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Growth Curves, Microscopic Morphology, and Subcultures of Beta-Lactamase-Positive and -Negative *Haemophilus influenzae* Under the Influence of Ampicillin and Cefamandole

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In contrast to the results obtained with ampicillin, the minimum inhibitory concentrations of cefamandole against *Haemophilus influenzae* were within the same range (0.5 to 1.5 μg/ml) whether or not the strains were beta-lactamase producers. The minimum bactericidal concentrations were somewhat higher for beta-lactamase-positive strains (6.4 μg/ml) than for negative strains (1.2 μg/ml). In a culture with high initial microbial density, monitored by recording optical densities, the addition of 10 μg of cefamandole per ml brought about rapid lysis of a beta-lactamase-negative strain. Observation of a beta-lactamase-positive strain revealed, in the early part of the growth curve, absence of lysis and an increase of biomass similar to that observed in a drug-free control curve. In contrast to the results obtained with ampicillin, the culture consisted uniformly of spherical forms, probably in the process of division, which were capable of generating colonies. When the microbial density exposed to cefamandole was increased still further, persistent bacillary forms were observed, and after 24 h hydrolysis had eliminated every trace of microbiologically active cefamandole.

The emergence of *Haemophilus influenzae* strains that produce beta-lactamase has made it necessary to evaluate the action of new antibiotics against this bacterial species, which used to be generally susceptible to ampicillin (24, 26).

Cefamandole acts efficiently in vitro against beta-lactamase-negative strains of *H. influenzae* (3, 5, 6), but it can be hydrolyzed by the beta-lactamase of producer strains (4, 14, 15, 17, 20, 23, 25). Study of the action of beta-lactam antibiotics on *H. influenzae* is complicated by the fact that these drugs quickly cause L-forms to appear, the morphology and viability of which depend on the composition and osmolarity of the medium. These findings account for the variety of criteria used to define the susceptibility of a strain (increase of biomass, microscopic appearance [12], or results of subculturing, sometimes in a hyperosmolar medium [13]). We report here a study of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of antibiotics, conducted on a small number of strains and complemented by a study of growth curves monitored by biophotometry. Microscopic examinations, subcultures, and antibiotic assays were performed at various times during bacterial growth in order to interpret as correctly as possible the significance of changes in the biomass. With increasingly widespread use of automatic equipment for monitoring biomass, this measurement has become a useful aid for assessing the susceptibility of microorganisms to antibiotics (19).

MATERIALS AND METHODS

Microbial strains. Six beta-lactamase-negative strains of *H. influenzae*, obtained by transtracheal puncture, and six beta-lactamase-positive strains (proven by Glaxo 87/312 chromogen method) kindly supplied by R. B. Sykes (Glaxo Research Ltd., England) and P. Piot (Tropical Institut, Antwerp, Belgium) were used. The two strains used for the monitored cultures were Brugmann no. C1 (beta-lactamase negative) and the Chisty strain from R. B. Sykes.

Culture media. Medium 1, Mueller-Hinton broth (Difco) with 2% Fildes enrichment (Difco), was used for determination of MIC in broth and for observations of growth curves. Medium 2, Mueller-Hinton agar (Difco) with 2% Fildes enrichment (Difco), was used for determination of MIC in agar, MBC, and subcultures of the monitored cultures.

Determination of MIC and MBC. (i) Dilution in agar (medium 2) was performed with a Dynatech multipoint inoculator by the method of Steers et al. (21). The "inoculum effect" was studied with a 24-h culture adjusted to a density of 0.5 McFarland no. 1 standard. This culture was used undiluted and in 100, 1,000, and 10,000 dilutions. Incubation took place for 18 h at 37°C in an atmosphere containing 10% CO_2.

The MIC was defined as the lowest concentration inhibiting macroscopic growth of the culture at the point of inoculation.

(ii) Dilution in broth (medium 1) was performed with Microtiter plates and a U-scoop (Cook Engineering Co.). Inoculum was a 24-h culture adjusted to a density of 0.5 McFarland no. 1 standard; the "heavy" inoculum consisted of 0.05 ml of this culture added to 0.05 ml of broth containing the various concentrations of antibiotic. The "light" inoculum consisted of 0.05 ml of this culture diluted 1,000 times. Incubation took place at 37°C in a 10% CO_2 atmosphere. The MIC was defined as that concentration in which there was absence of macroscopically visible growth; we left out of account, as recommended by Bottone et al. (2), a slight opacity which microscopic examination showed to be due to the presence of spheroplasts. To find the MBC we subcultured 0.01 ml from each well in spots spread out over a surface of about 2-cm diameter (on medium 2). The bactericidal threshold was defined by growth of less than five colonies per spot.

Growth curves. Culture growth was monitored by a biophotometer (Jobin & Ivon, Paris, France). Inoculum was 100 ml of broth sown with 1 ml of an overnight culture. A 5.5-ml portion of this suspension was placed in each cuvette; the cuvettes were balanced to 100% light transmission, converted automatically into optical density. Microbial density sufficient to permit a recording was reached after about 4 h. With this procedure reproducible growth curves were obtained. An optical density of 0.1 on the McFarland scale corresponded to approximately 10^8 bacteria per ml. Times were counted from the start of the recordable growth curve. We found that bacteria were adhering to the cuvette wall after 7 h of incubation. The cuvettes were therefore vigorously shaken by hand before samples were taken for microscopic examination and for culturing after 24-h incubation.

Microscopic examination. The cultures were examined by phase-contrast microscopy at the time of introduction of antibiotics, after 1 h, and after 2 h. The great variety of the forms observed and the continuous deformations that *H. influenzae* underwent under the influence of the beta-lactam antibiotics made it necessary to group them under a few morphological types, which are conventionally represented in Fig. 1 (in relative order of size). The bacillary form (B) denotes bacterial forms with greater length than breadth and displaying a characteristic bacillary morphology, sometimes with slight deformations. The term S1 denotes spherical forms not exceeding 2 μm, rather dense under phase-contrast microscopy, with chromatin masses that are sometimes arranged in an equatorial pattern suggestive of forthcoming bacterial division; their intense metabolic activity was demonstrated by straining the ribonucleic acid with acridine and by microscopic examination under ultraviolet light (27). The term S2 denotes large empty-centered forms in which lumps of chromatin can be seen at the periphery clinging to the cytoplasmic membrane; these features suggest degenerative forms. When stained with acridine these elements were slightly green or colorless. The terms B, S1, and S2 are used in Tables 2 to 5 only where microscopic examination revealed more than 10% of one of these forms.

Subcultures. From each densimetry cuvette, 0.01 ml was spread on the surface of a 9-cm petri dish containing medium 2 and was incubated for 48 h. Bacterial growth was assessed semiquantitatively: over 100 colonies or confluent colonies, +++; 10 to 100 colonies, ++; 1 to 10 colonies, +; no bacterial growth, 0. Samples were taken just before the introduction of antibiotics, after 1 h, and after 2 h.

Antibiotics. Cefamandole lithium (Lilly) and ampicillin sodium (Beecham) were dissolved in broth (120 μg/ml); 0.5 ml of this solution was added to the cuvettes (5.5 ml) at various points of the growth curve, giving a final concentration of 10 μg/ml. Ampicillin and cefamandole assays were done after 1, 2, and 24 h by the method of Bennett et al. (1).

RESULTS

MIC and MBC in agar and in broth. The extreme values shown in Table 1 represent the results obtained with the light inoculum of the most susceptible strain and the heavy inoculum of the least susceptible strain. For the beta-lactamase-negative strains the MICs of both antibiotics were of the same order. For the beta-lactamase-positive strains the MIC of ampicillin was more than 100 μg/ml. Cefamandole activity, on the other hand, remained virtually the same as against the negative strains, except that the MBC rose to 6.4 μg/ml for a heavy inoculum in broth.

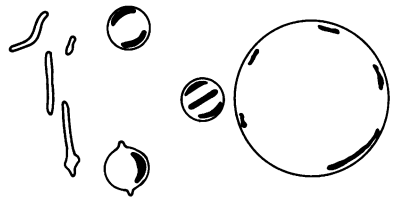

FIG. 1. *Codification of microbial forms observed under phase-contrast microscopy. See the text for explanation of designations.*

TABLE 1. *MIC in agar and broth and MBC in broth for six beta-lactamase-negative and six beta-lactamase-positive H. influenzae strains*

ANTIBIOTIC	MEDIUM	BETA-LACTAMASE NEGATIVE	BETA-LACTAMASE POSITIVE
AMPICILLIN	AGAR	0.10-0.40[a]	> 100
	BROTH (MIC)	0.20-0.80	> 100
	BROTH (MBC)	0.80-1.60	> 100
CEFAMANDOLE	AGAR	0.05-0.80	0.05-0.80
	BROTH (MIC)	0.20-1.60	0.40-0.80
	BROTH (MBC)	0.40-1.60	0.40-6.40

[a] The first number is the lowest value found for the light inoculum, and the second is the highest value found for the heavy inoculum (in micrograms per milliliter).

Monitored cultures. Figure 2 shows growth curves of beta-lactamase-negative and -positive *H. influenzae* exposed to 10 μg of ampicillin or cefamandole per ml. The antibiotic was introduced at various points of time during the growth curve, which also show the bacterial density on which the antibiotic was to act. The following comments can be made on these curves.

(i) Beta-lactamase-negative strains. The initial deflection corresponds to the diluting of the cultures by the broth containing the antibiotic; it has no microbiological significance.

After introduction of the antibiotic the biomass increased, following a curve parallel to the reference curve (same slope). The ascending phase of the curve was longer with ampicillin than with cefamandole (average on curves 1 to 5: ampicillin, 42 ± 3.3 min; cefamandole, 19 ± 6.6 min).

The descending phase of the curve, corresponding to lysis, was of fairly constant duration with ampicillin (about 15 min) and depended little on the initial bacterial density. With cefamandole the mean lysis time was more dependent on the initial bacterial density: 7.3 ± 5.3 min with lower densities (curves 1 to 4) and 13 ± 3.5 min with higher densities (curves 5 to 7).

Tables 2 and 3 give the following additional details. The samples taken before introduction of antibiotics showed the presence of B (bacillary) forms only. The bacillary forms persisted after 1 and 2 h of exposure to ampicillin or cefamandole. All the subcultures were positive with confluent colonies. Examinations after 24 h showed the presence of degenerative forms (S2) in all cultures exposed to ampicillin; forms of S1 type persisted in the cefamandole cultures (curves 5 to 7). The subcultures were positive with ampicillin (curve 7) and with cefamandole (curves 5 to 7). The ampicillin and cefamandole concentrations remained above the MIC throughout the experiment. Colonies that appeared after 24 h of growth retained the susceptibility of the initial strain.

(ii) Beta-lactamase-positive strains. With ampicillin no lysis was observed. Only the curve with low initial density (Fig. 2, curve 1) diverged from the reference curve. With cefamandole the culture biomass increased, but at a slower rate than the reference curve, with a difference in gradient observed as soon as the cefamandole was introduced.

Tables 4 and 5 give the following additional details. Microscopic examination revealed important differences between ampicillin and cefamandole. Cultures under ampicillin displayed numerous bacillary forms after 1 or 2 h. With cefamandole, microscopic examination showed that after 1 and 2 h the biomass consisted of spherical forms of S1 type. The morphological changes affected all the bacteria observed (no bacillary forms were present); subcultures, however, grew well (i.e., showed confluent growth). After 24 h the bacillary forms reappeared in cultures with high initial density, and again subcultures grew well. Both antibiotics were hydrolyzed by h 24, and no antibacterial activity could be detected.

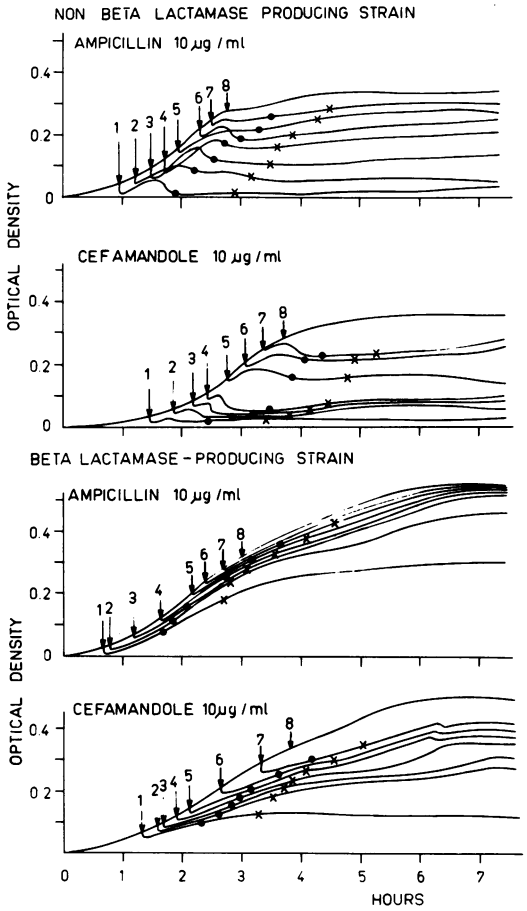

FIG. 2. *Growth curves of beta-lactamase-positive and -negative H. influenzae exposed to ampicillin and cefamandole. (1, 2, 3, 4, 5, 6, 7) Growth points at which antibiotic was introduced. (8) Control curve. The samplings, of which the results are shown in Tables 2 to 5, were performed 1 (●) and 2 (×) h after introduction of the antibiotic.*

DISCUSSION

The MIC of cefamandole for *H. influenzae* has been studied by a number of authors, who all report that the antibiotic is efficient against

this bacterial species. Their studies, however, were conducted on strains that do not produce beta-lactamase (3, 6). In this study we have shown that on beta-lactamase-negative strains in agar or in broth the MICs of cefamandole are similar to those observed with ampicillin. Even with a heavy inoculum (10^7 bacteria per ml) they did not exceed 1.6 μg/ml. The MICs for beta-lactamase-positive strains remained closely comparable to those for the negative strains. The only notable change was the MBC for heavy inoculum (10 bacteria per ml), which reached 6.4 μg/ml, a low value when compared with the ampicillin MBC, which was over 100 μg/ml.

To compare the antimicrobial activities of ampicillin and cefamandole, we used a recording biophotometer (7, 16) to monitor the optical density of cultures. The bacterial density needed for a good growth-curve recording was not less than 10^8 microorganisms per ml. Cefamandole and ampicillin were used in one concentration only, 10 μg/ml, chosen because it is easily achieved in human therapy (11). Since the MICs for the microorganisms tested are approximately the same, this single concentration enables the two substances to be compared. The two variables were therefore the time of introduction of the antibiotic and the presence or absence of beta-lactamase. The graphs show that when the optical density of the culture was low, cefamandole caused slightly more rapid lysis of the beta-lactamase-negative strains of *H. influenzae* than

TABLE 2. *Effect of ampicillin on beta-lactamase-negative H. influenzae*

GROWTH CURVES NO [a]		1	2	3	4	5	6	7	8
	TIME [b]								
MICROSCOPY	0	B [c]	B	B	B	B	B	B	B
	1	BS1	BS1	BS1	BS1	BS1	BS1	BS1	B
	2	BS1S2	BS1S2	BS1S2	BS1S2	BS1S2	BS1S2	BS1S2	B
	24	S2	S2	S2	S2	S2	S2	S2	BS2
SUBCULTURES	0	+++	+++	+++	+++	+++	+++	+++	+++
	1	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++
	24	0	0	0	0	0	0	+	++
AMPICILLIN	0	10	10	10	10	10	10	10	0
CONCENTRATION	1	10	10	10	10	10	10	10	0
(MCG/ML)	2	10	10	10	10	10	10	10	0
	24	9.2	9.6	9.4	8.2	8.6	8.4	8.6	0

[a] Growth curves are numbered in the order of time at which the antibiotic was added (see Fig. 2).

[b] Time of sampling in hours (see Fig. 2).

TABLE 3. *Effect of cefamandole on beta-lactamase-negative H. influenzae*

GROWTH CURVES NO [a]		1	2	3	4	5	6	7	8
	TIME [b]								
MICROSCOPY	0	B [c]	B	B	B	B	B	B	B
	1	BS1	BS1	BS1	BS1	BS1	BS1	BS1	B
	2	BS2	BS2	BS2	BS2	BS2	BS1	BS1	B
	24	S2	S2	S2	S2	S1	S1	S1	BS2
SUBCULTURES	0	+++	+++	+++	+++	+++	+++	+++	+++
	1	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++
	24	0	0	0	0	+	++	++	+++
CEFAMANDOLE	0	10	10	10	10	10	10	10	0
CONCENTRATION	1	10	10	10	10	10	10	10	0
(MCG/ML)	2	10	10	10	10	10	10	10	0
	24	± 9	± 9	± 9	± 9	± 9	± 9	± 9	0

[a] Growth curves are numbered in the order of time at which the antibiotic was added (see Fig. 2).

[b] Time of sampling in hours (see Fig. 2).

TABLE 4. *Effect of ampicillin on beta-lactamase-positive H. influenzae*

GROWTH CURVES NO [a]		1	2	3	4	5	6	7	8
	TIME [b]								
MICROSCOPY	0	B [c]	B	B	B	B	B	B	B
	1	BS1	BS1	B	B	B	B	B	B
	2	BS2	BS2	BS1	B	B	B	B	B
	24	BS2	BS2	BS2	BS2	BS2	BS2	BS2	B
SUBCULTURES	0	+++	+++	+++	+++	+++	+++	+++	+
	1	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++
	24	+++	+++	+++	+++	+++	+++	+++	+++
AMPICILLIN	0	10	10	10	10	10	10	10	0
CONCENTRATION	1	2.5	2	1.5	1	<1	<1	<1	0
(MCG/ML)	2	<1	<1	<1	<1	<1	<1	<1	0
	24	0	0	0	0	0	0	0	0

[a] Growth curves are numbered in the order of time at which the antibiotic was added (see Fig. 2).

[b] Time of sampling in hours (see Fig. 2).

TABLE 5. *Effect of cefamandole on beta-lactamase-positive H. influenzae*

GROWTH CURVES NO [a]		1	2	3	4	5	6	7	8
	TIME [b]								
MICROSCOPY	0	B [c]	B	B	B	B	B	B	B
	1	S1	S1	S1	S1	S1	BS1	BS1	B
	2	S1	S1	S1	S1	S1	BS1	BS1	B
	24	S2	S2	BS1S2	BS1S2	BS1S2	BS1S2	BS1S2	BS2
SUBCULTURES	0	+++	+++	+++	+++	+++	+++	+++	+++
	1	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++
	24	0	0	+	+	++	++	++	+++
CEFAMANDOLE	0	10	10	10	10	10	10	1.4	0
CONCENTRATION	1	5.4	3	2.6	2.6	1.6	1.5	1.4	0
(MCG/ML)	2	2.2	0.2	0.2	0.12	0.10	0.1	≤0.1	0
	24	0	0	0	0	0	0	0	0

[a] Growth curves are numbered in the order of time at which the antibiotic was added (see Fig. 2).

[b] Time of sampling in hours (see Fig. 2).

did ampicillin. Microscopic examination of subcultures confirmed the bactericidal activity of both antibiotics, each inducing the morphological changes typical of beta-lactam antibiotics. But the peculiarities observed with the beta-lactamase-producing strains made it necessary to schematize the microscopic findings, usually recorded as L-forms. We found that the addition of cefamandole to cultures of beta-lactamase-producing *H. influenzae* did not prevent the biomass from increasing, and after only 1 h microscopic examination revealed a population consisting wholly of spherical forms. Not a single bacillary form was seen. The subcultures, however, generated confluent colonies. These findings call to mind the action of mecillinam on *Escherichia coli* described by Greenwood et al. (9, 10): presence of spheroplasts, absence of lysis, and positive subcultures. As regards the cultures with low optical density at the time of introduction of the antibiotic, the subcultures were negative after 24 h of exposure, so it appears (subject to testing with special media, of variable osmolarity) that the morphological impairment observed is prelethal. The results obtained did not permit reconciliation of the various criteria used to gauge the threshold of activity of an antibiotic such as cefamandole acting against beta-lactamase-producing *H. influenzae*. (i) The presence of beta-lactamase did not greatly modify the MIC for cefamandole, although hydrolysis of the antibiotic was demonstrated. (ii) The biomass increased without lysis, and the microbial form that prevailed was spheroplasts capable of gen-

erating colonies after 1 and 2 h but not after 24 h. (iii) Modifications of the microscopic morphology, which some authors regard as the sole criterion for activity of a beta-lactam antibiotic on *H. influenzae* (2, 18), affected all the elements in a very dense culture, but the presence of those elements proved transient; the 24-h culture displayed bacillary forms. Bacterial population density influenced the results. The heaviest inoculum used for the MIC tests contained about 10^7 microorganisms per ml, whereas the highest densities in the monitored cultures reached 10^9 microorganisms per ml. This accounts for the discrepancy between the MIC and MBC results and the growth-curve results. The greater the initial microbial mass, the more rapid was the rate of hydrolysis of cefamandole. This rate was probably proportional to the amount of beta-lactamase released.

This study is not exhaustive, for it dealt with only a few strains. Measurement of the activity of a beta-lactam antibiotic against *H. influenzae* is complicated by the fact that there exist strains which form "stable spheroplasts" (22). Another difficulty is the heterogeneous character (variable percentages of beta-lactamase-positive bacteria in the same population) of ampicillin-resistant strains, demonstrated by Gray et al. (8).

In sum, the bacteriostatic and bactericidal activity of cefamandole was similar to that of ampicillin against beta-lactamase-negative *H. influenzae*; against producer strains its antibacterial activity proved to be less simple. As the MIC and MBC studies showed, the presence of beta-lactamase did not wholly suppress the action of cefamandole on *H. influenzae*, yet in tests based on bacterial density monitoring and a heavy bacterial inoculum, the increase in the biomass (consisting of abnormal microbial forms) is interpreted as characteristic of resistance. The question that remains to be answered is whether the morphological impairments we observed, which attest to antimicrobial activity, render this bacterium more vulnerable to the host's own defenses; if this were so, there might be a case for revising the criteria of resistance.

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