A NEW IN-VITRO MODEL TO INVESTIGATE ANTIBIOTIC PENETRATION OF THE INTERVERTEBRAL DISC

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We have studied the ability of a range of antibiotics to penetrate intervertebral disc tissue in vitro, using a mouse disc model. Equilibrium concentrations of antibiotics incorporated into the entire disc were determined by bioassay using a microbial growth-inhibition method.

Uptake was significantly higher with positively-charged aminoglycosides compared with negatively-charged penicillins and cephalosporins. Uncharged ciprofloxacin showed an intermediate degree of uptake.

Our results support the hypothesis that electrostatic interaction between charged antibiotics and negatively-charged glycosaminoglycans in the disc is an important factor in antibiotic penetration, and may explain their differential uptake.

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Infection of the disc space after surgery is a disabling illness with a high morbidity. It occurs in 0.75% to 4% of patients (Thibodeau 1968; Pilgaard 1969; El-Gindi et al 1976; Lindholm and Pylkkänen 1982; Staniforth et al 1984; Fernand and Lee 1986). Antibiotics have been shown experimentally to prevent infection (Fraser, Osti and Vernon-Roberts 1989) and are now used prophylactically in all branches of spinal surgery. There is still debate, however,

as to which antibiotics are capable of penetrating the disc to establish an effective inhibitory or therapeutic concentration (Gibson et al 1987).

The adult intervertebral disc is the largest avascular structure in the body. Nutrients and cellular metabolites enter and leave by way of passive diffusion through the adjacent bone, cartilaginous end-plates and the surrounding annulus fibrosus (Urban, Holm and Lipson 1990; Wallace et al 1994). Systemic antibiotics must also diffuse into the disc by this route.

The permeability of the disc to solutes is dependent on the sulphated glycosaminoglycan side-chains which form the aggregated proteoglycan matrix in both the annulus fibrosus and nucleus pulposus. These proteoglycans provide a high density of fixed negative charge in the matrix and also determine the matrix pore size (Urban and Maroudas 1979). Electrostatic interaction between the negatively-charged matrix and the charged solutes determines their penetration and diffusion. Thus, small neutral solutes, such as urea, have a partition coefficient of 1.0, while small positively-charged solutes, like the mono- or divalent cations Na^+ , Ca^{2+} and Mg^{2+} have partition coefficients >1.0 because they are concentrated within the disc. By contrast, negatively-charged solutes such as SO_4^{2-} have partition coefficients significantly <1.0 owing to their partial exclusion from the cartilage matrix (Maroudas 1976).

Urban et al (1990) found that negatively-charged sulphate was excluded from the nucleus pulposus while positive and uncharged solutes, such as calcium and glucose, were readily able to enter.

Substance diffusion into cartilage also depends on molecular weight (MW) and matrix pore size. Penetration decreases with increasing molecular weight. Glucose (MW 180) has a partition coefficient of 0.75, whereas albumin (MW 67 000) has a much lower penetration of cartilage and a partition coefficient of about 0.001 (Holm 1990). This may have important clinical implications for antibiotics with a high plasma-protein-binding capacity.

Our aim was to test the hypothesis that the charge on antibiotics due to their ionisable groups is important in determining their ability to diffuse into the disc. We wished to establish an in-vitro model which would allow study of the diffusion properties of a variety of commonly used antibiotics, and to measure the effect of antibiotic charge on diffusion into the disc for a range of potentially prophylactic antibiotics. We chose to use a mouse intervertebral

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disc model as this was already available in our laboratory (Brown and Batten 1994).

MATERIALS AND METHODS

Intervertebral discs were carefully excised from the lumbar region of six-week-old male BALB/C mice (mean weight 22 g). Through a dorsal incision, the lower thoracic and lumbar region was excised *en bloc* and the soft tissue, bony dorsal elements and corda equina removed under direct vision. Using magnification the vertebral end-plate was then carefully dissected off the vertebral body and excess bone removed, leaving the intact cartilaginous end-plate and intervertebral disc. Standard intravenous preparations of amoxycillin (Amoxil; Bencard, Brentford, UK), Augmentin (Beecham Research, Welwyn Garden City, UK), benzylpenicillin (Crystapen; Britannia Pharmaceuticals, Redhill, UK), ciprofloxacin (Ciproxin; Bayer, Newbury, UK), gentamicin (Cidomycin; Roussel, Uxbridge, UK) and tobramycin (Nebcin; Eli Lilly, Basingstoke, UK) were diluted in phosphate-buffered saline of pH 7.4.

Discs (nine replicates) were equilibrated overnight (16 hours) at 37°C in multiwell plates. Each well contained 1.0 ml of the antibiotic solution at a concentration of

100 µm/ml, which approaches the human peak plasma concentration for penicillins and cephalosporins used prophylactically (Bergan and Oydvin 1974; Staniforth et al 1984; Lang et al 1994).

After incubation overnight the pH of the solution was checked and each disc removed from the solution, blotted dry and washed free of any antibiotic on the surface by vortexing for 15 sec in distilled water. The disc was again blotted dry, weighed accurately to 0.1 mg and homogenised manually in 0.5 ml of sterile water using a glass homogeniser.

A bacterial growth-inhibition bioassay was used to measure the concentration of antibiotic in the homogenised solution. A standard curve was established for each antibiotic by plotting the diameter (mm) of the zone of inhibition (y-axis) against the antibiotic concentration (log g/ml) (x-axis).

The organisms used for assay and their sensitivity to each antibiotic tested are shown in Table I.

Zones of inhibition from the disc homogenates were used to calculate their antibiotic concentration by reference to the standard curve. The antibiotic solution remaining in the multiwell plates was also assayed to ensure that there was no loss of activity during overnight incubation.

RESULTS

The pH of the antibiotic solution after overnight incubation was 7.3.

Table II shows the incorporation of the antibiotics into the disc. Penetration of the disc of the positively-charged aminoglycosides, tobramycin and gentamicin, was significantly ($p < 0.001$) higher than that of the uncharged quinolone, ciprofloxacin. The negatively-charged betalactam antibiotics benzylpenicillin and amoxycillin, showed significantly poorer penetration ($p < 0.001$).

The negatively-charged cephalosporins, cefuroxime and ceftriaxone, showed no measurable penetration of the disc.

Table I. Details of the microbial inhibition bioassay

Antibiotic	Indicator organism	Range of working standards (mg/l)
Amoxycillin	<i>Sarcina lutea</i>	0.006 to 1.0
Benzylpenicillin	<i>Sarcina lutea</i>	0.006 to 1.0
Ceftriaxone	<i>Escherichia coli</i>	1.5 to 250
Cefuroxime	<i>Escherichia coli</i>	0.6 to 10
Ciprofloxacin	<i>Escherichia coli</i>	0.05 to 10
Clavulanic acid	<i>Klebsiella aerogenes</i>	0.3 to 5.0
Gentamicin	<i>Klebsiella edwardsii</i>	1.0 to 16
Tobramycin	<i>Klebsiella edwardsii</i>	1.0 to 16

Table II. Disc uptake of antibiotics (µm/mg wet weight of disc; mean ± SD of nine replicates)

Antibiotic	Uptake	Molecular charge	MW*
Aminoglycosides			
Tobramycin	32.71 ± 5.31	Positive	1425
Gentamicin	8.57 ± 2.03	Positive	
Quinolone			
Ciprofloxacin	0.58 ± 0.20	Neutral (pH 7.4)	421
Penicillins			
Benzylpenicillin	0.062 ± 0.0007	Negative	373
Amoxycillin	0.036 ± 0.014	Negative	387
Amoxycillin (as Augmentin)	0.042 ± 0.008	Negative	387
Cephalosporins			
Cefuroxime	Not detected	Negative	446
Ceftriaxone	Small zone below standard	Negative	662
Betalactamase inhibitors			
Clavulanic acid	Not detected	Negative	237

* molecular weight

Table III. Previous publications of comparable data

Authors	Antibiotic	Penetration of nucleus pulposus
Aminoglycosides		
Eismont et al (1987)	Tobramycin	Yes
Currier et al (1994)	Gentamicin	Yes
	Quinolone	
	Ciprofloxacin	No published data
Penicillins		
Eismont et al (1987)	Oxacillin	No
Gibson et al (1987)	Flucloxacillin	No
Riley et al (1994)	Benzylpenicillin	No
	Amoxycillin	No published data
Cephalosporins		
	Cefuroxime	No published data
Lang et al (1994)	Ceftriaxone	Yes
Gibson et al (1987)	Cephadrine	No
Eismont et al (1987)	Cephalothin	No
Fraser et al (1989)	Cephazolin	No

Small inhibitory zones were seen with ceftriaxone, but these were below the limits of detection for the assay. The betalactamase inhibitor component of Augmentin, clavulanic acid, also failed to penetrate the disc.

DISCUSSION

We describe an in vitro mouse model using an intact intervertebral disc for the study of antibiotic penetration. Previous studies have been carried out in vivo using animal models (Eismont et al 1987; Fraser et al 1989; Scuderi et al 1993; Currier, Banovac and Eismont 1994) and patients (Gibson et al 1987; Lang et al 1994). Although useful in assessing the potential prophylactic value of an antibiotic they introduce other pharmacokinetic variables which are independent of the physicochemical properties of the drug. This makes it difficult to identify and evaluate the factors which actually determine the ability of the antibiotic to penetrate the disc.

Recently, Riley et al (1994) described the penetration of the intervertebral disc by gentamicin and penicillin in a rabbit model and concluded that the charge effect influenced their penetration and distribution.

In vitro studies allow us to identify individual factors responsible for antibiotic diffusion into the disc. Knowledge of these may be important in the selection of an antibiotic for effective prophylactic use. With our model more than 30 discs can be easily harvested from mice in one session allowing several antibiotics to be investigated together.

The markedly greater degree of penetration of disc tissue by the positively-charged aminoglycoside antibiotics, compared with the negatively-charged penicillins and cephalosporins, supports the hypothesis that the charge due to ionisable groups on the antibiotic molecule is an important factor in determining the ability to diffuse into the intervertebral disc. We equilibrated the discs with antibiotic solution at peak plasma concentration, and our results

probably represent the upper limit for uptake. Equilibration rates are relatively slow in human discs and if plasma clearance times are a significant proportion of the equilibration time then uptake into the disc will be reduced. Significant plasma-protein binding, as seen with some antibiotics, will also inhibit uptake.

Our results are consistent with previous studies carried out in vivo (Table III). Aminoglycosides have been found to penetrate the nucleus in rabbits (Eismont et al 1987; Currier et al 1994; Riley et al 1994), whereas the first-generation cephalosporins, cephadrine and cephazolin, showed no evidence of penetration in patients (Gibson et al 1987) or in a sheep model (Fraser et al 1989).

The latter group found that in four out of five discs cephazolin was present only in the annulus fibrosus and suggested that antibiotic penetration of the normal intervertebral disc occurred through the outer annulus fibrosus and not by the central end-plates. Because of the small size of the mouse disc we were unable to differentiate between the nucleus and annulus, but this explanation may account for the accumulation of the positively-charged aminoglycosides within the nucleus pulposus at steady state, as occurs with other cations such as calcium (Urban 1990), with the negatively-charged betalactams penetrating only the annulus fibrosis. Ciprofloxacin has positively-charged ionisable groups at a lower pH such as occurs within the anaerobic disc (Holm 1990).

High-molecular-weight proteins such as albumin have a low partition coefficient. Some of the cephalosporin and penicillin groups have a high plasma-protein-binding capacity (60% to 85%) compared with gentamicin (20% to 30%) and this will inhibit uptake into the disc.

The newer, third-generation ceftriaxone has been shown, using gas-liquid chromatography, to penetrate the human disc (Lang et al 1994). Although we did find small zones of inhibition in the discs equilibrated with ceftriaxone, these were too low to quantitate by the less sensitive bioassay technique.

We have shown that the mouse disc model demonstrates comparable antibiotic penetration of intervertebral discs as in previous studies in vivo. There is a significant and marked increase in penetration with the positively-charged aminoglycosides, gentamicin and tobramycin, compared with penicillins and cephalosporins, suggesting that antibiotic charge is the principal factor influencing uptake into the disc. The aminoglycosides may be a better choice of antibiotic for prophylactic use in spinal surgery.

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