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Susceptibility of *Bordetella* Species to Growth Inhibition and Killing by Chlorpromazine

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Chlorpromazine, the prototype phenothiazine tranquilizer, inhibited the growth and killed organisms of the genus *Bordetella*. There were striking differences, however, among the three *Bordetella* species. *Bordetella pertussis* was most susceptible, with some inhibition of growth at ≥4 μg/ml and killing at 16 μg of chlorpromazine per ml. *Bordetella parapertussis* and *Bordetella bronchiseptica* were less susceptible, with killing at 32 and 256 μg/ml, respectively. Although the phenothiazines were inhibitory for *Bordetella* extracytoplasmic adenylate cyclase, the lethal effect occurred at a lower concentration and did not appear to involve modification of the enzyme activity. Exposure of *B. pertussis* to combinations of chlorpromazine and erythromycin resulted in impaired growth at concentrations lower than that of either drug alone, but there was no evidence that the two drugs interacted either synergistically or antagonistically.

Bordetella species are unique in their production of adenylate cyclase, which is predominantly extracytoplasmic in location and is dependent upon the eucaryotic, calcium-dependent regulatory protein calmodulin for activation (2–5). As with other calmodulin-mediated effects, activation of the enzyme is inhibited by phenothiazine tranquilizers, such as chlorpromazine and trifluoperazine (2, 3, 23). While investigating the pharmacological effect of these agents on cultures of *Bordetella pertussis*, we discovered that the organisms were inhibited and killed. In the present study, the characteristics of the inhibitory and lethal effects of chlorpromazine for *Bordetella* species are presented and contrasted with previously observed effects of phenothiazines on other bacteria (9–14, 21).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Four strains of *B. pertussis* and two each of *Bordetella parapertussis* and *Bordetella bronchiseptica* were studied. The characteristics of each strain are given in Table 1. Organisms were stored frozen at −70°C in skim milk and reconstituted for study. Primary culture for 48 h was carried out on Bordet-Gengou agar containing 20% sheep blood. Thereafter, each strain was inoculated into modified synthetic Stainer-Scholte medium (5) and cultured for an additional 24 h. At that point, the mid-exponential-phase cultures were used to inoculate nephelometer flasks for growth studies. Starting inocula ranged from 1.0×10^6 to 5.2×10^6 organisms per ml in all studies cited. Fifty-milliliter flasks containing 20 ml of medium were incubated in a New Brunswick environmental chamber oscillating at 150 rpm with room air at 35.5°C.

Drug effects on bacterial growth and viability. Chlorpromazine and trifluoperazine, kindly provided by Smith Kline & French Laboratories, Philadelphia, Pa., were dissolved in deionized, glass-distilled water. Serial twofold dilutions were added to duplicate culture flasks described above either immediately after inoculation with organisms or later as indicated. Bacterial growth was monitored by turbidimetric readings on a Bausch & Lomb Spectronic 20 spectrophotometer at 650 nm.

To quantitate the phenothiazine effects in a manner that could be compared to those of other antibiotics (18), susceptibility tests to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of chlorpromazine were performed by a modification of standard methods to accommodate the slow growth of *Bordetella* species. As described above, serial twofold dilutions of drug were added to duplicate cultures (*B. pertussis*, 1 to 16 μg/ml; *B. parapertussis*, 16 to 64 μg/ml; and *B. bronchiseptica*, 16 to 512 μg/ml) immediately after inoculation with organisms (1.0×10^6 to 5.2×10^6 organisms per ml). Cultures were incubated for 70 ± 2 h and at that time observed visually for the presence or absence of turbidity. Absence of visible turbidity corresponded to an absorbance at 650 nm of <0.1. The MIC was defined as the lowest concentration of drug that inhibited visible growth.

The number of organisms surviving at 70 ± 2 h was determined by agitating and sampling all flasks without visible growth and the first flask with growth for plating on Bordet-Gengou agar. One hundred microliters of culture medium, or a dilution thereof, was plated on Bordet-Gengou agar and incubated at 35°C for 72 h. Colonies were counted on a Quebec colony counter. The MBC was defined as the lowest concentration of phenothiazine yielding a 10^3 or greater reduction in viable organisms.

TABLE 1. Characteristics of *Bordetella* strains studied

Species and strain	Pertussis toxin[a]	Extracytoplasmic[b] adenylate cyclase	Other features
B. pertussis			
114	+[c]	+	Stable, laboratory-adapted strain
UVa-1	+	+	Third-passage clinical isolate
M3	NT	+	Third-passage clinical isolate
UT 25-80	−	−	Derived from clinical isolate by 80 passages in vitro
B. parapertussis			
501	−	+	Laboratory strain
M2	−	+	Third-passage clinical isolate
B. bronchiseptica			
469	−	+	Canine clinical isolate
213	−	+	Laboratory strain

[a] Histamine-sensitizing activity.
[b] Measured as described in the text.
[c] +, Present; −, not detected; NT, not tested.

Interaction between chlorpromazine and erythromycin was evaluated by carrying out the MIC and MBC assays as described above, except twofold dilutions of both drugs were added at the time of culture inoculation.

Adenylate cyclase assay. The extracytoplasmic adenylate cyclase of *B. pertussis* was assayed as described previously (5). Enzyme activity was determined by incubating whole organisms (2 to 10 μg of protein) with $MgCl_2$ (10 mM) and ATP (1 mm with 0.5 μCi of [α-^{32}P]ATP per assay tube) in Tricine buffer (50 mM, pH 8.0) containing 0.1% bovine serum albumin. Additions (phenothiazine or NaF) were dissolved in Tricine buffer and added before initiation of the assay by addition of substrate. Incubation was terminated by addition of "stopping mix" containing cyclic AMP (cAMP) (10 mM), ^{3}H-cAMP (10^4 cpm), and sodium dodecyl sulfate and by chilling the tube on ice. cAMP produced in 10 min of incubation was separated by the double-column method of Salomon et al. (20) and quantitated by counting on a Beckman scintillation counter.

RESULTS

When *B. pertussis* was cultured in synthetic Stainer-Scholte medium, the addition of chlorpromazine produced a dose-dependent inhibition of growth (Fig. 1). Whereas 2 μg of chlorpromazine per ml was without apparent effect, 4 μg/ml resulted in reduced growth and 8 μg/ml totally prevented growth of the culture. Trifluoperazine (MIC, 8 μg/ml), but not the chlorpromazine metabolite, chlorpromazine-5-oxide (no effect up to 64 μg/ml), caused growth inhibition and killed *B. pertussis* (data not shown).

Because chlorpromazine inhibited the extracytoplasmic adenylate cyclase of *B. pertussis* (23), it was important to determine whether the observed inhibitory effects might be mediated through alteration of the adenylate cyclase activity. A concentration of chlorpromazine which produced maximal inhibition of growth (16 μg/ml) reduced the adenylate cyclase activity by only 8 to 10% (data not shown). Furthermore, sodium fluoride, a potent inhibitor of *B. pertussis* adenylate cyclase at 10 mM (5), had no effect on the growth of the organism. Finally, *B. pertussis* UT25-80, which is avirulent, nontoxigenic, and produces no detectable extracytoplasmic adenylate cyclase, exhibited chlorpromazine susceptibility equivalent to that of the prototype strains. These data suggest strongly that chlorpromazine inhibition of *B. pertussis* growth occurs independently of its effects on adenylate cyclase.

The inhibitory effect of chlorpromazine was further evaluated by adding the drug during exponential growth rather than at the time of culture initiation (latent phase). As shown in Fig. 2, exponentially growing *B. pertussis* cells were less susceptible to the inhibitory effect of chlorpromazine. A lag of approximately 10 h was seen before 16 μg of chlorpromazine per ml caused inhibition of growth, and 6.4 μg/ml was without effect. The decrease in absorbance with the higher doses of drug suggested that bacterial lysis was occurring, and this possibility was evaluated by quantitating viable cell counts at the termination of culture.

The effect of chlorpromazine on viability of *B. pertussis* paralleled its effect on growth (Fig. 3). Final culture density of viable organisms was reduced by 4 μg/ml, and there was a net decrease in viable counts as compared to the starting inoculum at 8 and 16 μg/ml.

When *B. parapertussis* and *B. bronchiseptica* were evaluated in this assay, they were found to be markedly less susceptible to the inhibitory and lethal effects of chlorpromazine (Fig. 3). Killing of these two organisms required drug

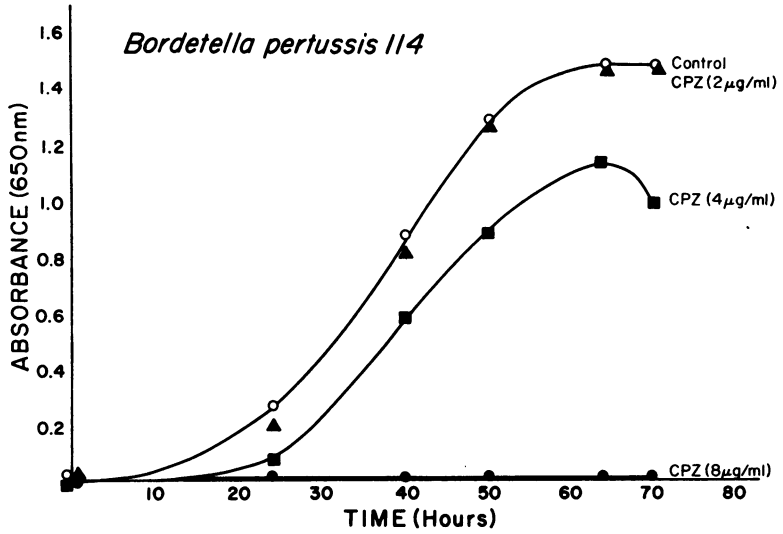

FIG. 1. Dose-dependent inhibition of *B. pertussis* growth by chlorpromazine. *B. pertussis* was cultured in synthetic Stainer-Scholte medium from an initial inoculum of 10^6 CFU/ml. Chlorpromazine dissolved in medium was added immediately after culture inoculation. Growth was followed by nephelometry at 650 nm. Symbols: ○, control organisms without drug. Chlorpromazine was used at 2 μg/ml (▲), 4 μg/ml (■), and 8 μg/ml (●).

concentrations that were 8- and 32-fold higher, respectively, than those required for *B. pertussis*.

To make these data comparable to previously studied antibiotic susceptibilities of the *Bordetella* species (1), rigorous MIC and MBC determinations were carried out (Table 2). The *B. pertussis* strains tested were most susceptible to chlorpromazine, with an MIC of 8 μg/ml and an MBC of 16 μg/ml. In contrast, *B. parapertussis* had an MIC and MBC of 32 μg/ml and *B. bronchiseptica* had an MIC and MBC of 256 μg/ml.

Because *B. pertussis* is recognized to be susceptible to several antibiotics (1) and because membrane-active agents such as the phenothiazines have been observed to enhance permeability of other bacteria (9, 13), the effect of the

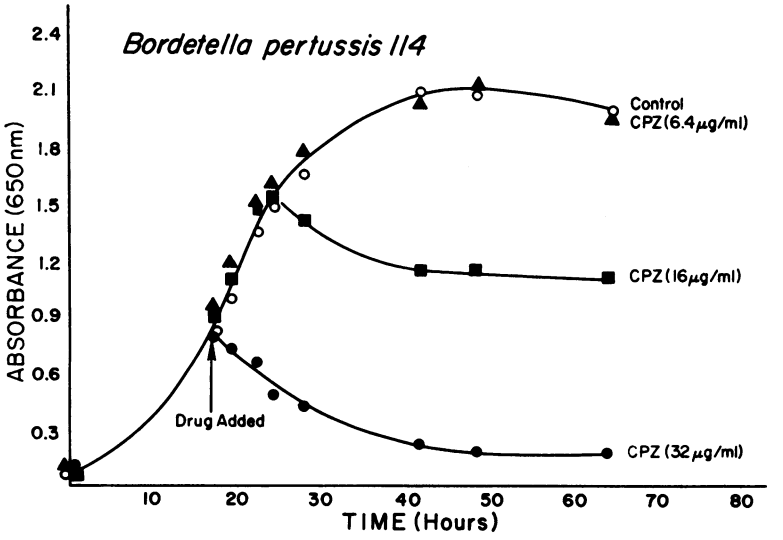

FIG. 2. Response of exponentially growing *B. pertussis* to chlorpromazine. Culture of *B. pertussis* was carried out as described, except chlorpromazine was added (arrow) during exponential growth at 17 h after culture inoculation. Symbols: ○, control organisms without drug. Chlorpromazine was used at 6.4 μg/ml (▲), 16 μg/ml (■), and 32 μg/ml (●).

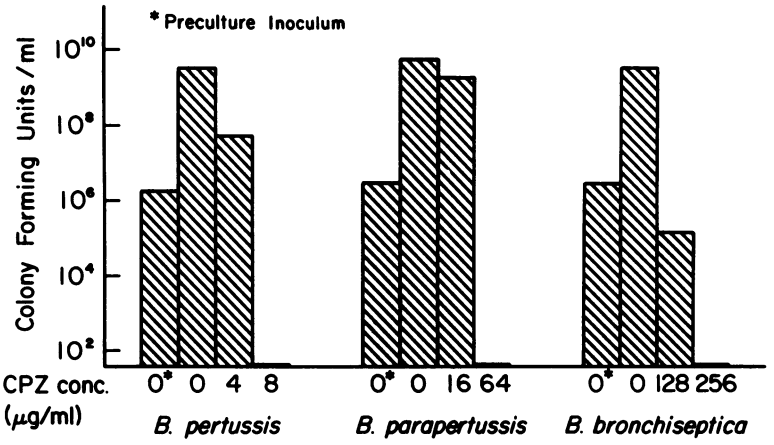

FIG. 3. Differential susceptibility of *Bordetella* species to the lethal effect of chlorpromazine. *Bordetella* organisms (*B. pertussis* 114, *B. parapertussis* 501, and *B. bronchiseptica* 469), cultured as described in the text were exposed to concentrations of chlorpromazine as indicated. At the end of 72 h, culture medium (100 μl or dilutions thereof) was inoculated onto Bordet-Gengou agar plates to determine viable cell count. Colonies were counted after 72 h of incubation. The first bar in each group (*) represents the viable cell count in the inoculum before culture. The second bar in each group represents the viable cell count reached at 72 h in the absence of drug.

combination of chlorpromazine and erythromycin on *B. pertussis* was studied. As demonstrated above, the MIC for chlorpromazine alone was 8 μg/ml. The MIC for erythromycin was 0.05 μg/ml (Table 3). The MICs of the two drugs combined, however, were 0.025 μg/ml for erythromycin and 4 μg/ml for chlorpromazine. In addition, there was impaired growth at sub-MICs (chlorpromazine, 1 μg/ml, plus erythromycin, 0.025 μg/ml). These data suggest that, while not strictly synergistic, the combination of these two drugs at sub-MICs is not deleterious and may result in an enhanced effect on *B. pertussis*.

DISCUSSION

Phenothiazines, most widely employed for their neuroleptic effects, have been recognized to possess antimicrobial activity (9–14). Chlorpromazine, the prototype of this class of drugs, is inhibitory and lethal for an array of microorganisms (11, 12). The general pattern which has emerged from prior studies shows gram-positive organisms to be much more susceptible to these agents than gram-negative organisms. For example, the MICs of chlorpromazine for *Streptococcus pneumoniae, Streptococcus pyogenes*, and *Staphylococcus aureus* range from 20 to 40 μg/ml, whereas those for the gram-negative organisms *Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are substantially higher at 130 to 1,250 μg/ml (12). In light of these data, the susceptibilities of the gram-negative organisms *Bordetella pertussis* (MIC, 8 μg/ml) and *Bordetella parapertussis* (MIC, 32 μg/ml) are particularly striking. In contrast, the relative insusceptibility of *B. bronchiseptica* (MIC, 256 μg/ml) is as would be expected. The interspecies differences are of importance, since Kloos et al. have shown that the three *Bordetella* species are sufficiently similar by DNA hybridization to be combined into one species (8). The biochemical basis for their differential chlorpromazine susceptibility remains to be determined but, when understood, may be useful in helping to elucidate the mechanism of the drug effect.

The reduced susceptibility of exponentially grown *B. pertussis* organisms to the inhibitory effect of chlorpromazine (Fig. 2) is in contrast to

TABLE 2. MICS and MBCS of chlorpromazine for *Bordetella* species

Species and strain	MIC[a]	MBC
B. pertussis		
114	8	16
UVa-1	8	16
M3	8	16
UT 25-80	8	16
B. parapertussis		
501	32	32
M2	32	32
B. bronchiseptica		
469	256	256
213	256	256

[a] MIC and MBC were measured as described in the text.

TABLE 3. Effect of chlorpromazine and erythromycin on the growth of *B. pertussis* 114

Chlorpromazine concn (μg/ml)	*B. pertussis* growth at following erythromycin *concn* (μg/ml)[a]:				
	0.1	0.05	0.025	0.0125	0
8	NT	NT	NT	NT	0
4	0	0	0	++	+
2	0	0	+	++	++
1	0	0	+	++	++
0	0	0	++	++	++

[a] NT, Not tested; 0, no detectable growth; +, submaximal growth (absorbance less than control in the absence of drug); ++, maximal growth (absorbance equal to control in the absence of drug).

the effect seen with a number of other antibiotics. Many antimicrobial agents, especially those which alter membrane or cell wall synthesis, are most effective against exponentially growing organisms. The alternative explanation for this observation is that increased bacterial density reduced either the effective drug concentration or the susceptibility of the organisms to these agents. Nevertheless, it is anticipated that this change in susceptibility of *B. pertussis* to chlorpromazine is another feature which may be important in providing insight into the mechanism of antimicrobial activity of these drugs.

Although chlorpromazine and other phenothiazines have been shown to have suppressive and lethal effects on mycobacteria in vitro (10, 11), preliminary studies failed to show the efficacy of these agents alone in mice infected with *Mycobacterium tuberculosis*. Anecdotal clinical observations have suggested, however, that chlorpromazine may enhance the efficacy of other antibiotics, perhaps by facilitating their entry into target cells (7). This suggestion is supported by in vitro studies in which chlorpromazine was shown to increase the permeability of several microorganisms (9, 13). The demonstration of a combined effect of chlorpromazine and erythromycin in the present study is consistent with such an effect. Concentrations of each drug that were subinhibitory alone caused total inhibition of *B. pertussis* growth when combined. In light of the previous suggestion that chlorpromazine be used for its antiemetic effects to treat patients with whooping cough (19), these data provide support for a clinical evaluation of its efficacy in conjunction with erythromycin in children with clinical pertussis. Furthermore, these data suggest that at least one locus of chlorpromazine action may be at the membrane level.

Phenothiazines are well recognized to have a number of diverse effects on eucaryotic cells, such as modification of cyclic nucleotide metabolism (15), association with DNA (6), and alteration of membrane structure and function (22). Which, if any, of these effects are primary in the clinical neuroleptic activities of these drugs is unresolved. Demonstration of a lethal effect of phenothiazines for the eucaryotic protozoal parasite *Leishmania donovani* (17) suggests some possible mechanisms relevant to the present data. At a relatively high drug concentration (50 μg/ml), the parasite is rapidly immobilized and its O_2 consumption falls markedly (16). Because effects are not reversible with removal of the drug and because the drugs are less potent at reduced oxygen tension, it has been hypothesized that oxidized metabolites or free radicals may be involved, perhaps acting at the parasite membrane.

As with the phenothiazine effects on eucaryotic cells, the mechanism by which these drugs cause their inhibitory and lethal effects on bacteria is unknown. Since the present study was prompted by interest in the unique *Bordetella* extracytoplasmic adenylate cyclase (2–5, 23), it was important to determine whether inhibition of the enzyme is responsible for the chlorpromazine growth effects. First, concentrations of the drug which fully inhibit growth (16 μg/ml) have only a slight inhibitory effect on adenylate cyclase activity. Second, a known inhibitor of the *Bordetella* cyclase, NaF, had no effect on *B. pertussis* growth or viability at a concentration that inhibited the enzyme greater than 50%. Third, UT25-80, an avirulent, nontoxigenic strain of *B. pertussis* which has no measurable extracytoplasmic adenylate cyclase activity, was equally susceptible to growth inhibition by chlorpromazine. Finally, the three *Bordetella* species all possess extracytoplasmic adenylate cyclases which are equally susceptible to chlorpromazine inhibition (E. Hewlett, unpublished data), yet their susceptibilities to the lethal effects of the drug differ by 40-fold. Therefore, it appears that inhibition of the cyclase is not involved.

Inhibition of growth and killing of other bacteria by chlorpromazine is associated with a variety of effects, including inhibition of respiratory enzymes (14), enhancement of membrane permeability (9, 13), and modification of membrane ultrastructure (21). Since these drugs are lipophilic and membrane active, it is not surprising that these observed effects all involve structure and function of membrane or membrane-associated proteins. As with the effects of eucaryotic cells, however, it is impossible to know at the present time which, if any, of these effects is responsible for the bactericidal activity. It is hoped that the differential susceptibility among the *Bordetella* species may provide a useful model for studying phenothiazine effects on bacteria in general.

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