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Postantibiotic and Post-β-Lactamase Inhibitor Effects of Amoxicillin plus Clavulanate

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The postantibiotic effect (PAE) of amoxicillin-clavulanate was studied for strains of *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella pneumoniae*, and *Escherichia coli*. A PAE of approximately 2 h was seen for β-lactamase-positive and -negative strains of *S. aureus* following 2 h of exposure to twice the MIC and did not increase at 16 times the MIC. The PAE observed with *H. influenzae* was clearly related to the growth rate of the organism. A PAE of 0.8 h was found for amoxicillin (four times the MIC) against a β-lactamase-negative strain of *H. influenzae* (generation time, 26.3 min) and a PAE of 1.74 h was found for amoxicillin-clavulanate (twice the MIC) against a β-lactamase-positive strain (generation time, 32.2 min). When the β-lactamase-positive strain was growing more slowly (generation time, 120 min), the PAE of amoxicillin-clavulanate increased to >3.32 h. The PAE of amoxicillin-clavulanate at 2/1 μg/ml on a β-lactamase-producing strain of *M. catarrhalis* was >2.9 h, and, as expected, the PAEs of twice the MIC on *K. pneumoniae* and *E. coli* were generally short (<1 h). The post-β-lactamase inhibitor effect (PLIE), determined after removal of only clavulanate, was also examined for β-lactamase-positive strains. This was more prolonged (approximately 3 to 4 h) than the corresponding PAE for *S. aureus*, *H. influenzae*, and *M. catarrhalis*. The PLIE was related to the amount of β-lactamase produced and required the presence of amoxicillin in the initial exposure period. These data may have implications for reducing the dosage of amoxicillin-clavulanate.

The postantibiotic effect (PAE), or persistent suppression of bacterial growth after brief exposure of a bacterial culture to an antimicrobial agent, is a well-established phenomenon (3, 4). For example, aminoglycosides and 6-fluoroquinolones are noted for prolonged suppression of growth of gram-negative bacilli, such as *Escherichia coli* (11) and *Pseudomonas aeruginosa* (3), while erythromycin and vancomycin display PAEs for *Staphylococcus aureus*. β-Lactam antibiotics characteristically produce PAEs of short duration (1.5 to 2 h) on staphylococci (and streptococci) but show little effect on gram-negative bacteria (3), except in the case of carbapenems, which demonstrate a PAE against *P. aeruginosa* (2). More recently, Gould et al. (8) reported that amoxicillin and clavulanate produced PAEs up to 8 h against β-lactamase-negative strains of *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *E. coli*. Unexpectedly, the addition of clavulanate to amoxicillin produced an extended PAE, except with *E. coli*. These observations prompted us to investigate the extent of the PAE produced by amoxicillin-clavulanate against β-lactamase-positive bacteria, since this combination is frequently used for the treatment of infections caused by amoxicillin-resistant organisms.

In addition, the effect of removing clavulanate after brief incubation of the β-lactamase-producing strains in the presence of amoxicillin-clavulanate and continuing incubation in the presence of amoxicillin alone was investigated. This was done to simulate the clinical situation, where amoxicillin-clavulanate formulations contain more amoxicillin (two to seven times more) than clavulanate. Consequently, amoxicillin is present in the patient after elimination of the clavulanate. The phenomenon of continuing suppression of bacterial growth after removal of the β-lactamase inhibitor was termed the post-β-lactamase inhibitor effect (PLIE).

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MATERIALS AND METHODS

Compounds. Amoxicillin trihydrate, lithium clavulanate, and ampicillin trihydrate were supplied as laboratory reference materials by SmithKline Beecham Pharmaceuticals, Worthing, United Kingdom. Gentamicin sulfate (Genticin; Nicholas Laboratories Ltd., Slough, United Kingdom) was used as a commercially available preparation.

Organisms. Two β-lactamase-negative strains and two β-lactamase-positive strains of *S. aureus*, one β-lactamase-negative strain and two TEM-1 β-lactamase-producing strains of *H. influenzae*, one BRO-1 β-lactamase-producing strain of *M. catarrhalis*, three strains of *Klebsiella pneumoniae* which produce chromosomally mediated class IV β-lactamase, and two TEM-1 β-lactamase-producing strains of *E. coli* were used in these studies. The susceptibilities of these organisms are shown in Table 1.

Inocula. Antibiotic concentrations were prepared in 20-ml volumes of Mueller-Hinton broth (BBL) and were inoculated with 200 μl of an overnight tryptone soya broth (Oxoid) culture for tests with *S. aureus* and 40 μl of an overnight culture for *K. pneumoniae* and *E. coli*. In the case of *H. influenzae*, a 50-fold dilution of a logarithmic-phase culture was made into *Haemophilus* test medium, and for *M. catarrhalis*, 19 ml of antibiotic-containing brain heart infusion broth (Oxoid) was inoculated with 1 ml of a rapidly growing culture. All starting inocula were 10^6 or 10^7 CFU/ml.

PAE and PLIE determinations. The organisms were exposed to the antibiotics (usually at twice the MIC, but occasionally at higher concentrations) for 2 h before removal of the antibiotics by filtration of the culture through a 0.2-μm-pore-size nylon filter followed by two washes with 10 ml of drug-free, prewarmed test medium. The bacteria on the filter were resuspended in 20 ml of drug-free, prewarmed medium to determine the PAE and 20 ml of prewarmed test medium containing amoxicillin, at the same concentration as originally present, to determine the PLIE. Hourly determinations of the numbers of viable bacteria were carried out during the 2-h exposure period, for 6 to 10 h thereafter, and at 24 h. Samples were also counted immediately after filtration. During the experiments, the cultures were incubated at 37°C in an environmental orbital shaker, and the PAE and PLIE were calculated as $T - C$, where T is the time for the number of viable organisms in the test culture to increase 1 $\log_{10}$ above the number observed immediately after filtration and C is the time for the number of viable

TABLE 1. Susceptibilities of the test organisms to amoxicillin (AMX), clavulanate (CA), and amoxicillin-clavulanate (AMX/CA)

Organism	MIC (μg/ml) of:		
	AMX	CA	AMX/CA[a]
S. aureus NCTC 6571[b]	0.125	8	0.125
S. aureus 1555[b]	0.125	16	0.125
S. aureus NCTC 11561	>512	16	1.0
S. aureus WB112	128	16	1.0
H. influenzae H2[b]	0.5	64	0.5
H. influenzae LH2803	16	128	1.0
H. influenzae NEMC1	32	64	1.0
M. catarrhalis Ravasio	8	4	0.03
K. pneumoniae NCTC 9633	64	32	2
K. pneumoniae I112	64	64	2
K. pneumoniae T767	128	32	2
E. coli ATCC 35218	>512	16	8
E. coli NCTC 11560	>512	16	16

[a] Expressed as the concentration of amoxicillin present in a 2:1 ratio.
[b] β-Lactamase-negative strain.

organisms in the untreated control culture to increase 1 $\log_{10}$ above the number observed immediately after filtration.

Microbiological assays were used to confirm removal of the compounds to below 0.02 μg of amoxicillin per ml (*Micrococcus luteus* NCTC 8340) and below 0.08 μg of clavulanate per ml (*K. pneumoniae* NCTC 11228 in agar containing 60 μg of benzylpenicillin per ml).

In some tests with *H. influenzae*, alternative methods of antibiotic removal were used. These were dilution of the culture 100-fold into drug-free, prewarmed medium and the addition of 2.5% (vol/vol) β-lactamase (Genzyme; Whatman) to cultures containing amoxicillin or ampicillin alone.

Viable bacterial counts. Samples were diluted 10-fold serially in sterile, drug-free medium, and three 20-μl drops of each dilution were dispensed onto nutrient agar (Lab M Ltd.) containing 0.4% (vol/vol) β-lactamase (Penicillinase; Difco) and supplemented with 5% sterile horse blood (Tissue Culture Services) for *M. catarrhalis*, which was preheated to "chocolate" in the case of *H. influenzae*. The numbers of CFU were determined following 24 h of incubation at 37°C.

Measurement of β-lactamase activity. Cultures of *S. aureus* were grown in drug-free Mueller-Hinton broth and broth containing amoxicillin and amoxicillin-clavulanate for 2 h at 37°C, following which they were filtered, washed, and resuspended in drug-free medium, as described above for the PAE determinations. The absorbance of each culture was measured at 450 nm with a PU8735 spectrophotometer (Philips; Unicam). The amoxicillin-treated and untreated control cultures were then diluted to give an absorbance reading similar to that of the culture treated with amoxicillin-clavulanate and thus similar inoculum levels (confirmed by viable count). Nitrocefin (0.4 ml of a 500-μg/ml solution) was added to 3.6 ml of each culture in a conventional cuvette, and the rate of change of absorbance was monitored at 493.4 nm for up to 3 h at 37°C. This rate should reflect the amount of active β-lactamase present following the previous 2-h antibiotic exposure. An uninoculated control was also included.

RESULTS

PAE and PLIE. (i) *S. aureus*. A PAE of 1.74 h was observed following incubation of a β-lactamase-negative strain, *S. aureus* NCTC 6571, in the presence of 0.25 μg of amoxicillin per ml (twice the MIC) for 2 h at 37°C and removal of the antibiotics by filtration (Fig. 1). The addition of clavulanate (0.125 μg/ml) to the amoxicillin produced no increase in activity (Fig. 1), and the PAE of the amoxicillin-clavulanate combination (1.87 h) was similar to that seen for amoxicillin alone. Increasing the concentration of amoxicillin (2 μg/ml) and amoxicillin-clavulanate (2/1 μg/ml) to 16 times the MIC resulted in a PAE of 2.38 h for the β-lactamase-negative strain, *S. aureus* 1555, which was similar to the effect produced by twice the MIC against *S. aureus* NCTC 6571 (Table 2).

In tests against a β-lactamase-producing strain, *S. aureus* NCTC 11561, amoxicillin-clavulanate at twice the MIC (2/1 μg/ml) produced PAEs of 1.54 to 2.55 h, similar to those observed with the β-lactamase-negative strains, whereas amoxicillin (2 μg/ml) was much less active (Fig. 2a). In this case, however, the PLIE was also measured and shown to be considerably longer (3.77 and 4.90 h [Table 2]) than the PAE. The prolonged effect is apparent from the viable-count graph (Fig. 2a), where it can be seen that the culture from which both amoxicillin and clavulanate were removed started to regrow between 2 and 3 h after antibiotic removal, whereas the culture from which only clavulanate was removed did not regrow until 4 to 5 h after removal of the β-lactamase inhibitor. The difference between the PAE and the PLIE was even more marked in the case of another β-lactamase-producing strain, *S. aureus* WB112, where the culture from which only clavulanate was removed did not start to regrow until 5 to 6 h after antibiotic removal (Fig. 2b). In these experiments it was considered possible, particularly in the case of *S. aureus*, that the filtration process may remove enough of the extracellular β-lactamase to render the organism susceptible to amoxicillin. This could cause inhibition of growth after removal of amoxicillin-clavulanate and readdition of the amoxicillin (as in determination of the PLIE). Control cultures, in which the organisms were exposed to either drug-free medium (no β-lactamase induction) or medium containing amoxicillin (likely to induce staphylococcal β-lactamase production) for 2 h, filtered, washed, and then reexposed to amoxicillin, were therefore included. If the growth of this culture was not inhibited by the amoxicillin added after filtration, the PLIE was considered a valid effect. For all the PLIE studies included in Table 2, no more than a transitory inhibition of growth was seen following exposure to drug-free medium or amoxicillin alone, filtration, and reexposure to amoxicillin, and PLIE values calculated by using these cultures as the control fell within the quoted ranges.

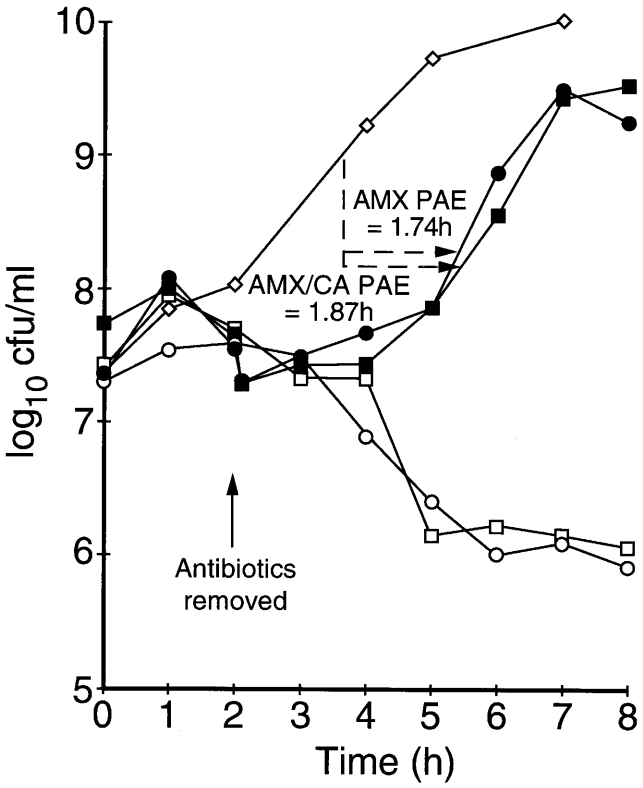

FIG. 1. Bactericidal activities of 0.25 μg of amoxicillin (AMX) per ml (○) and 0.25/0.125 μg of amoxicillin-clavulanate (AMX/CA) per ml (□) and the effect of removing these antibiotics at 2 h (● and ■, respectively), compared with an untreated control culture (◇) of *S. aureus* NCTC 6571.

TABLE 2. Duration of the PAE and the PLIE of amoxicillin-clavulanate (AMX/CA)

Organism	β-Lactamase	AMX/CA concn (μg/ml)[a]	PAE (h)	PLIE (h)
S. aureus NCTC 6571	Negative	0.25/0.125 (2)	1.87	NA[b]
S. aureus 1555	Negative	2/1 (16)	2.38	NA
S. aureus NCTC 11561	Plasmid	2/1 (2)	1.54–2.55	3.77–4.90
S. aureus WB112	Plasmid	2/1 (2)	0.69–1.24	>3.28–4.30
H. influenzae H2	Negative	2/1 (4)	0.63	NA
H. influenzae LH2803	TEM-1	2/1 (2)	1.67	3.23
M. catarrhalis Ravasio	BRO-1	2/1 (64)	>2.87	ND[c]
K. pneumoniae NCTC 9633	Chromosomal	4/2 (2)	0.77	1.18
K. pneumoniae I112	Chromosomal	4/2 (2)	0.37	0.78
K. pneumoniae T767	Chromosomal	4/2 (2)	0	1.30
E. coli ATCC 35218	TEM-1	16/8 (2)	0.78	1.56
E. coli NCTC 11560	TEM-1	32/16 (2)	0.87	0.38

[a] Numbers in parentheses are multiples of the MIC.
[b] NA, not applicable.
[c] ND, not determinable.

(ii) *H. influenzae*. In initial experiments, no regrowth of the β-lactamase-positive strains *H. influenzae* LH2803 and *H. influenzae* NEMC1 was seen by 8 to 12 h in cultures where twice the MICs of amoxicillin and clavulanate were removed at 2 h. This meant that exact values for the PAE and PLIE could not be calculated, although the PAE was at least 2.26 to 7.16 h. Table 3 includes results from two examples of these experiments, where the PAE and PLIE of amoxicillin-clavulanate on *H. influenzae* LH2803 (generation time, 120 min) and *H. influenzae* NEMC1 (generation time, 50 min) were >3.32 and >2.26 h, respectively. These data were in contrast to published data, where the PAE of penicillin against β-lactamase-negative *H. influenzae* has been reported as <0.5 to 1.5 h (15), and 1.25 μg of ampicillin per ml (five times the MIC) was shown to produce no PAE, whereas a twofold-higher concentration gave a PAE of 1.5 h (7). To assess whether methodology was the reason for this discrepancy, the PAE of ampicillin against a β-lactamase-negative strain, *H. influenzae* H2, was measured by using three methods for antibiotic removal: filtration, dilution, and the addition of β-lactamase.

As with the β-lactamase-producing strains, in an initial experiment with *H. influenzae* H2, the growth rate was low (generation time, 73 min), such that the PAE could not be calculated. In a subsequent experiment, however, the growth rate of *H. influenzae* H2 was considerably increased by repeated subculture on agar, and PAEs could be calculated for ampicillin (2 μg/ml [eight times the MIC]), amoxicillin (2 μg/ml [four times the MIC]), and amoxicillin plus clavulanate (2/1 μg/ml [four

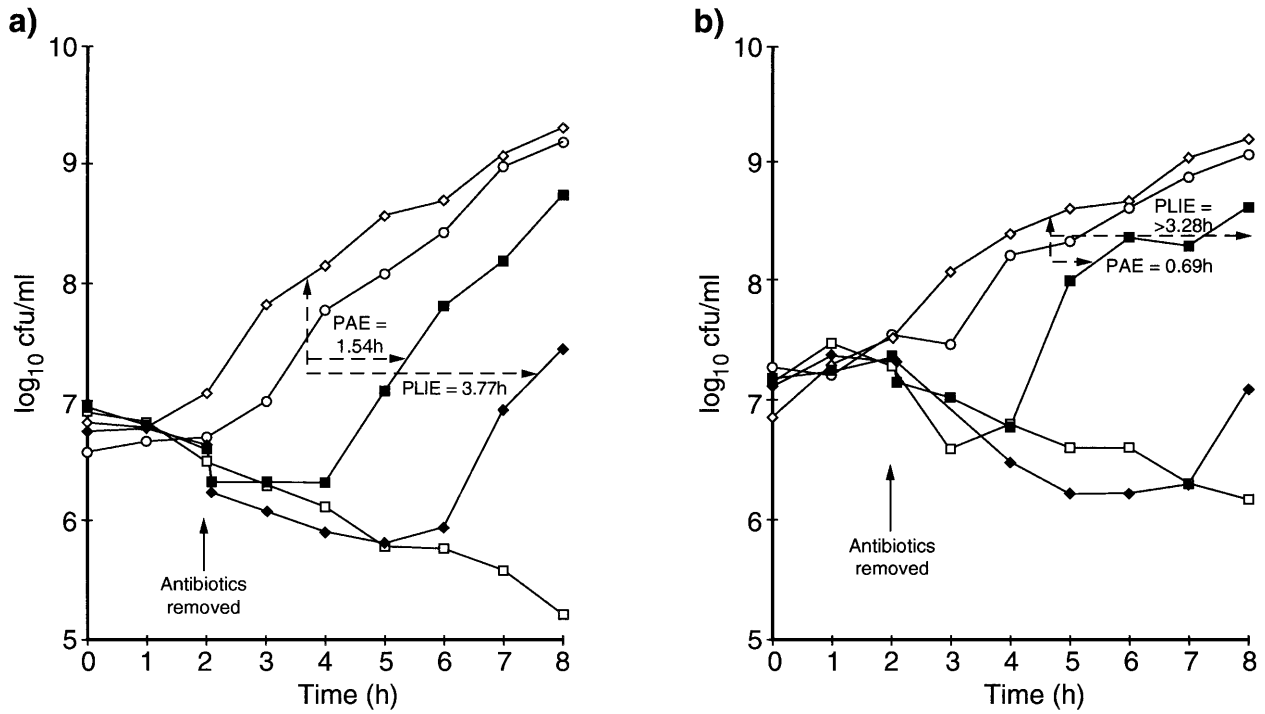

FIG. 2. Bactericidal activities of 2 μg of amoxicillin per ml (○) and 2/1 μg of amoxicillin-clavulanate per ml (□) and the effect of removing both amoxicillin and clavulanate (■) or only clavulanate (◆) after 2 h of incubation with 2/1 μg of amoxicillin/clavulanate per ml, compared with untreated control cultures (◇) of *S. aureus* NCTC 11561 (a) and *S. aureus* WB112 (b).

TABLE 3. Effect of growth rate and method of removal of antibiotics on the PAE of ampicillin (AMP), amoxicillin (AMX), and amoxicillin-clavulanate (AMX/CA) against *H. influenzae*

H. influenzae strain	Generation time (min)	Antibiotic (concn [μg/ml])	Method of removal	PAE (h)	PLIE (h)
H2[a]	26.3	AMP (2)	Filtration	0.84	NA[b]
	26.3	AMP (2)	Dilution	0.84	NA
	26.3	AMP (2)	β-Lactamase	2.37	NA
	26.3	AMX (2)	Filtration	0.79	NA
	26.3	AMX (2)	β-Lactamase	1.89	NA
	26.3	AMX/CA (2/1)	Filtration	0.63	NA
LH2803	32.2	AMX/CA (2/1)	Filtration	1.74	3.33
	120	AMX/CA (2/1)	Filtration	>3.32	>3.32
NEMC1	50	AMX/CA (2/1)	Filtration	>2.26	>2.26

[a] β-Lactamase-negative strain.
[b] NA, not applicable.

times the MIC]). The PAEs for ampicillin and amoxicillin (with or without clavulanate) obtained by the filtration method (as used against all other organisms) and by dilution (used only for ampicillin) were similar (0.6 to 0.8 h [Table 3]). Moreover, these values tied in well with published data (7, 15). When the addition of β-lactamase was used to remove ampicillin and amoxicillin, however, longer PAEs (approximately 2 h) were obtained, which may have been due to the slower removal of the penicillins by the β-lactamase enzyme.

Since this study showed that the filtration methodology gave results similar to published data, provided that the growth rate was adequate, the PAE and PLIE of amoxicillin-clavulanate were reinvestigated against the β-lactamase-producing strain *H. influenzae* LH2803, which was subcultured to increase the growth rate in vitro (Fig. 3a). The growth rate was similar (generation time, 32.2 min) to that seen with the β-lactamase-negative strain (26.3 min) (Table 3), and the PAE obtained for amoxicillin-clavulanate was 1.74 h, closer to published data than in previous tests. The PLIE obtained was 3.33 h, similar to the values seen for *S. aureus* (Table 2).

(iii) *M. catarrhalis.* Amoxicillin-clavulanate was tested at 2/1 μg/ml (64 times the MIC), concentrations readily achievable in serum and tissues of humans following oral dosing (6), against a β-lactamase-producing strain, *M. catarrhalis* Ravasio, and produced substantial bactericidal effects, whereas amoxicillin (2 μg/ml) failed to inhibit growth (Fig. 3b). After removal of amoxicillin-clavulanate from the culture, regrowth occurred after 2 to 3 h, and a PAE of >2.87 h was calculated. The PLIE was not considered valid, since the control culture exposed to amoxicillin alone (2 μg/ml), filtered, and reexposed to amoxicillin also showed inhibition of growth.

(iv) Members of the family *Enterobacteriaceae.* Variable PAE results were obtained for strains of *K. pneumoniae* which produce class IV, chromosomally mediated β-lactamase, ranging from 0 h for *K. pneumoniae* T767 to 0.77 h for *K. pneumoniae* NCTC 9633. Despite a calculated PAE of 0 h, on examination of the viable counts of *K. pneumoniae* T767, a 1-h lag (PAE) was apparent after removal of amoxicillin-clavulanate. The PLIE on this organism appeared to be longer than the PAE from the graph and was calculated as 1.30 h (Table 2). In general, the PLIE of twice the MIC of amoxicillin-clavulanate on *K. pneumoniae* was longer than the PAE and was similar for the three strains (0.78 to 1.30 h [Table 2]).

PAEs and PLIEs measured for amoxicillin-clavulanate (twice the MIC) against *E. coli* ATCC 35218 (0.78 and 1.56 h [Table 2]) were similar to those seen with *K. pneumoniae*,

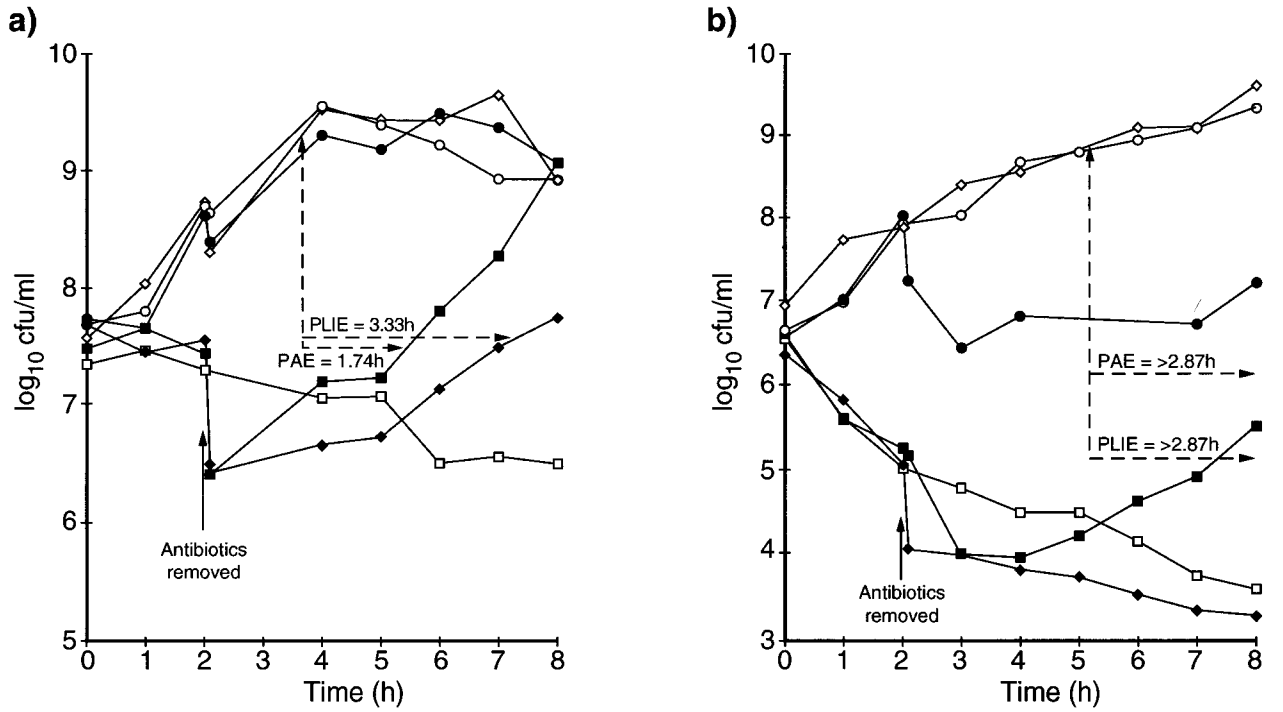

FIG. 3. Bactericidal activities of 2 μg of amoxicillin per ml (○) and 2/1 μg of amoxicillin-clavulanate per ml (□) and the effect of removing both amoxicillin and clavulanate (■) or only clavulanate (◆) after 2 h of incubation with 2/1 μg of amoxicillin-clavulanate per ml, compared with untreated control cultures (◇) of *H. influenzae* LH2803 (a) and *M. catarrhalis* Ravasio (b). Also shown (●) is the activity of 2 μg of amoxicillin per ml, removed and replaced at 2 h.

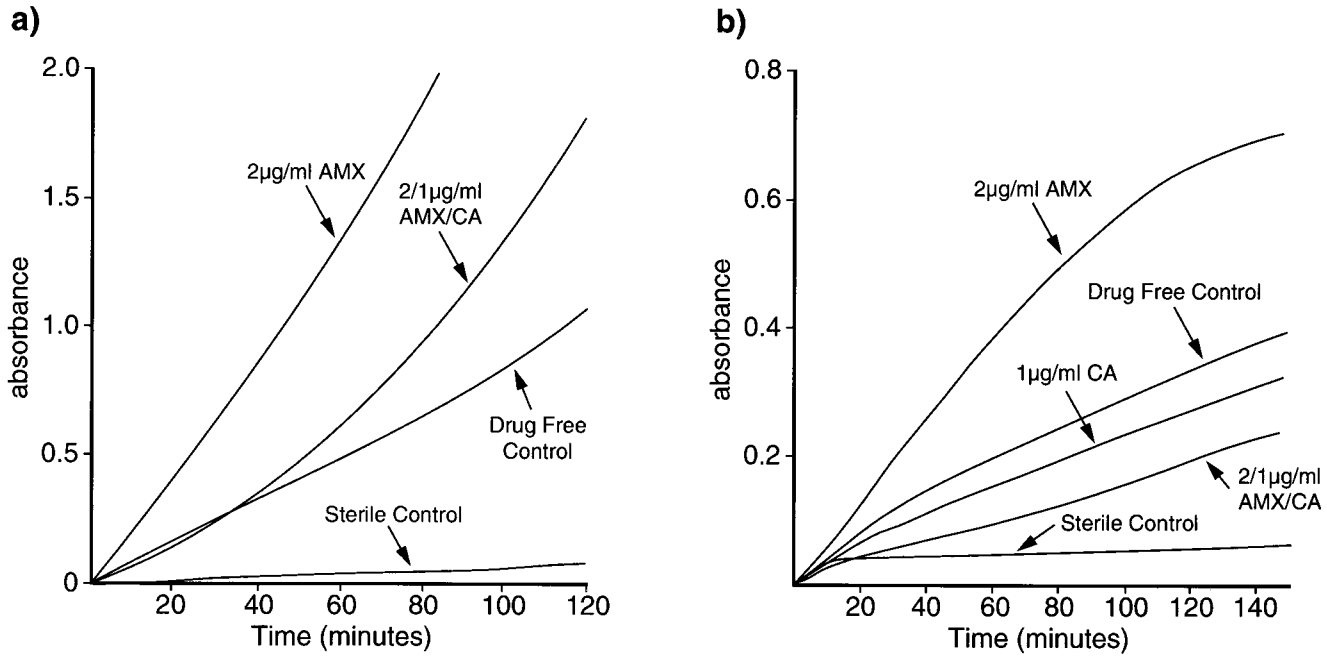

FIG. 4. β-Lactamase activity at 37°C following exposure to amoxicillin (AMX), amoxicillin-clavulanate (AMX/CA), clavulanate (CA), or no antibiotic for 2 h, removal of the antibiotics by filtration, and resuspension in prewarmed, drug-free media for cultures of *S. aureus* NCTC 11561 (a) and *S. aureus* WB112 (b).

whereas although the PAE on *E. coli* NCTC 11560 was similar (0.87 h), the PLIE was shorter.

Mechanism of action of PLIE. A possible mechanism for the PLIEs determined in these studies could involve a recovery period of β-lactamase production after exposure to amoxicillin-clavulanate. To investigate this, the β-lactamase activities of drug-free cultures of *S. aureus*, preincubated in the presence of amoxicillin, amoxicillin-clavulanate, or clavulanate, were determined by spectrophotometric measurement of the rate of hydrolysis of nitrocefin. The data in Fig. 4 show that the rate of inactivation of nitrocefin was highest in the cultures of *S. aureus* NCTC 11561 and *S. aureus* WB112, which were preincubated with amoxicillin, probably because of induction of the staphylococcal β-lactamase by the penicillin. Both cultures previously exposed to amoxicillin-clavulanate displayed lower β-lactamase activities than the amoxicillin-treated cultures, and in the case of *S. aureus* WB112, this activity was lower than that of the untreated control culture. In the test with *S. aureus* WB112, a culture was also preincubated in the presence of clavulanate alone (1 μg/ml), and this culture inactivated nitrocefin more slowly than the untreated control culture but more rapidly than the culture preexposed to amoxicillin-clavulanate (2/1 μg/ml).

DISCUSSION

The PAEs found for amoxicillin, with or without clavulanate, for β-lactamase-negative strains of *S. aureus* (approximately 2 h following exposure to twice the MIC) were in agreement with data reported for other β-lactams (3, 7, 14). No increase in the PAE was seen when the concentration of amoxicillin was increased further (up to 16 times the MIC), also in agreement with published data (5). The lack of enhancement of the amoxicillin PAE by clavulanate differs from the findings of Gould et al. (8), but those workers used much higher concentrations of clavulanate, which exhibited antibacterial activity in their own right.

The removal of amoxicillin-clavulanate from cultures of β-lactamase-positive *S. aureus* and replacement with amoxicillin resulted in inhibition of growth which was more prolonged than that seen in the PAE studies, despite these strains being resistant to amoxicillin. This phenomenon, reported briefly in 1992 (12), was termed the PLIE, analogous to the PAE. The persistent inhibition of growth of *S. aureus* after removal of amoxicillin-clavulanate or clavulanate appeared, from the time-kill studies, to be due to a prolonged lag phase rather than an increase in generation time. Although the PAE of amoxicillin-clavulanate on *S. aureus* WB112 was shorter than that on *S. aureus* NCTC 11561, the PLIE was longer. This was most likely due to the larger amount of β-lactamase produced by *S. aureus* NCTC 11561, as demonstrated by the MICs of amoxicillin alone for these two organisms.

It is generally perceived that β-lactam antibiotics, other than carbapenems (2), do not exhibit a PAE on gram-negative organisms, but in preliminary studies of amoxicillin-clavulanate against *H. influenzae* we observed long PAEs and PLIEs (12). The data reported here showed that both PAEs and PLIEs were influenced significantly by the growth rate of the cultures—the lower the growth rate, the longer the PAE and PLIE. It has been noted by other workers (9) that growth conditions have an effect on the duration of PAEs, and since bacteria are considered to grow more slowly in vivo than in nutritious media in vitro, the prolonged PAEs and PLIEs initially reported for *H. influenzae* may be more relevant to the clinical situation.

In the study with *M. catarrhalis* Ravasio, a PAE of >2.87 h was measured, although a precise value could not be calculated. A longer time of inhibition of growth (>6 h) was seen following removal of the clavulanate with retention of amoxicillin, but growth was also inhibited to some extent by preexposure to amoxicillin alone, filtration, and postexposure to amoxicillin. This was probably because the concentrations of amoxicillin-clavulanate used (2/1 μg/ml), although easily achievable in humans with oral dosages (6), were far in excess of the MIC

(64-fold) and close (1/4) to the MIC of amoxicillin alone. Other workers (13) have reported a PAE of 3 h and a PLIE of 5 to > 7 h for amoxicillin-clavulanate at four times the MIC for a β-lactamase-producing strain of *M. catarrhalis*, which is in agreement with our results.

The PAEs obtained for strains of *K. pneumoniae* and *E. coli* were generally short, which was consistent with published data for penicillins against members of the *Enterobacteriaceae* (3). The PLIEs against these strains were similar to or longer than the PAEs and appeared to be related to the amount of β-lactamase produced. For example, *E. coli* NCTC 11560 produces larger amounts of TEM-1 β-lactamase than *E. coli* ATCC 35218, and the PLIE was shorter for *E. coli* NCTC 11560, despite the PAE value being similar for the two strains.

If the amount of β-lactamase produced does determine the length of the PLIE, this suggests that one likely mechanism for the PLIE is the lag time before production of sufficient quantities of new enzyme following inhibition by clavulanate. Spectrophotometric studies with *S. aureus* showed the cultures previously exposed to amoxicillin to contain elevated levels of β-lactamase, most likely because of induction of the staphylococcal enzyme, whereas cultures previously exposed to amoxicillin-clavulanate or clavulanate alone showed diminished β-lactamase activity. These findings suggest either a lag phase before further β-lactamase production or remaining cell-associated clavulanate. Data reported by Balcabao et al. (1), who simulated concentrations achieved in the serum of humans in vitro, also showed a reduction in β-lactamase production by *S. aureus* which lasted up to 4 h after removal of clavulanate from cultures previously exposed to amoxicillin-clavulanate. Although these data and our spectrophotometric results also showed a reduction in β-lactamase activity after removal of clavulanate from a culture previously exposed to clavulanate alone, in the studies described here a PLIE could not be demonstrated in cultures preincubated with clavulanate (1 μg/ml) for 2 h and reincubated with amoxicillin (2 μg/ml) (data not shown). Thus, to demonstrate a PLIE, the culture must be previously exposed to both amoxicillin and clavulanate.

In clinical settings, it is likely that the infecting bacteria will be initially exposed to high concentrations of both amoxicillin and clavulanate, following which the concentration of both agents will decrease, but, because of the lower unit dose, the clavulanate concentration will fall below a detectable level first. This is when the PLIE becomes relevant. Subinhibitory concentrations of both agents will be present for some time and these may further prolong the antibacterial activity (4). Odenholt-Tornqvist et al. (10) showed that sub-MIC concentrations of β-lactam antibiotics substantially prolonged the regrowth of cultures previously exposed to high concentrations of the same antibiotic, in contrast with the effect seen with cultures which were not previously exposed. This was particularly marked for antibiotic-bacterium combinations which showed a PAE. All these factors may have implications for reducing the dosage of amoxicillin-clavulanate.

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