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EFFECT OF DOXYCYCLINE ON OXYGEN-DEPENDENT KILLING MECHANISMS OF HUMAN NEUTROPHILS

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Abstract—The effects of doxycycline on neutrophil adhesivity, ingestion rate, and oxidative burst by particle and soluble compounds have been analyzed. The rate of bacterial ingestion by neutrophils as well as its subsequently particle-induced oxidative burst comprising oxygen uptake, hydrogen peroxide and superoxide anion productions, and iodination were all inversely correlated to doxycycline concentration included in the assay medium. The neutrophil oxidative burst induced by phorbol myristate (a soluble stimulant) was also inversely correlated to doxycycline concentration. Drug effect was observed at lower concentrations when the neutrophil stimulant was a soluble compound than when it was particles. In contrast doxycycline did not affect neutrophil adhesivity to either nylon fibers or Petri dishes. Further studies are needed to assess whether the activity of the drug on the neutrophil is due only to its ability to chelate calcium and magnesium or to other properties.

Human polymorphonuclear leukocytes (PMN) play a crucial role in non-specific defence against infectious agents, particularly bacteria [1, 2]. It has become increasingly apparent that some antimicrobial agents may adversely affect PMN function, and this may hamper recovery from infection [3–6]. Doxycycline (Dx) is a tetracycline known to possess bacteriostatic and not bactericidal activity [7]. This suggests that Dx would be all the more efficient as it did not reduce PMN bactericidal activity. However, Dx has been shown to diminish PMN particle-ingesting capacity [8–11] and bacteria-killing ability [12]. It is known that stimulated PMN generate toxic oxygen species essential for the killing of bacteria, and that particle and soluble compounds stimulate PMN in different ways [1, 2, 13]. In this connection, the effect of Dx on the oxygen-dependent PMN killing mechanisms has recently been reported to inhibit PMN luminescence induced by particles [14]. However, these reports neither assessed the effect of Dx on PMN stimulated by soluble compounds nor tested the most potent oxygen-dependent killing mechanism of PMN, i.e. myeloperoxidase-hydrogen peroxide-halide [1]. Consequently the effect of Dx on PMN oxidative burst induced by both soluble and particle compounds was analyzed here, directly, by measuring oxygen uptake, the production of superoxide anion and hydrogen peroxide, as well as iodination.

MATERIALS AND METHODS

Human PMN were isolated from venous blood anticoagulated with preservative-free lithium heparinate (10 UI per ml of blood) by one step centrifugation of whole blood on mono-poly resolving medium (Flow Lab. Puteaux, France) which separates mononuclear cells and PMN into two distinct bands [15]. Erythrocytes were removed by lysis with ammonium chloride [16]. PMN were washed twice and suspended in Ca^{2+} and Mg^{2+}-free Krebs Ringer Phosphate, pH 7.4, with 0.72 mM glucose, referred to hereafter as KRP. Dx hydrochloride ($C_{22}H_{25}Cl_2O_8$; M.W.: 480) a gift from Pfizer Corp. was prepared just before use in KRP.

PMN adhesivity was measured by two different techniques on nylon fibers and plastic Petri dishes. Adhesivity to nylon fibers was measured according to MacGregor *et al.* [17] using various amount of fibers, and at various effluent flow speeds. PMN (2×10^6 in 2 ml) were preincubated with Dx or its solvent for 30 min at 37° before being filtered through the nylon fibers, which contained the same amount of Dx. The percentage of adhering PMN was calculated from the percentage recovered. PMN adhesivity to Petri dishes was measured as follows: 2 ml of a PMN suspension (10^6 per ml) in KRP was poured into plastic Petri dishes (Falcon 10 × 35 mm Lab. Express Ser. France) in the presence of various amounts of Dx. Where indicated, 1 mM Ca^{2+} and 0.5 mM Mg^{2+} were added 10 min later. The dishes were then washed twice with 1 ml of preheated KRP per dish. The washing solutions were then pooled with the original supernatant. We checked by a further washing that the solution no longer contained any PMN. The PMN recovered were counted in a Coulter Counter (Coultronics, France), and the percentage of adhering PMN was then calculated.

The rate of PMN ingestion of heat-killed ^{14}C-labelled *Klebsiella pneumoniae* was measured as previously described [18] in the presence of 100 microorganisms per cell. Before these microorganisms were added, PMN were incubated for 10 min at 37° with various concentrations of Dx in KRP. Dx was not removed during the assays. Oxygen uptake by

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unstimulated PMN or by PMN stimulated with either opsonized zymosan or phorbol myristate acetate (PMA) (Sigma Chem. Co., St Louis, MO) was measured polarographically as previously described [19]. Hydrogen production by PMN stimulated with opsonized zymosan or PMA was also measured [19]. Before stimulation, PMN were incubated in KRP for 10 min at 37° with various concentrations of Dx which was not removed during the assays. Superoxide anion production by PMN stimulated by PMA was measured according to Cohen and Chovianec [20] by continuous recording of cytochrome *c* reduction with a Uvikon 810:820 spectrophotometer. The incubation medium, placed in a thermostated cuvette (37°), contained 80 μm cytochrome *c*, 10% decomplemented human serum and 10^6 PMN per ml. PMN were preincubated in KRP for 10 min at 37° with Dx. When used, superoxide dismutase was at a final concentration of 10 μg per ml. The effects of Dx on oxygen uptake and superoxide anion production by the xanthine-xanthine-oxidase system, were measured as in PMN, except that the latter were replaced by the enzymatic system. Iodination was measured as previously described [16] with 20 μM sodium iodide in the incubation medium and preincubation of the PMN with Dx for 10 min at 37° in KRP. In all control experiments, PMN were incubated in KRP for the same period as with Dx.

Results used for statistics are the means of assays performed in duplicate, after subtraction of appropriate blanks. Results were discarded when a variation of more than 10% was observed between the duplicate values. Statistics were calculated on a Compucorp 445 Statistician. The significance of differences between control and assay values was assessed by the paired or unpaired Student-*t*-test. Linear regression parameters were calculated by the least square method.

RESULTS

PMN adhesivity to nylon fibers was not altered by preincubation of PMN with Dx concentrations ranging from 10 to 100 μg per ml. Under experimental conditions in which the amount of nylon fibers and liquid output speed varied, PMN adhesivity was similar for Dx-treated PMN and controls (Fig. 1). PMN adhesivity to the plastic Petri dishes was also unaltered by Dx. The percentage of control PMN which adhered to the dishes was 30 ± 7.2 (mean ± 1 S.D.; N = 6). Dx (50 or 100 μg per ml) did not modify this percentage (29.4 ± 8.3 and 31.6 ± 9.1 respectively). Addition of 1 mM Ca^{2+} and 0.5 mM Mg^{2+} to the incubation medium increased the adhesivity of both control and Dx-treated PMN (more than 90% of the PMN were adherent).

Dx inhibited the rate of ingestion by PMN of heat-killed opsonized *Klebsiella pneumoniae*. The inhibition was linearly correlated to Dx concentration (Fig. 2A). To exclude any direct toxic effect of Dx on PMN, 50 or 100 μg per ml Dx was incubated for 30 or 60 min at 37° with whole blood or isolated PMN. The ultrastructural morphology of the cells (results not shown) as well as their ability to exclude trypan blue (more than 90% of the PMN) did not alter.

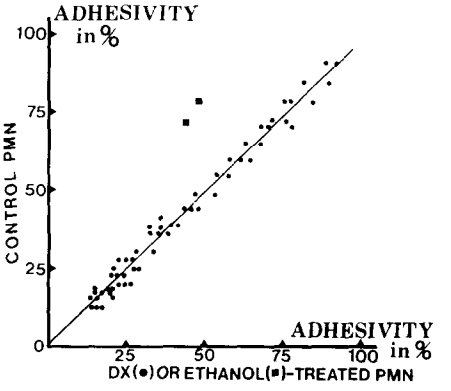

Fig. 1. Effect of doxycycline (Dx) on PMN adhesivity to nylon fibers. PMN adhesivity to nylon fibers was measured in the presence of various amounts of fibers, at different flow rates and in the presence of Dx concentrations ranging from 10 to 100 μg per ml. The correlation between control values (abcissa) and Dx-treated PMN (ordinate) was linear ($r = 0.986$; $P < 0.001$). The slope was close to one ($s = 0.985$) and intercepted the ordinate at 1. The technique was checked with PMN treated with ethanol which reduces adhesivity (■).

Oxygen uptake by unstimulated PMN (0.52 ± 0.22 nmoles per min per 10^6 PMN) was not affected by Dx at concentrations ranging from 5 to 100 μg per ml. As expected from the ingestion rate results [18], Dx inhibited oxygen uptake by PMN stimulated with opsonized zymosan. The inhibition was linearly correlated to Dx concentrations ranging from 5 to 100 μg per ml (Fig. 2B). Dx inhibited oxygen uptake by PMN, not only when they were stimulated by particles, but also by a soluble stimulus such as PMA. Here again, the inhibition was linearly correlated to the Dx concentration and was detectable at low Dx concentrations (Fig. 2B). Like oxygen uptake, hydrogen peroxide production by PMN stimulated either by opsonized zymosan or PMA was inhibited by Dx (results not shown).

Superoxide anion production by PMN stimulated by PMA was also inhibited by Dx, similarly to oxygen uptake (Fig. 2A). The lag time preceding the reduction of cytochrome *c* (75 ± 6 sec for 20 different control assays) was not altered by Dx. It was checked that Dx impeded neither oxygen uptake nor superoxide anion production by xanthine-xanthine oxidase system used instead of the PMN.

Dx reduced iodination by PMN stimulated with opsonized zymosan (Fig. 2A). This reduction was proportional to the hydrogen peroxide production, suggesting that myeloperoxidase activity was not inhibited, and that this enzyme was released in sufficient amounts from the azurophilic granules into the phagosomes [18, 21].

DISCUSSION

Our results show that Dx inhibits not only the ability of PMN to ingest particles and mount a subsequent oxidative burst but also the oxidative burst induced by a soluble compound such as PMA. They also show that Dx does not alter PMN adhesivity

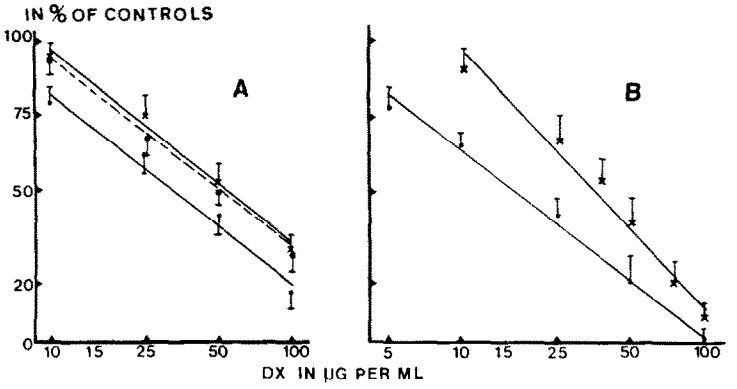

Fig. 2. Effect of doxycycline on ingestion rate, oxygen uptake, superoxide anion production and iodination. PMN were incubated with Dx at concentrations ranging from 5 to 100 µg per ml for 10 min before performing function tests. Results are expressed as percentages of control values (mean ± 1 S.D. of 7 to 10 experiments). Log scale for Dx concentrations. Part A represents ingestion rate of heat-killed *Klebsiella pneumoniae* (×), superoxide anion production induced by PMA (■) and iodination induced by zymosan (●). Correlation coefficients were higher than 0.98 and slopes were −63.8, −63.9 and −62.4 per log of Dx concentration respectively. Part B represents oxygen uptake by PMN stimulated either with zymosan (×) or PMA (●). Correlation coefficients were higher than 0.98 and slopes were −84.5 and −61 respectively.

either in the absence of Ca^{2+} and Mg^{2+} added to the medium or when these dications are added after contact of PMN with the drug.

The diminished rates found here for ingestion by PMN of heat-killed opsonized *Klebsiella pneumoniae* confirm previous investigations with other particles showing that Dx impedes engulfment by PMN [8–11]. The drug target of Dx was the PMN and not the opsonins, because when PMN were washed after incubation with Dx, they behaved like unwashed PMN. Inhibition of particle ingestion was linearly correlated to the drug concentration. The IC_{50} of the drug was about 50 µg per ml. Because the PMN oxidative burst induced by particles is closely correlated to the ingestion rate [18], we observed, as expected, that Dx inhibited the PMN oxidative burst induced by opsonized zymosan. The oxidative burst of stimulated PMN comprises an increase in cyanide-insensitive oxygen uptake, and the generation of toxic oxygen by-products such as superoxide anion and hydrogen peroxide [1, 13]. In the presence of a halide and of hydrogen peroxide, myeloperoxidase then forms highly toxic products which kill bacteria, and the overall activity involved can be measured by the iodination test [1, 16]. Here, oxygen uptake and iodination by the PMN stimulated with zymosan were linearly correlated to Dx concentrations. For both activities, the IC_{50} of the drug was about 40 µg per ml. These results confirm and extend the finding of previous studies which used the unspecific and non-quantitative test of histochemical reduction of nitroblue tetrazolium dye reduction to show that the PMN oxidative burst was altered by Dx [22, 23]. Iodination was also depressed by Dx. Its decline was, however, completely accounted for by the decrease in hydrogen peroxide production by the zymosan-stimulated PMN, suggesting that degranulation, at least of azurophilic granules, occurred normally. Furthermore, we observed that PMN stimulated by PMA, a soluble stimulus, also had a depressed oxidative burst which was proportional to the Dx concentration. Inhibition of oxygen uptake and superoxide anion production by PMN stimulated with PMA was noted for low Dx concentrations such as those observed during the treatment of humans with this drug [7]. It is known that PMA is a potent stimulus of oxygen uptake and superoxide anion production, that it does not need to be ingested and that it acts on the PMN by binding to specific receptors [20]. PMA binding to its specific receptors can be measured by the lag time between its addition to the medium and the beginning of superoxide anion production [20]. Here, superoxide anion production declined in the presence of Dx, but the lag period did not alter, suggesting that PMA bound normally to the PMN membrane. This normal binding, combined with the fact that oxygen uptake by PMN stimulated by opsonized zymosan, which possesses other specific receptors on the PMN, suggests that Dx acts on some step involved in the oxidative burst subsequent to the binding of the stimuli to the PMN membrane.

The mechanisms of action of Dx on the PMN have not been analyzed here and remains speculative. It is known that Dx, a lipophilic compound, chelates dications and Goodhart suggested that chelation of cellular calcium was responsible for inhibition of PMN locomotion [24]. If this is the case, our results for PMN adhesivity that are similar in the presence or absence of Dx would suggest that cellular dications do not play a major role in cell adhesivity. However, in this study, the cellular pool(s) of calcium which is (are) altered by Dx has (have) not been defined and we cannot rule out that a pool of cellular calcium unaltered by Dx could act on adhesivity. In contrast, extracellular dications, as expected, increased PMN adhesivity and are crucial for this function. In regard to the effects of DX on PMA induced oxidative burst the inhibition may not be connected to dications chelation because previous experiments have

demonstrated that O_2^- production by PMA-stimulated PMN is not accompanied by changes in cytosolic-free calcium [25, 26]. Further studies are needed to analyse the mechanisms of the effects of Dx in order to assess whether it involves calcium chelation only, or other components of PMN functions.

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