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Influence of Tetracyclines on Human Polymorphonuclear Leukocyte Function

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Low concentrations of oxytetracycline, doxycycline, or minocycline (< 10 μg/ml) did not influence in vitro polymorphonuclear leukocyte random migration, chemiluminescence, or glucose oxidation. At high concentrations of doxycycline or minocycline (> 10 μg/ml), chemiluminescence and glucose oxidation were impaired. High concentrations of doxycycline also reduced random migration. Oxytetracycline did not influence these functions in concentrations up to 100 μg/ml. The inhibiting effect of doxycycline and minocycline was abolished when 4 mM Mg^{2+} was added to the reaction mixture, and 4 mM Ca^{2+} partly restored minocycline-inhibited polymorphonuclear leukocyte functions. This indicates that the major effect of tetracyclines on in vitro polymorphonuclear leukocyte functions is mediated by their divalent cation chelating effect and that the results of in vitro experiments are highly dependent on the concentration of divalent cations in the reaction mixtures. The difference between the tetracyclines may be due to differences in lipid solubility, with solubility being highest for minocycline and lowest for oxytetracycline, or to different divalent cation chelating ability.

Polymorphonuclear leukocytes (PMNLs) represent cornerstones of host defense against infections (2). The possibility that antimicrobial agents may impair PMNL functions is of particular interest since these drugs are frequently administered to patients with enhanced susceptibility to infection. Several studies have focused on the effect of tetracyclines on PMNL function, but the results of these studies are not consistent. In some studies, (4, 8, 12, 18, 24) doxycycline has been shown to reduce PMNL chemotaxis (4, 12), phagocytosis (8, 19), or bactericidal activity (18, 24) or all three of these. However, in another study, Forsgren and Gnarpe have failed to demonstrate any effect on these functions (7). Oxytetracycline has been reported not to inhibit PMNL functions (7). We have previously shown that doxycycline does not reduce in vivo PMNL migration to skin chambers (10) and have indicated that the impairment of PMNL functions measured in vitro may be due to photo-induced production of toxic oxygen species or due to the divalent cation chelating effect of tetracyclines or both. The aim of the present study was to examine the influence of oxytetracycline, doxycycline, and minocycline on human PMNL functions in the presence of high and low divalent cation concentrations.

MATERIALS AND METHODS

Leukocytes. Leukocytes were obtained by dextran sedimentation of heparinized blood (18 U of heparin per ml of blood) from healthy volunteers. After centrifugation of the supernatant (500 × *g*, 5 min) the red cells were lysed with ammonium chloride solution (0.15 M). The leukocytes used for chemiluminescence (CL) and glucose oxidation assays were washed twice in phosphate-buffered saline. A differential count was made, and the cells were resuspended in Hanks balanced salt solution, containing 20 mg of bovine serum albumin per ml, to make concentrations of 10^7 PMNLs per ml. For tube migration assays, the cells were washed twice in 0.15 M NaCl and suspended in autologous plasma.

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Tube migration. Migration of leukocytes in capillary tubes was studied by a method described by Ketchel and Favour (15) and modified by Schreiner and Hopen (22). Briefly, leukocyte suspensions (ca. 10^7 cells per ml) in autologous plasma were aspirated halfway into hematocrit capillary tubes, which were sealed at the bottom end. The tubes were centrifuged at 2,500 × *g* for 3 min in a hematocrit centrifuge and mounted in five parallels on microscope slides. After incubation for 2 h at 37°C, the zone of migrating leukocytes on the tube wall was measured with an ocular micrometer. Tube migration was expressed in millimeters (mean value of five tubes for each test condition).

CL. The reaction mixture contained 2.8 ml of phosphate-buffered saline, 0.2 ml of opsonized zymosan, and 0.5 ml of leukocyte suspension which made a total volume of 3.5 ml and a ratio of ca. 100 zymosan particles per PMNL (23). The mixtures were prepared in polyethylene scintillation vials (Hostalen; Werner Zinsser, Frankfurt/Main, Federal Republic of Germany) which were kept in the dark before use. The vials were immediately capped upon introduction of the cells and placed in a Beckman LS-100c scintillation counter, which was out of phase and which had one photomultiplier disconnected. Duplicate vials for each test condition were counted in 10-min intervals for 60 min at ambient temperature, and the results were recorded in mean counts per minute.

Oxidation of [1-^{14}C]glucose. The reaction mixture contained 2.8 ml of phosphate-buffered saline, 0.2 ml of opsonized zymosan, and 0.5 ml of leukocyte suspension. The glucose concentration in the reaction mixture was 0.72 mM (0.1 μCi, [1-^{14}C]glucose included; Radiochemical Centre, Amersham, England). The incubations were carried out in 20-ml scintillation vials at 37°C. The reaction was terminated after 1 h by the addition of 0.1 ml of 9 M H_2SO_4. [^{14}C]CO_2 was absorbed into filter paper wetted with 0.5 ml of 25% (vol/vol) β-phenylethylamine in methanol (16). After shaking for 30 min, 10 ml of scintillation fluid (Scint Hei 3; Koch Light Laboratories, Colnbrook, Buckinghamshire, England) was added, and the samples were counted in a Beckman LS-100c scintillation counter to a precision of 2%.

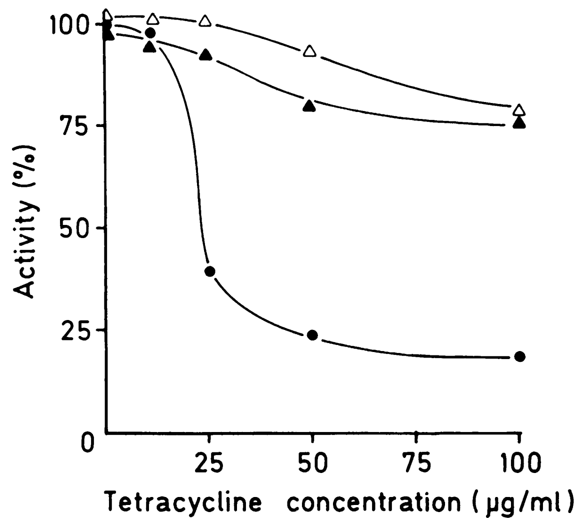

FIG. 1. Effect of oxytetracycline (△), doxycycline (●), and minocycline (▲) on PMNL random migration. Results are given as the percentage of the activity without tetracycline and represent the mean value of three experiments. The range was within 7% of the mean.

Fluorescence assay. The relative fluorescence of the tetracyclines was measured with a Mark I spectrofluorometer (Farrand Optical Co., Inc.) with a xenon lamp as the power supply. The tetracyclines were dissolved in 0.15 mM NaCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer (pH 7.4) to a concentration of 100 μg/ml. In some experiments, 4 mM divalent cations (Ca^{2+} or Mg^{2+}) were included in the solutions.

Antibiotics. Oxytetracycline and doxycycline (crystalline chloride) were supplied by Pfizer Corp., Brussels, Belgium, and minocycline was supplied by Lederle Laboratories, Pearl River, N.Y. In the CL and glucose oxidation tests, the tetracyclines were dissolved in phosphate-buffered saline and added to the reaction mixtures. In the tube migration test, the tetracyclines were dissolved in 0.9% NaCl and added to the leukocyte plasma suspensions. The tetracyclines were added to the cell suspensions immediately before the test procedure, and all suspensions containing drugs were kept in the dark. The results are given as the percentage of the control samples without drug added.

Media. The CL and glucose oxidation reaction mixtures contained 0.12 mM Mg^{2+} and 0.18 mM Ca^{2+} when not otherwise stated. In some experiments, the divalent cation concentration was increased by adding 4 mM $MgCl_2$ or 4 mM $CaCl_2$ to the reaction mixture.

RESULTS

Doxycycline (10 μg/ml) had no effect on PMNL random migration (Fig. 1). At higher concentrations, a significant reduction occurred; and at 100 μg/ml, only 25% of the initial activity was left. Oxytetracycline and minocycline had only a minor effect on random migration. In the presence of doxycycline and minocycline, PMNL CL and glucose oxidation rapidly declined, with minocycline being the most potent inhibitor (Fig. 2 and 3). At 100 μg of minocycline per ml, these functions were abolished. At this concentration, doxycycline only partly inhibited CL and glucose oxidation, and oxytetracycline had no effect.

The inhibitory effect of tetracyclines on PMNL functions was abolished when excess Mg^{2+} (4 mM) was added to the reaction mixture (Table 1). Even an increase above the initial CL production was observed when the test was carried out with 4.3 mM Mg^{2+} in the presence of 100 μg of doxycycline or oxytetracycline per ml. Ca^{2+} (4 mM) only partly abolished the inhibiting effect of minocycline, whereas no effect of Ca^{2+} was observed on doxycycline-inhibited PMNL functions (Table 1).

The fluorescence intensity of minocycline was low compared with doxycycline and oxytetracycline (Table 2). An increase in fluorescence intensity occurred when 4 mM

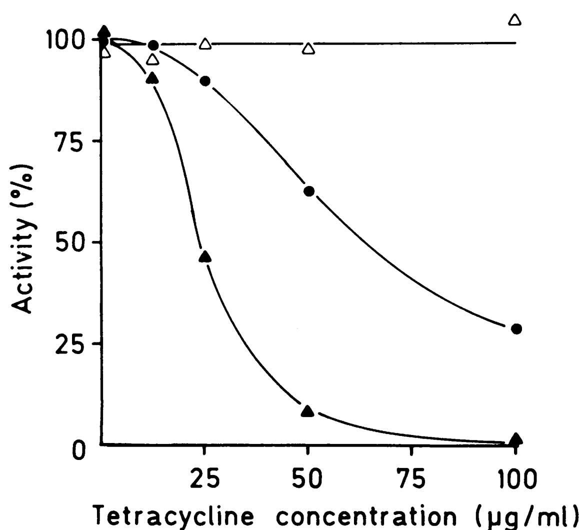

FIG. 2. Effect of oxytetracycline (△), doxycycline (●), and minocycline (▲) on CL from PMNLs exposed to zymosan. Results are given as the percentage of the activity of cells without tetracycline and represent the mean value of three experiments. The range was within 10% of the mean.

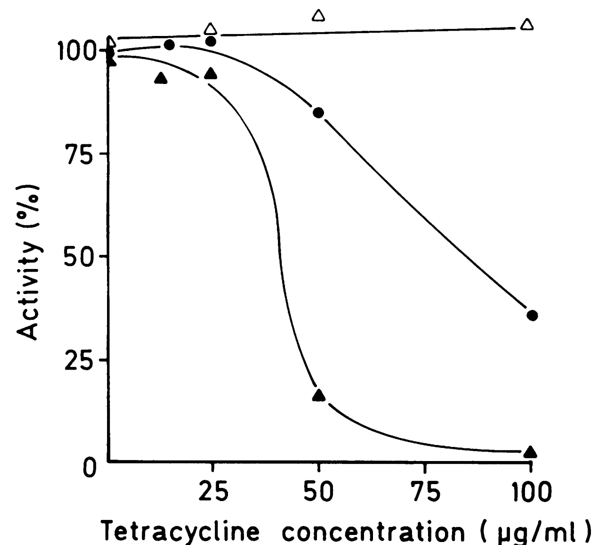

FIG. 3. Effect of oxytetracycline (△), doxycycline (●), and minocycline (▲) on neutrophil glucose oxidation. Results are given as the percentage of the activity of cells without tetracycline and represent the mean value of three experiments. The range was within 10% of the mean.

Mg^{2+} or Ca^{2+} was added to solutions of 100 μg of doxycycline or oxytetracycline per ml. Also, an excitation maximum shift toward higher wavelengths was observed when divalent cations were added to oxytetracycline or doxycycline solutions.

DISCUSSION

Despite great similarities in chemical structure, tetracyclines differ markedly in their ability to influence PMNL functions (4, 7, 8, 12, 14). In our study, high concentrations of doxycycline reduced PMNL random migration, CL, and glucose oxidation, whereas minocycline had a marked effect on CL and glucose oxidation. Oxytetracycline had no marked effect on any of these functions. The reasons for these differences between closely related drugs are not obvious. However, CL and glucose oxidation each reflects the phagocytic and bactericidal activity of the PMNLs (1, 2, 5, 6, 17). In our study, this was indicated by the close correlation between the results of these two tests. Random migration, however, is independent of the bactericidal activity of the PMNLs. Accordingly, CL and glucose oxidation may be reduced without simultaneous impairment of cell migration, as demonstrated in our experiments with minocycline. On the other hand, doxycycline influences both groups of PMNL functions. The minor effect of oxytetracycline on any of these functions may be due to the poor lipid solubility of this drug (3) or to the lower divalent cation chelating effect.

In the present study, a marked reduction of PMNL functions occurred when doxycycline or minocycline was added to the test suspensions. The reason for this reduction may be binding of these lipid-soluble tetracyclines to membrane-associated Mg^{2+} and Ca^{2+} (9, 20, 21). The divalent cations are necessary for normal PMNL functions (11, 13), and marked binding to tetracyclines may result in impaired cell functions (13). In fact, the decrease in fluorescence of membrane-bound drug has been used to monitor Ca^{2+} release from the membrane to the cytosol during stimulation (20). However, an excess of divalent cations in the medium should result in saturation of the tetracycline chelating effect, and no subsequent binding to cell-associated ions should occur. In our study, this was demonstrated by the restoration of normal cell function when excess Mg^{2+} was present in the reaction mixture. Excess Ca^{2+}, however, did not reverse doxycycline-inhibited functions and only partly reversed those inhibited by minocycline. This may be due to the requirement for Mg^{2+} for normal PMNL response to opsonized zymosan. Nevertheless, these results indicate that the inhibiting effect of doxycycline and minocycline on PMNL functions, at least in part, is mediated by their divalent cation chelating effect.

TABLE 1. Effect of tetracyclines (100 μg/ml) on PMNL functions at low and high divalent cation concentrations

Drug	Divalent cation[a] added to 0.3 mM Mg^{2+} + Ca^{2+}	Mean (range) % activity of control of the following tests[b]	
		CL	Glucose oxidation
Oxytetracycline	0	110 (106–116)	100 (96–108)
	Mg^{2+}	256 (232–279)	112 (101–119)
	Ca^{2+}	214 (166–262)	90 (89–91)
Doxycycline	0	32 (27–34)	25 (18–32)
	Mg^{2+}	247 (204–285)	104 (85–117)
	Ca^{2+}	31 (27–38)	23 (19–30)
Minocycline	0	0	5 (2–10)
	Mg^{2+}	105 (86–126)	122 (109–132)
	Ca^{2+}	24 (7–41)	35 (28–47)

[a] Indicated cations were added at a concentration of 4 mM.

[b] Results (mean value of three experiments and range) are given as the percentage of the activity of cells suspended in medium containing low (0.3 mM) divalent cation concentrations and no tetracycline.

TABLE 2. Fluorescence intensity and excitation maxima of tetracyclines (100 μg/ml) in the absence and presence of divalent cations

Drug and added divalent cation	Fluorescence intensity[a]	Fluorescence excitation maximum (nm)
Oxytetracycline	2.5	402
Oxytetracycline + 4 mM Ca^{2+}	14	412
Oxytetracycline + 4 mM Mg^{2+}	29	403
Doxycycline	1	394
Doxycycline + 4 mM Ca^{2+}[b]	83	412
Doxycycline + 4 mM Mg^{2+}	30	407
Minocycline	0.006	436
Minocycline + 4 mM Ca^{2+}	0.019	400
Minocycline + 4 mM Mg^{2+}	0.003	440

[a] Fluorescence intensity is recorded in arbitrary units, with the fluorescence of doxycycline in buffer considered as 1 unit. The fluorescence emission wavelength was 520 nm.

[b] Precipitation of calcium doxycycline.

In the present study, CL production was doubled, as compared with initial values, in the presence of doxycycline or oxytetracycline and excess divalent cations. Most likely, this was due to reinforcement of the PMNL light production by the tetracycline fluorescence rather than an increase in PMNL activity. This is supported by the observation that CL was only restored to control levels in the presence of excess divalent cations and a tetracycline, i.e., minocycline, with low fluorescence intensity. Furthermore, no increase above initial levels of glucose oxidation activity was observed when 4 mM Mg^{2+} was added to doxycycline or oxytetracycline-containing reaction mixtures.

Results of in vitro PMNL function tests carried out in the presence of tetracycline are highly influenced by the concentration of divalent cations, particularly Mg^{2+}, in the reaction mixtures. It is likely that the discrepancy in results of studies concerning the influence of tetracyclines on PMNL functions may, at least in part, be due to methodological differences in test procedures, primarily variations in divalent cation concentration in the reaction mixture.

Therapeutic concentrations of tetracyclines caused no reduction in PMNL functions in our test systems. From this point of view, the effect of tetracyclines on PMNL functions measured in vitro does not necessarily reflect in vivo effects.

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