Comparative Activity of Ampicillin and Cefuroxime Against Three Types of *Haemophilus influenzae*

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On the basis of their susceptibility to ampicillin, strains of *Haemophilus influenzae* can be divided into three types: type 1 are normally susceptible strains, type 2 produce stable spheroplasts from low inocula, and type 3 are β -lactamase producers. Because of the production of spheroplasts, standard broth and agar dilution techniques have failed to differentiate between the responses of type 2 and 3 strains to ampicillin, or to identify the superiority of cefuroxime over ampicillin against the β -lactamase-producing strains. Disk susceptibility tests with heavily seeded plates were also difficult to interpret. To overcome these problems, we developed a medium that supports the growth of *H. influenzae*, but not survival of spheroplasts, thereby reducing the complicating influence of spheroplast formation. Utilization of the medium made it possible to identify β -lactamase-producing strains via both minimal inhibitory concentration and disk susceptibility techniques, as well as the superiority of cefuroxime over ampicillin against such strains. In vivo experiments showed that cefuroxime and ampicillin are equally active against infections with type 1 and 2 strains, but that cefuroxime is superior to ampicillin against infections with type 3 β -lactamase-producing strains.

Until relatively recently, *H. influenzae* has remained remarkably stable in regard to its susceptibility to antibiotics (22). Ampicillin, chloramphenicol, and the tetracyclines have proved generally effective against the organism. However, over the last few years, an increasing number of reports have described the appearance of antibiotic-resistant strains (3, 5, 6, 8-10, 16, 19).

Infection due to ampicillin-resistant strains of *H. influenzae* has become an increasingly serious and widespread problem (1, 4, 18). The primary cause of ampicillin resistance appears to be the production of a β -lactamase that is capable of hydrolyzing a number of penicillin and cephalosporin substrates (7, 13, 17).

We have also found strains of *H. influenzae* which, tested by the conventional minimal inhibitory concentration (MIC) technique, show increased resistance to ampicillin but do not produce a β -lactamase. The apparent resistance of these strains resides in their ability to form osmotically stable spheroplasts at low inoculum concentrations. Thus, on the basis of their susceptibility to ampicillin, strains of *H. influenzae* are of three types. Type 1 includes normally susceptible strains, type 2 strains produce stable spheroplasts at low inocula, and type 3 strains are β -lactamase producers.

By using representative strains of the three

types of *H. influenzae*, we compared the activities of ampicillin and cefuroxime, a β -lactamase-stable cephalosporin (14). Tests have been carried out using a commercially available nutrient medium and a contrived medium of low osmolality in which the spheroplasts of *H. influenzae* fail to survive.

MATERIALS AND METHODS

Organisms. All studies were carried out using six strains of each type of *H. influenzae*. Isolates were all derived from clinical material and given to us by P. Cavanagh, Public Health Laboratory, Stafford, England.

Media. The nutrient broth was Oxoid no. 2 (osmolality, 260 mOsmol/kg). The nutrient agar was Oxoid blood agar base. Dextrose phosphate broth contained bacteriological peptone (Difco), 4 g, dipotassium hydrogen phosphate, 5 g, and 4 g of glucose per liter (osmolality, 115 mOsmol/kg). To prepare the agar, agar (Difco) was added at a concentration of 1.5%. All media were supplemented with 5% Fildes and yeast extract (osmolality, 30 mOsmol/kg), prepared by adding equal volumes of Fildes extract (Oxoid) and 20% yeast extract (Difco). Cultures on solid media were incubated at 37°C in a CO₂ incubator (CO₂ concentration, 5 to 8%). Cultures in broth were grown in an air incubator at 37°C.

Susceptibility tests. Dilutions of antibiotics were prepared in the appropriate medium. Organisms grown for 6 h in liquid culture in a rotary shaking incubator at 37°C were diluted to give the required

inoculum levels. Results were determined by both macroscopic and microscopic examination after 18 h of incubation at 37°C.

Osmolality. Osmolality was determined using a digimatic osmometer (Advanced Instruments Inc., Needham Heights, Mass.).

Protection tests. The animals used were albino Charles River female mice, weighing 18 to 20 g. Mice were challenged by the intraperitoneal route with the test organisms suspended in 0.2 ml of 2% dried bakers' yeast. The challenge dose was approximately 10 to 100 times the mean lethal dose, and the majority of untreated animals died within 24 h of challenge. Five animals were used at each of the serial threefold dose concentrations of antibiotic tested, which were given subcutaneously (0.2 ml per animal) at 1 and 5 h after challenge. The number of animals surviving on day 5 was used to calculate the median effective dose by logit transformation of the dose-response curve.

RESULTS

(i) **MIC determinations.** The activity of ampicillin and cefuroxime against the three types of *H. influenzae* is shown in Table 1. Both antibiotics were highly active against the type 1 strains but, above inocula of 10^5 colony-forming units (CFU), spheroplast formation was observed. With the type 2 strains, spheroplast formation was observed at low inoculum levels, leading to an apparent increase in the MIC.

Cefuroxime was highly active against the type 3 strains, but spheroplasts were present at the high inoculum levels. Ampicillin, however, showed a response similar to that seen against the type 2 strains, with increased resistance at the low inoculum levels but, in the case of the type 3 strains, no spheroplast formation was observed.

(ii) **Disk susceptibility tests.** Two sets of nutrient agar plates were inoculated by flooding with 6-h broth cultures of *H. influenzae*, undiluted and diluted 1:100, respectively, and antibiotic disks (30 µg) of ampicillin and cefuroxime were applied to each. Type 1 strains gave

large, clear zones with ampicillin and cefuroxime on the lightly seeded plates but hazy zones on the heavily seeded plates. With the type 2 strains, zones were difficult to read at both inoculum levels (Fig. 1a and b), spheroplasts being responsible for the hazy zones. Against the β -lactamase-producing strain (Fig. 2a and b), a reduced zone was seen for ampicillin on the lightly seeded plate, compared with a large, clear cefuroxime zone. No zones were visible on the heavily seeded plates.

The spheroplasts of *H. influenzae* induced by β -lactam antibiotics are almost unique in their remarkable osmotic stability. Roberts and colleagues (16) reported that *H. influenzae* spheroplasts were highly resistant to osmotic lysis. They suggested that to overcome the "spheroplast problem," a light inoculum should be used in testing *H. influenzae* for susceptibility to β -lactam antibiotics. With the emergence of resistant strains, such a method may have many pitfalls.

With these points in mind, we set out to find a medium that could support the growth of *H. influenzae* but in which spheroplasts could not survive. Dextrose phosphate medium fulfilled these requirements. With this medium, we repeated the comparison between ampicillin and cefuroxime against the three types of *H. influenzae*.

(i) **MIC determinations.** Ampicillin and cefuroxime showed high antibacterial activity against *H. influenzae* type 1 and 2 strains even at the high inoculum levels (Table 2). Against the type 3 strains, cefuroxime was highly active, whereas ampicillin resistance was still apparent (Table 2).

(ii) **Disk susceptibility tests.** Ampicillin and cefuroxime disks produced clear zones against *H. influenzae* type 1 and 2 strains at both inoculum concentrations (Fig. 3a and b). Against the β -lactamase-producing strain (Fig. 4a and b), ampicillin showed reduced activity on the lightly seeded plate and no activity at the

TABLE 1. Activity of ampicillin and cefuroxime against *H. influenzae*—nutrient broth and nutrient agar

<i>H. influenzae</i>	MIC ampicillin/MIC cefuroxime (µg/ml) with CFU in inoculum:				
	10	10^2	10^5	10^6	10^7
Nutrient broth					
Type 1	0.25/0.25	0.25/0.5	0.5/0.5	16 ^a /2 ^a	>250 ^a / ^a >250 ^a
Type 2	0.5/0.5	4 ^a /8 ^a	8 ^a /16 ^a	31 ^a /31 ^a	>250 ^a / ^a >250 ^a
Type 3	0.5/0.25	2/0.5	31/0.5	>250/2 ^a	>250/>250 ^a
Nutrient agar					
Type 1	0.25/0.5	0.5/0.5	31 ^a /31 ^a	>250 ^a / ^a >250 ^a	>250 ^a / ^a >250 ^a
Type 2	0.5/0.5	2 ^a /2 ^a	31 ^a /31 ^a	>250 ^a / ^a >250 ^a	>250 ^a / ^a >250 ^a
Type 3	4/0.5	4/0.5	250/62 ^a	>250/>250 ^a	>250/>250 ^a

^a Spheroplasts predominant cell type.

FIG. 1. Disk susceptibility of *H. influenzae* type 2 to ampicillin (A) and cefuroxime (C) on enriched nutrient agar. (a) Light inoculum; (b) heavy inoculum.

TABLE 2. Activity of ampicillin and cefuroxime against *H. influenzae*—dextrose phosphate medium

<i>H. influenzae</i>	MIC ampicillin/MIC cefuroxime ($\mu\text{g/ml}$) with CFU in inoculum:				
	10	10^3	10^5	10^6	10^7
Dextrose phosphate broth					
Type 1		0.5/0.25	0.5/0.5	1/0.5	1/1
Type 2		0.25/0.25	1/0.25	1/0.25	2/1
Type 3		4/0.25	16/0.5	125/0.5	>250/1
Dextrose phosphate agar					
Type 1	0.1/0.25	0.1/0.25	0.25/0.5	0.25/1	0.25/1
Type 2	0.25/0.25	0.25/0.5	0.5/1	1/1	2/1
Type 3	2/0.5	4/0.5	16/0.5	125/0.5	>250/0.5

heavy inoculum. Cefuroxime, on the other hand, showed clear zones at both inoculum levels.

In vivo studies. Protection tests in mice infected with each of the *H. influenzae* types showed ampicillin and cefuroxime to be equally effective against type 1 and 2 strains and cefuroxime to be 20 times more effective than ampicillin against the β -lactamase producer (Table 3).

DISCUSSION

Susceptibility testing of *H. influenzae* to β -lactam antibiotics with the conventional macroscopic determination of end point can lead to highly misleading results. Technical difficulties result from the ability of *H. influenzae* to form osmotically stable spheroplasts (2, 11, 12, 15).

The isolation of β -lactamase-producing

strains of *H. influenzae* and the necessity of detecting these strains at an early stage of investigation has put greater emphasis on susceptibility testing procedures. With conventional techniques, we have encountered difficulties in differentiating between β -lactamase-producing strains and those strains of *H. influenzae* that form stable spheroplasts at low inoculum concentrations. Also, the superior activity of cefuroxime over ampicillin against β -lactamase-producing strains is not obvious by such techniques.

These difficulties have been overcome by the use of a medium in which the normal bacillary form of the organism can grow, but in which the spheroplasts do not survive. With this medium, either in a liquid or solid form, the importance of the inoculum size is greatly reduced, since spheroplasts no longer interfere with the interpretation of results. In the presence of am-

FIG. 2. Disk susceptibility of *H. influenzae* type 3 to ampicillin (A) and cefuroxime (C) on enriched nutrient agar. (a) Light inoculum; (b) heavy inoculum.

FIG. 3. Disk susceptibility of *H. influenzae* type 2 to ampicillin (A) and cefuroxime (C) on dextrose phosphate medium. (a) Light inoculum; (b) heavy inoculum.

picillin, β -lactamase-producing strains of *H. influenzae* are easily recognized on this medium, both by MIC and disk susceptibility tests. The superior activity of cefuroxime over ampicillin against such strains is also readily evident.

The in vitro results correlate well with the in vivo findings, in which cefuroxime, equally ac-

tive as ampicillin against the type 1 and 2 strains of *H. influenzae*, is superior in activity to ampicillin against the β -lactamase-producing strain.

Cefuroxime should be an extremely useful β -lactam antibiotic for treating infections caused by *H. influenzae*, irrespective of their susceptibility to ampicillin.

FIG. 4. Disk susceptibility of *H. influenzae* type 3 to ampicillin (A) and cefuroxime (C) on dextrose phosphate medium. (a) Light inoculum; (b) heavy inoculum.

TABLE 3. Protection tests on experimentally infected mice

<i>Haemophilus</i> type	Median effective dose in mg/kg dose of:	
	Ampicillin	Cefuroxime
1	3	5
2	3	3
3	30	1.5

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