Concentration of amoxycillin and clavulanate in lung compartments in adults without pulmonary infection

P J Cook, J M Andrews, J Woodcock, R Wise, D Honeybourne

Abstract

Background - The efficacy of an antibiotic is usually predicted from serum levels and MIC\textsubscript{90} values for likely pathogens, but in the lung tissue concentrations may be more informative. This study compares concentrations of amoxyccillin and clavulanate in serum, epithelial lining fluid (ELF), alveolar macrophages, and bronchial mucosa in 15 adults.

Methods - Amoxycillin 500 mg and clavulanic acid 250 mg were given 1-2 hours before diagnostic bronchoscopy for haemoptysis or radiological abnormality. Mucosal biopsy samples were taken from macroscopically normal sites, alveolar macrophages harvested by lavage, and ELF volume derived from urea concentrations in bronchial lavage fluid and blood. Amoxycillin was assayed by inhibition of growth of Micrococcus luteus, and clavulanate (in serum, ELF, and bronchial mucosa) by inhibition of growth of Klebsiella pneumoniae; in macrophages clavulanate was measured by high performance liquid chromatography.

Results - The median concentrations in serum were 6.90 mg/l for amoxycillin and 5.25 mg/l for clavulanate. The median bronchial mucosal concentration of amoxycillin was 2.99 mg/l and of clavulanate was 1.65 mg/l; the median concentrations in ELF were 0.89 and 0.96 mg/l, and in macrophages 0 and 0.76 mg/l, respectively. In macrophages amoxycillin levels were undetectable in 10 of 14 subjects (71%); by contrast, only 6 of 14 subjects (43%) had no detectable clavulanate.

Conclusions - Clavulanate levels exceeded quoted MIC\textsubscript{90} values (around 0.25 mg/l) for Legionella pneumophila both in ELF and in macrophages. Amoxycillin-clavulanate may therefore have a clinical role in infections with Legionella pneumophila.

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During the last decade 20–32% of Haemophilus influenzae type b and about 15% of non-encapsulated H influenzae isolates have been resistant to ampicillin, mainly as a consequence of the non-inducible β-lactamase TEM-1.1 A similar increase in the production of β-lactamases has been observed, particularly in the hospital setting, among clinical isolates of several other organisms including enterobacteria (especially Escherichia coli), Moraxella catarrhalis, and some Gram positive cocci.3 These enzymes now provide the commonest mechanism of resistance to β-lactam antibiotics among clinical bacterial isolates. A number of β-lactam compounds, including the oxapenamic clavulanic acid and the penicillin sulphones tazobactam and sulbactam, inhibit a wide range of β-lactamases by binding to their active sites, thus increasing the efficacy of β-lactam antibiotics. Amoxycillin-clavulanate, a broad spectrum antibiotic comprising mixtures of clavulamic acid with the modified amino-penicillin amoxycillin is used in both lower and upper respiratory tract infections, particularly community acquired pneumonias and acute purulent exacerbations of chronic bronchitis or bronchiectasis.5,6

To be effective against respiratory tract pathogens an antibiotic must achieve bactericidal concentrations at sites of infection. The present study compares concentrations of amoxycillin and clavulanate in serum, bronchial mucosa, epithelial lining fluid (ELF), and alveolar macrophages in adults undergoing diagnostic bronchoscopy for chest radiographic abnormalities or haemoptysis.

Methods

PATIENTS

Fifteen patients (12 men and three post-menopausal women) were recruited into the study. These patients had a mean age of 58.7 years (range 24–75) and mean weight of 67.0 kg (range 47.2–97.0). None had significant renal or hepatic impairment and all were free of lung infection. Thirteen patients had abnormal chest radiographs; the remaining two presented with haemoptyses. Each gave informed consent before entry into the study, the protocol of which had been approved by the hospital ethical committee.

BRONCHOSCOPY

One to two hours before bronchoscopy each patient (having fasted for the previous eight hours) swallowed 750 mg amoxycillin-clavulanate (comprising amoxycillin 500 mg and clavulamic acid 250 mg) with 200 ml water. A standard premedication of 600 μg atropine was given by intramuscular injection 60 minutes before bronchoscopy, 4 ml of 4% lignocaine by nebuliser 40 minutes later, and up to 5 mg midazolam intravenously at the time of bronchoscopy. At that time up to 4 ml of 4% lignocaine was administered to the pharynx and
Concentration of amoxycillin and clavulanate in lung compartments

vocal cords and 6 ml of 2% lignocaine to the carina and main bronchi.

All antibiotic assays, except those in the ELF, were performed on the day of bronchoscopy. Venous blood was collected before the administration of amoxycillin-clavulanate and at bronchoscopy, and centrifuged at 1500 g for 10 minutes at 4°C.

Bronchoalveolar lavage (BAL) was performed in the right middle lobe or the lingula. The first 50 ml of BAL fluid was discarded to avoid contamination with cells and secretions from the proximal airways, and the next three 50 ml aliquots were collected into a Teflon container (thus preventing adherence of aspirated cells). Lavage time was reduced to a minimum to limit the concentration-dependent efflux of solutes into BAL fluid. The total leucocyte count was estimated immediately with an improved Neubauer haemocytometer. Within five minutes of lavage the pooled BAL fluid was centrifuged at 400 g for five minutes: the supernatant was then freeze dried and reconstituted in one tenth of the original volume of distilled water, making a tenfold concentration.

Assuming that urea is present at the same concentration in ELF and blood, the volume of ELF in bronchoalveolar lavage aspirate can be calculated from urea concentrations in BAL fluid and serum,7 and concentrations of antibiots derived as follows:

\[ V_{\text{ELF}} = V_{\text{OIBAL}} \times \frac{[\text{urea}]_{\text{BAL}}}{[\text{urea}]_{\text{ELF}}} = V_{\text{OIBAL}} \times \frac{[\text{urea}]_{\text{ELF}}}{[\text{urea}]_{\text{BAL}}} \times \frac{[\text{antibiotic}]_{\text{BAL}}}{[\text{antibiotic}]_{\text{SERUM}}} \times \frac{[\text{urea}]_{\text{SERUM}}}{[\text{urea}]_{\text{BAL}}} \]

In health approximately 95% of cells in the alveoli are alveolar macrophages8 whose mean (SE) volume has previously been estimated9 by velocity gradient centrifugation10 at 2-42 (0-41) μl/10⁶ cells. The total volume of macrophages in the BAL aspirate was calculated, assuming that the leucocyte count and macrophage count were equal, as follows:

\[ V_{\text{OIBAL}} = 2-42 \times 10^{-6} \times \text{leucocyte count per ml } \times V_{\text{OIBAL}} \]

The cell pellet was suspended in a measured volume of cold phosphate buffer (pH 7-0) and homogenised by ultrasonication on ice before antimicrobial concentrations were assayed. Concentrations of antimicrobials in macrophages were therefore calculated from the formula:

\[ [A]_{\text{OIBAL}} = [A]_{\text{OIBAL}} \times \frac{V_{\text{OIBAL}}}{V_{\text{OIBAL}}} \]

where \([A]_{\text{OIBAL}}\) is the concentration measured and \(V_{\text{OIBAL}}\) is the volume of macrophages and buffer.

Superficial biopsy samples for antimicrobial assay were taken from macroscopically normal subcarinal sites – that is, without erythema, oedema, induration, or abnormal friability – before diagnostic biopsy samples. Visibly bloodstained specimens were discarded. The tissue was transferred immediately to a humidity chamber, weighed, and homogenised by ultrasonication in a known volume of phosphate buffer (pH 7-0) on ice.

**MICROBIOLOGICAL ASSAYS**

Amoxycillin was measured by inhibition of growth in an overnight broth culture of *Micrococcus lutea* (ATCC 9341) diluted to an OD₅₅₀ of 0.05 and poured over a 200 ml No. 1 agar plate (Unipath, UK). Aliquots of all serum samples, concentrated ELF, mucosal tissue, and macrophages were placed in 5 mm wells cut in this agar and the plate was incubated for 24 hours at 37°C. Zones of inhibition of bacterial growth around patient serum samples were compared with zones produced by standard amoxycillin concentrations in human serum (pH 7-0): zones around mucosal tissue and macrophage samples were compared with those produced by amoxycillin in phosphate buffer (pH 6-6). For assays in concentrated ELF standard amoxycillin preparations were made in 9% sodium chloride. The lower limit of sensitivity for these assays was 0.03 mg/l.

Assay for clavulanic acid in serum, ELF, and mucosa was performed in a similar manner; 16 ml of an overnight broth culture of *Klebsiella pneumoniae* (ATCC 29665), diluted to an OD₅₅₀ of 0-8, was incorporated into a 200 ml No. 2 agar plate (Oxoid, Basingstoke, UK) containing piperacillin at a concentration of 80 mg/l. Samples were incubated in 5 mm wells for 24 hours at 37°C. As described above, zones of inhibition were compared with those produced by standard clavulanate concentrations in human serum (pH 7-0) and 9% sodium chloride. The lower limit of sensitivity for these assays was 0-02 mg/l.

In these systems negative control preparations had no inhibitory effect on the assay organisms. The between assay coefficients of variation were 11.4% for both assays.

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

Clavulanic acid was assayed in alveolar macrophages by high performance liquid chromatography (HPLC), after reaction with imidazole, which produces a derivative absorbing at 311 nm.11,12 The mobile phase was composed of 10 mM disodium hydrogen orthophosphate (BDH), titrated to pH 2-5 with orthophosphoric acid (BDH), 15 mM octanesulphonic acid (BDH) as pairing ion, 23 mM tetraethylammonium bromide (Sigma) as organic counter ion, and 5% acetonitrile (Rathburn Chemicals) passed through a 0-22 μm filter: this was pumped at 0-4 ml/min.

All compounds were analytical grade or higher. 500 μl of each alveolar macrophage homogenate or 500 μl of phosphate buffer standard (pH 6-6) was mixed with 50 μl imidazole (BDH ≥ 99% pure) in 5 M hydrochloric acid (pH 6-8) and incubated in a light proof cabinet at room temperature for 60 minutes. 200 μl of
Table 1  Calculations of amoxycillin and clavulanic acid concentrations (in mg/l) in epithelial lining fluid

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NDL = no detectable level.

Table 2  Concentrations (mg/l) of amoxycillin and clavulanic acid in lung compartments

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A 2 mg/l solution of salicylamide (Sigma) was added to provide an internal standard immediately before solid phase extraction. Extraction was carried out using 100 mg (1 ml) C-18 Bond Elut cartridges (Varian), pre-conditioned with 1 ml methanol, followed by 1-5 ml water. Samples were added to the pre-conditioned cartridges which were then washed with 1-5 ml water and allowed to dry. The adsorbed compounds (clavulanate derivative and salicylamide) were eluted into glass tubes with 500 μl methanol, dried under a stream of air, and reconstituted in 50 μl HPLC grade water by vortex mixing. 25 μl of the eluate was then injected into a Gc, Hypersil-ODS (100 × 2.1 mm, 3 μm) reverse phase steel chromographic column (HPLC Technology Ltd, UK). Samples were read with ultraviolet light at 311 nm using a Kontron 432 detector with an 8 μl flow cell.

In this system the amoxycillin does not interfere with the assay for clavulanic acid. Recovered salicylamide gave a coefficient of variation of 10-0% and clavulanic acid 12-5%, with satisfactory resolution of the two compounds. The between-run coefficient of variation was 6-8% (at 0-25 and 0-1 mg/l) and the lower limit of sensitivity was 0-0125 mg/l.

Results

Table 1 shows the urea concentrations measured in BAL fluid and serum, assayed concentrations of amoxycillin and clavulanic acid in the lavage fluid (10 × concentrated), and calculated ELF concentrations for each of the patients studied. The BAL fluid aspirated from one patient (no. 11) was unsuitable for analysis so that ELF and alveolar macrophage concentrations of these antibiotics could be determined in only 14 patients.

Table 2 shows concentrations of amoxycillin and clavulanic acid (in mg/l) measured in each lung compartment for each subject.

The calculation of mean values quoted takes account of those subjects in whom antibiotics were undetectable in one or more compartments; in macrophages, for example, amoxycillin levels were not detected in 11 of 14 subjects (79%), while six of 14 subjects (43%) had no detectable clavulanate.

Discussion

Methods for the measurement of total antibiotic concentrations in biopsy specimens are now well established.13 14 The concentrations of antibiotics at the site of infection may be more informative than serum concentrations15 – for example, antibiotics may be concentrated in the urine and hence effective in urinary infections even where serum concentrations are so low as to predict clinical failure46 but renal tissue concentrations have been shown to be good predictors of efficacy in ascending urinary infections.17 18 Differences between serum and tissue concentrations of antibiotics occur in discrete compartments of the lung (mucosa and submucosa, alveolar macrophages, epithelial lining fluid, and sputum). The alveolar membrane is thus relatively impermeable to antibiotics owing to the presence of many tight junctions (zonulae occludentes)16–21 and the
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capillary endothelium which separates the sub-
mucosa from blood, being non-fenestrated, 22,23
is also less permeable than the fenestrated ca-
pillaries of other organs. 24 Measured con-
centrations in each compartment depend on
the rates of penetration and clearance: the lipo-
philicity, pH, molecular weight, and protein
binding of the drug also influence these pro-
cesses. 25

In acute bronchitis and acute exacerbations
of chronic obstructive airways disease and
bronchiectasis bacteria are found within the
lumen of the airways, at the mucosal cell sur-
face, and within bronchial mucosal tissue. 26 In
pneumonia they are also chiefly intraluminal:
the ELF and alveolar macrophages appear to
be important locations. 27 These organisms are
therefore separated from blood by significant
barriers to antibiotic diffusion but, in some
cases, antibiotics may be secreted into the bron-
chial lumen by epithelial cells. Measurement
of antibiotic concentrations in sputum is tech-
nically difficult – for example, sputum is fre-
quently contaminated by saliva into which antibiot-
cs such as β-lactams penetrate only poorly,
or by blood. 28 Bronchial secretions col-
lected by direct aspiration via an endotracheal
tube or fibreoptic bronchoscope may have been
pooling for some hours, allowing time for de-
gradation by the β-lactamases of commensal
bacteria; 29 and concentrations of clavulanic
acid, which is unstable at physiological tem-
peratures, are particularly unreliable. 30 Never-
thess the reported concentrations of β-
lactams in directly harvested sputum samples
are only 5–25% of serum levels. 31 This may
increase somewhat in the presence of active
inflammation. By contrast, in all β-lactam anti-
biotics studied to date penetration into the
bronchial mucosa is 35–50% 32,33 whether mul-
tiple or single dosing is used.

Bronchoalveolar lavage allows the meas-
urement of antibiotic levels in ELF and alveolar
macrophages, as well as in bronchial secretions,
sputum, and lung tissue. 34,35 For practical pur-
poses the ELF is defined as the fluid that
takes the small airways distal to the point
of impaction of the tip of the bronchoscope that
is recovered by BAL. It is a complex mixture
of solutes and anti-inflammatory cells, present
in both healthy and infected people, bathing
the terminal bronchioles, alveoli, and alveolar
macrophages.

According to our data serum antibiotic con-
centrations show a partial correlation with those
in bronchial mucosa, but not with ELF or mac-
rophage levels. There is no reason, as far
as we know, to expect a correlation between
serum and macrophage concentrations, but in
the case of ELF such a relationship might
certainly be predicted. However, technical
problems in processing BAL fluid cause a sig-
ificant variation in antibiotic levels, par-
ticularly resulting from the movement of solute
and solvent across the alveolar-capillary mem-
brane. For example, vigorous suction during
lavage promotes the efflux of urea into the
aspirate, artificially increasing the estimated
volume of ELF and thus depressing the derived
drug concentrations; the antibiotics, especially
clavulanic acid, are unstable at physiological
temperatures. Despite the care that we have
taken to standardise the procedures used in
this study to keep dwell times to a minimum
and to freeze dry all specimens (or store them
at ~70°C) before assay, we therefore believe
that a correlation would always be difficult to
demonstrate in ELF and macrophages.

However, the other drugs used during bron-
choscopy (atropine, lignocaine and midazolam)
are unlikely to have influenced the distribution
or activity of the antibiotics. Atropine certainly
reduces the volume of bronchial secretions but
is not known to have any effect on ELF, while
lignocaine, though exerting its pharmacological
effects on cell membranes, has not been re-
ported to alter antibiotic uptake by pulmonary
tissues.

There is now considerable interest in the
importance of intracellular infections in the
lung with such pathogens as Legionella pne-
umophila, Chlamydia pneumoniae, and atypical
mycobacteria, and in the special problems of
antimicrobial delivery to these sites of infection.
In such infections penetration of macrophages
is an important property of antibiotics, but it
is not clear whether this penetration occurs
in the alveolar lumen, or whether circulating
monocyte-derived macrophages acquire the
drug before migrating to the alveolar com-
partment. Beta-lactams have generally been
shown to be taken up only poorly by phagocytic
cells compared with macrolides and quino-
lones. If particulate carriers such as liposomes
and polyisohexylcyanoacrylate nanoparticles
are used to transport β-lactam agents into
macrophages, their bactericidal action is sig-
nificantly enhanced: thus nanoparticle-bound
ampicillin is more effective than free ampicillin
against Listeria monocytogenes in mouse peri-
toneal macrophages. 36 In infections with Leg-
ionella pneumophila clavulanate and β-lactam-
clavulanate combinations are both bactericidal
in vitro and in animal models. 37 The present
study indicates that, at least in some patients,
clavulanate is taken up to a significant degree
by bronchial mucosa, ELF, and macrophages,
and exceeds quoted MIC90 values (around
0.25 mg/l) for Legionella pneumophila. 38,39 In
these compartments. Its results are therefore
consistent with those of the animal experiments
mentioned above.

Interest in the treatment of Mycobacterium
tuberculosis infection with β-lactam antibiotics
has also been aroused recently by the discovery
of an inducible, clavulanate-sensitive β-lac-
tamase produced by this bacterium, active
against penicillins and cephalosporins, and of
a synergistic bactericidal effect of penicillin-
clavulanic acid combinations against M tuber-
culosis. 40

A potential clinical role for amoxycillin-
clavulanate in a variety of infections with
intracellular pathogens is suggested. Further
clinical studies to evaluate such a role may be
warranted.

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