Clin. exp. Immunol. (1979) 35, 443–446.

Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues

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(*Received* 20 *December* 1977)

SUMMARY

The effect of tetracyclines on mitogen-induced proliferative responses of human lymphocytes was examined. The results showed that of the three tetracycline analogues studied, doxycycline possessed the most potent inhibiting effects. This occurred at drug concentrations (1–10 μg/ml) easily attainable in serum during conventional dosage schedules. Other investigations have shown that tetracyclines also interfere with neutrophil function. Taken together, these findings may have clinical significance. Recovery from serious infections generally requires some minimal host immune responses, and the immunosuppressive side-effects of tetracyclines may have detrimental effects on patients with debilitating illnesses or impaired immunological defence mechanisms. Furthermore, tetracyclines may share some common properties of conventional immunosuppressive drugs, such as cytotoxicity, teratogenicity and cancerogenicity. The long-term use of tetracyclines for conditions such as chronic bronchitis, bronchiectasis and acne vulgaris needs to be re-examined.

INTRODUCTION

The tetracyclines are a group of antibiotics with broad spectrum antimicrobial activity enjoying widespread clinical use (Weinstein, 1975). Previous studies have shown that tetracyclines exert inhibitory effects on neutrophil function (Munoz & Geister, 1950; Forsgren, Schmeling & Quie, 1974; Martin *et al.*, 1973; 1974; Rubinstein & Pelet, 1973; Hill *et al.*, 1974). The effect of tetracyclines on lymphocyte function has not been studied. We have therefore studied the effects of tetracycline analogues on mitogen-induced lymphocyte proliferative responses, the *in vitro* correlates of immune responsiveness.

MATERIALS AND METHODS

The tetracycline analogues (tetracycline, doxycycline and oxytetracycline) were kindly provided by Pfizer Prop. Ltd., New South Wales, Australia. They were dissolved in RPMI 1640 tissue culture medium on the day of each experiment.

Lymphocytes were purified from heparinized blood of healthy adult donors by Hypaque–Ficoll centrifugation (Böyum, 1968). The cells were washed three times and resuspended in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum. Preliminary studies have established that the tetracycline analogues, at the concentrations used in these experiments, were not toxic to lymphocytes for up to 4 days in culture, as assessed by trypan blue dye exclusion.

Lymphocyte transformation studies were performed by a microtechnique described previously (Thong *et al.*, 1973). Briefly, sterile microtitre plates were used for cell cultures. Each round-bottom well received 2×10^5 lymphocytes in 0·1 ml volume and either 0·05 ml of phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). To the test wells were added 0·05 ml of one of the tetracycline analogues to reach a final concentration of 1·0 μg, 4·0 μg or 10 μg/ml. Control wells received 0·05 ml of medium only. The final concentrations for PHA (1·0 μg/ml) and PWM (50 μg/ml) used in these experiments were previously determined to produce optimal stimulation.

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0099-9104/79/0030-0443$02.00

The microtitre plates were incubated for 72 hr at 37°C in a 5% CO_2-air atmosphere and high humidity. 6 hr prior to harvesting, 1·0 μCi of ^{3}H-thymidine was added to each well. Harvesting was performed with the aid of a Skatron multiple sample harvester. The cells were aspirated onto glass-fibre filters, automatically washed with 0·015 M saline and then dried for quantification of radioactive uptake in a Packard Tricarb liquid scintillation spectrometer.

Additional experiments were performed to examine the effect of delayed addition of doxycycline on lymphocyte proliferative responses. A separate set of experiments was performed to determine whether the inhibitory effects of doxycycline on lymphocyte transformation could be reversed by washing. A further set of experiments was also conducted to assess blastogenic responses by direct microscopy of stained samples, in order to confirm that inhibition of ^{3}H-thymidine uptake correlates with suppression of blastogenesis.

RESULTS

Among the three tetracycline analogues, doxycycline was found to produce the greatest suppression of ^{3}H-thymidine uptake in both PHA-stimulated (Table 1), and PWM-stimulated cultures (Table 2).

TABLE 1. Effect of tetracycline analogues on PHA-induced human lymphocyte proliferative responses

	Tetracycline		Doxycycline		Oxytetracycline	
Drug concentration (μg/ml)	Ct/min ^{3}H-thymidine uptake	Percentage inhibition	Ct/min ^{3}H-thymidine uptake	Percentage inhibition	Ct/min ^{3}H-thymidine uptake	Percentage inhibition
1	35,990±10,425	15·4	34,592±10,541	18·7	35,696±6705	14·4
4	31,569±8644*	25·0	26,849±8299†	35·8	30,749±8977*	26·4
10	32,030±8864*	24·0	2282±560†	94·4	31,945±8395*	23·0

Results represent mean±s.d. of nine experiments using cells from nine different donors. Uptake of ^{3}H-thymidine in control cultures was 42,288±8127 ct/min (mean±s.d.).

* $P<0·01$.

† $P<0·001$.

TABLE 2. Effect of tetracycline analogues on PWM-induced human lymphocyte proliferative responses

	Tetracycline		Doxycycline		Oxytetracycline	
Drug concentration (μg/ml)	Ct/min ^{3}H-thymidine uptake	Percentage inhibition	Ct/min ^{3}H-thymidine uptake	Percentage inhibition	Ct/min ^{3}H-thymidine uptake	Percentage inhibition
1	17,749±6478	4·8	17,834±7041	5·0	20,975±9468	0
4	13,888±5063*	27·3	12,378±4193*	31·0	17,217±7602	9·1
10	12,042±5349	36·2	1423±1071	92·2	16,295±7537	13·6

Results represent mean±s.d. of nine experiments using cells from nine different donors. Uptake of ^{3}H-thymidine in control cultures was 19,358±9665 ct/min (mean±s.d.).

* $P<0·01$.

† $P<0·001$.

At a concentration of 10 μg/ml, percentage inhibition of ^{3}H-thymidine uptake in PHA-stimulated cultures was 94·4 in doxycycline-treated cultures, compared to 24·0 in tetracycline-treated and 23·0 in oxytetracycline-treated cultures. In PWM-stimulated cultures percentage inhibition of ^{3}H-thymidine uptake was 92·2 in the presence of doxycycline, compared to 36·2 in the presence of tetracycline, and 13·6 in the presence of oxytetracycline.

The inhibitory effect of doxycycline was also dose-dependent (Tables 1 and 2). This inhibitory effect was found to be due to a direct, inhibition of blast transformation. At 10 μg/ml concentration, the blasto-

TABLE 3. Effect of immediate and delayed addition of doxycycline on mitogen-induced human lymphocyte proliferative responses

Time of addition of doxycycline (10 μg/ml)	PHA ct/min ^{3}H-thymidine uptake	PWM ct/min ^{3}H-thymidine uptake
0	3102± 198	1100± 170
24 hrs	3808± 575	492± 38
48 hr	5489± 683	1508± 419
None added	36,946± 6601	16,063± 3252

Results represent mean± s.d. of three experiments using three different donors.

genic index in PHA-stimulated cultures was 0·5 in the presence of doxycycline compared to 26·1 in the control; in PWM-stimulated cultures it was 2·1 in the presence of doxycycline compared to 17·0 in the control.

Some characteristics of this inhibitory effect have been defined. In both PHA- and PWM-treated cultures, marked suppression of ^{3}H-thymidine uptake occurred even when doxycycline was added 48 hr after the start of experiments (Table 3). The inhibitory effect of doxycycline could be reversed completely by washing (Table 4) suggestive of a lack of tight binding to membrane receptors.

TABLE 4. Reversibility of inhibition of mitogen-induced human lymphocyte responses by doxycycline

Treatment (doxycycline 10 μg/ml)	PHA ^{3}H-thymidine uptake	PWM ^{3}H-thymidine uptake
Unwashed	2158± 631	1377± 660
Washed	22,618± 3519	11,042± 1619
Untreated control	26,922± 4236	13,633± 2754

Results represent mean± s.d. of triplicate samples.

In these experiments, lymphocytes were incubated in the presence of doxycycline for 1 hr, and then washed three times prior to culture with mitogens.

DISCUSSION

The results of the present studies indicate that doxycycline, and to a much lesser extent, tetracycline and oxytetracycline, exert a potent inhibitory effect on mitogen-induced lymphoproliferative responses of human lymphocytes. Other investigators have also reported that tetracyclines adversely affect neutrophil chemotactic (Munoz & Geister, 1950; Forsgren *et al.*, 1974), phagocytic and metabolic function (Rubinstein & Pelet, 1973; Hill *et al.*, 1974), as well as the bactericidal effect of serum (Forsgren & Gnarpe, 1973). Taken together, these observations may have clinical relevance. Since recovery from serious infections generally requires at least some minimal host immune responses, these immunosuppressive properties of tetracyclines may be detrimental to patients with debilitating diseases or impaired immunological defence mechanisms.

The tetracyclines may also share some common properties of conventional immunosuppressive drugs, such as cytotoxicity, teratogenicity and cancerogenicity (Meischer, Gerebtzoff & Lambert, 1976). The clinical observation of acute hepatic necrosis in patients treated with tetracyclines may be a manifestation of cytotoxic side-effects (Schultz *et al.*, 1963; Lloyd-Still, Grand & Vawter, 1974). Tetracyclines traverse the placenta readily and teratogenic effects on rats and humans have been documented (Carter & Wilson,

1962; Cohlan, Beverlander & Tiamsic, 1963). The cancerogenic potential of tetracyclines has not yet been realised clinically. These potential hazards of tracyclines are increased, especially in clinical situations of chronic bronchitis and acne vulgaris, where tetracyclines are prescribed for long-term usage.

The mechanism by which tetracyclines adversely affect immune function is not well understood. This may be related to its metabolic effects on microorganisms. In this respect, its principal action appears to be the inhibition of protein synthesis (Gale *et al.*, 1972). At higher concentrations (50–100 μg/ml), however, tetracycline also inhibits DNA synthesis and alters the membrane properties of *Eschericia coli* and *Bacillus subtilis* (Pato, 1977). The relevance of this observation to human lymphocytes is not clear, since the effects on human lymphocytes were seen with low concentrations of 1–10 μg/ml. Further studies are needed to elucidate the mechanism of immunosuppression by tetracyclines.

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