The extent of cellular contamination in the lymphocytes recovered from the control cultures was determined by volume spectroscopy (Potts *et al.*, 1980).

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 G_1-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 minimize 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 68h, 0·5μCi [³H]-TdR (Radiochemical Centre, Amersham, England) was added to each culture well: the cells were harvested 4h 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.

Observational microscopy and autoradiography. The influence of doxycycline on DNA synthesis by individual cells in established cultures was studied by adding 0·5μCi [³H]-TdR (Radiochemical Centre, Amersham, England) to each well after culture for 68h and the cells were harvested 4h later. The effect of doxycycline on the recruitment of cells into S-phase was studied by the addition of 0·125μCi [³H]-TdR to 18h PHA stimulated cultures and the cells were harvested at hourly intervals over the next 10h. In both sets of experiments, the cells were washed in plain TC199, fixed in methanol-acetic (3:1) and smeared on gelatinized slides. For observational microscopy the preparations were stained with Giemsa. For autoradiography they were dipped in K2 emulsion (Ilford Ltd., Mobberley, England) with grade 2 sensitivity, exposed for 1 or 2 weeks developed in DK50 Developer (Kodak, Hemel Hempstead, England), stained with Giemsa or haemalum and mounted in DPX.

Flow cytofluorimetric measurement of cellular nucleic acid content. The resuspended cells were stained with acridine orange (Polysciences Inc., Warrington, Pennsylvania, USA) by the method of Traganos *et al.* (1977). Fluorescence emission at 530nm (green) and 640nm (red) was measured in an Ortho 50H cytofluorimeter with illumination from a 8W argon-ion laser.

RESULTS

Addition of doxycycline inhibited the growth of cells from all subjects at, and above, $3·75\mu g/ml$ but the presence of 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 (Fig. 1).

The effect of doxycycline and tetracycline on cell volume growth during the first 24h of culture was 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 ($1·85–30·0\mu g/ml$) or in control medium. Neither drug (even at $30\mu g/ml$) altered the size distribution profile of either the unstimulated cells or modified that of PHA stimulated cells. Analysis of the profiles by computer confirmed that neither the numbers of cells responding to stimulation, nor their averaged rate of volume growth in the pre-S-phase period was modified by either of the drugs.

In flow cytofluorimeter studies of cells grown in the absence of drug, the RNA content of unstimulated cells remained relatively unchanged during the first day of culture, but the profile was shifted to the right in PHA stimulated cultures (Fig. 2). This lectin-induced change was reduced only very slightly by doxycycline at $5\mu g/ml$ or $15\mu g/ml$, indicating a relatively minor reduction in RNA growth: tetracycline did not modify the PHA-induced change in cellular RNA.
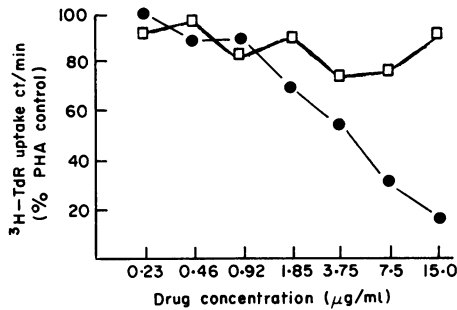

Fig. 1. Comparison between effects of doxycycline and tetracycline on PHA-stimulated growth of peripheral blood mononuclear cells from a healthy young adult male. The cultures were grown for 3 days and pulsed with [^{3}H]-TdR for the last 4h of culture. □——□ tetracycline; ●——● doxycycline.
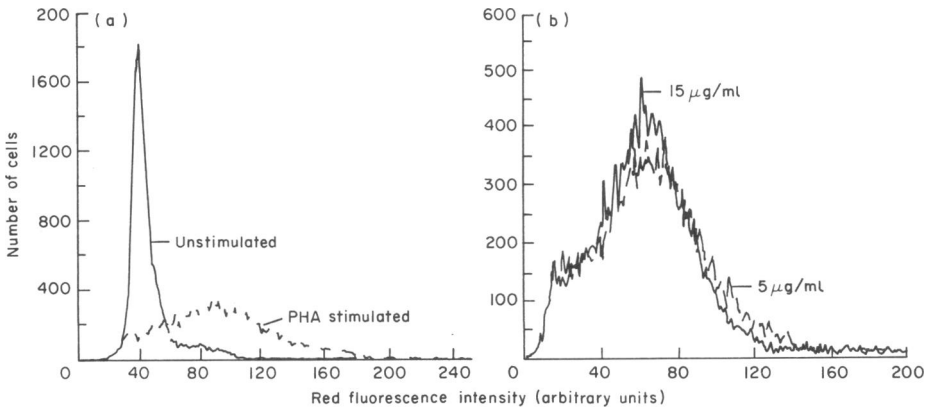

Fig. 2. Cytofluorogram of acridine orange stained cells to demonstrate cellular RNA content unstimulated and PHA stimulated 24h cultures grown in the absence of drug: doxycycline ($5\mu g/ml$ and $15\mu g/ml$) did not alter either distribution.
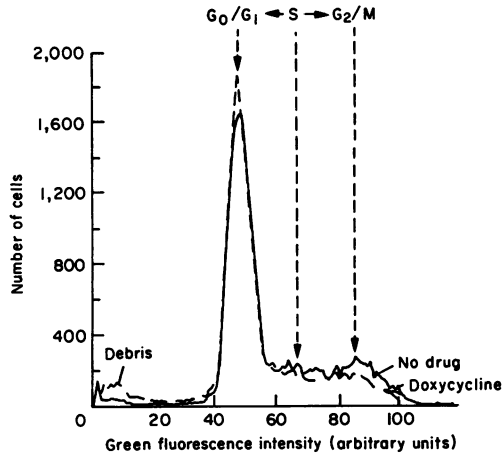
Fig. 3. Cytofluorograms showing distribution of intensity of green fluorescence in acridine orange stained cells from 72h PHA stimulated cultures: this is a measure of cellular DNA content. The measurements in the absence of drug are shown with the solid line and those in the presence of doxycycline (15μg/ml) with the interrupted line. Culture with doxycycline results in a fall in the S-phase plateau and the G_2/M peak with an increase in debris in the lower channels.
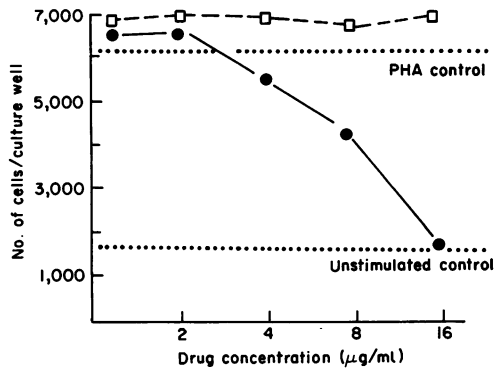

Fig. 4. Numbers of cells recovered from 3 day PHA stimulated cultures of peripheral blood mononuclear cells from a healthy adult male grown in the presence of various concentrations of drugs. Doxycycline (●——●) interfered with cellular proliferation in a dose-dependent manner, whereas tetracycline (□——□) did not inhibit growth in number of cells.

The cellular DNA content of 3 day PHA stimulated cultures was distributed as a major peak of G_0/G_1 cells with diploid 2c DNA content, a plateau of S-phase cells and a minor peak of G_2/M cells with 4c DNA content. In the presence of doxycycline the G_2/M cells did not form an identifiable peak, the S-phase plateau was reduced slightly and there were cells or fragments with less than 2c DNA (Fig. 3). These changes indicate that DNA replication is continuing, albeit at a reduced rate, in surviving cells, but the presence of cell fragments suggests that the fall in cell count (Fig. 4) in doxycycline treated cultures is due to increased cell death. The DNA histogram of PHA stimulated cells was not modified by tetracycline.

The effect of doxycycline on DNA synthesis by individual cells was studied by autoradiography afer exposure to [³H]-TdR. In one experiment, the PHA stimulated cells were cultured with the drug for 3 days and the label added for the last 4h: it is clear the doxycycline caused a dose related reduction in the proportion of cells synthesizing DNA (Fig. 5). In another experiment, the [³H]-TdR
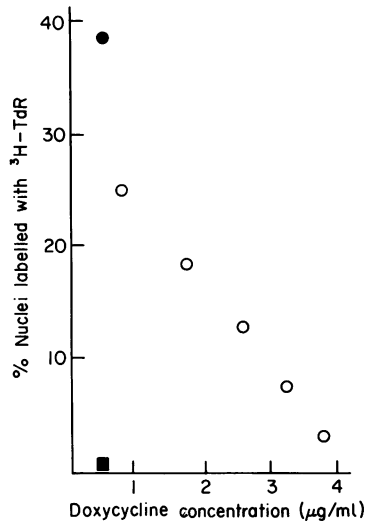
Fig. 5. Frequency of autoradiographic labelling of interphase nuclei in 3 day cultures of PHA stimulated peripheral blood mononuclears exposed to [³H]-TdR for the last 4h of culture. Doxycycline causes a dose related fall in the proportion of labelled cells. ● = PHA alone; ○ = PHA + doxycycline; ■ = non-stimulated.

was added after the PHA stimulated cells had been grown in tissue culture for 18h and the cells were harvested at hourly intervals: Fig. 6 shows that the recruitment of cells into the first S-phase in these cultures is retarded in the presence of doxycycline.

Microscopy of the 3 day PHA stimulated cultures showed that at moderate doxycycline concentration (1·0–3·0µg/ml) the cytological appearances are unmodified. At higher concentrations (4–15µg/ml) there were still healthy blast cells (many of which have taken up [³H]-TdR) but a few cells have cytoplasmic vacuolation or are undergoing apoptotic change (Fig. 7). Tetracycline at concentrations up to 15µg/ml produced little cytological change.
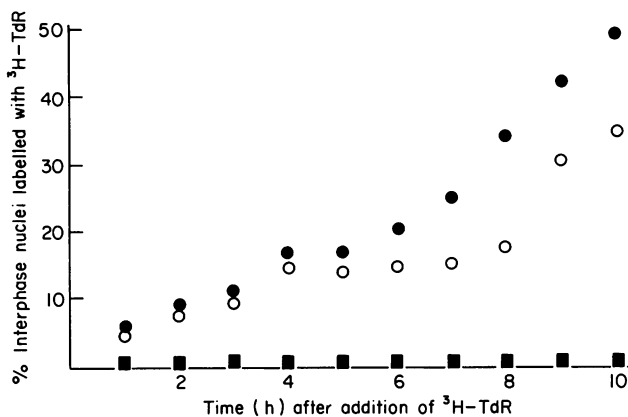

Fig. 6. Frequency of autoradiographic labelling of interphase nuclei in cultures harvested at hourly intervals after pulsed exposure to [³H]-TdR: the cells had been grown under optimal PHA stimulation for 18h before addition of label. The recruitment of cells into the first S-phase is retarded in the presence of doxycycline (5µg/ml). ● = PHA alone; ○ = PHA + doxycycline; ■ = non-stimulated.
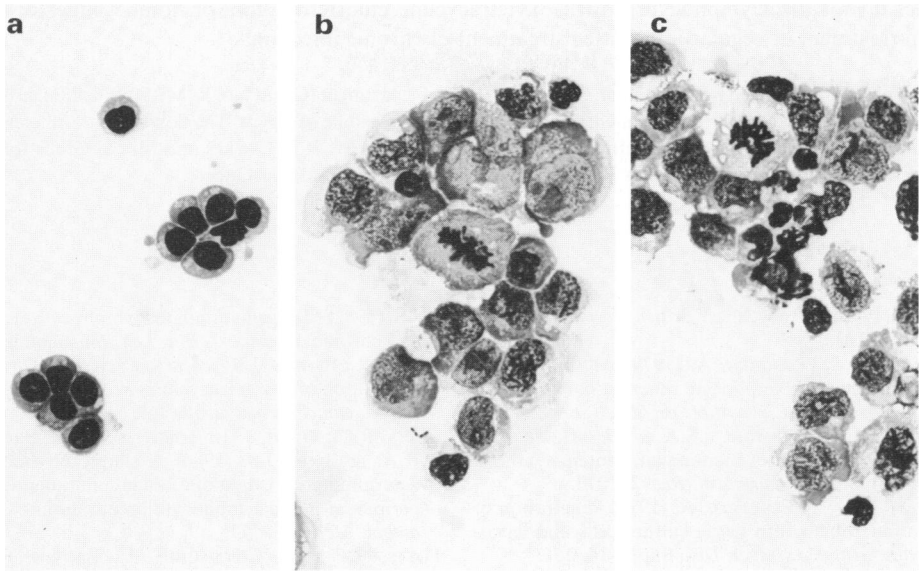
Fig. 7. Cytocentrifuge preparations of 3 day cultures of peripheral blood mononuclear cells from healthy adult male. (a) Unstimulated cells; (b) PHA stimulated cells without drug; (c) PHA stimulated cells with doxycycline (5μg/ml). Cultures grown with doxycycline show more unstimulated cells than the controls; the blast cells, although less frequent, do not appear abnormal. Geimsa stain, × 400.

DISCUSSION

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 amino-acyl-s-RNA to the RNA ribosome complex (Beard, Armentrout & Weisberger, 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.

In this study of the growth inhibiting action of doxycycline we have shown that the drug 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 numbers 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. These defects cannot be attributed solely to interference with protein synthesis. We have not identified the functional defect, but in view of the results of our comparative studies with various tetracycline analogues, it would not be very surprising if the lymphocyte growth inhibiting effect had a different biochemical basis from that of the bacteriostatic effects (Potts *et al.*, 1983).

When normal subjects take the usual recommended dose of doxycycline orally (100mg daily), the plasma levels are in the range 3–5μg/ml (Green, Brown & Calvert, 1976). Clearly, even higher blood levels may be found in excessive dosage (e.g., doxycycline in daily doses of 200mg or more). We have found that the DNA synthesis rate of stimulated lymphocytes may be reduced by as much as 50% with concentration doxycycline that correspond to therapeutic blood levels. In other studies (Potts *et al.*, 1983) we have shown that other tetracycline analogue drugs (demeclocycline, methacycline and minocycline) can reduce DNA synthesis of PHA stimulated lymphocytes by 20–40% at *in vitro* concentrations equivalent to therapeutic blood levels. Since this effect might interfere with the immune response *in vivo*, it would be prudent to use tetracycline or other drugs

which do not affect lymphocyte growth, oxytetracycline, chlortetracycline or clomocycline, for long term treatment or regular intermittent treatment of chronic infections.

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