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SPECIFIC INACTIVATION OF ANTIMICROBIAL AGENTS AND ITS INTERINDIVIDUAL DIFFERENCES

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INTRODUCTION

ß-Lactamases are enzymes of bacterial origin which hydrolyse the C-N bond in the ß-lactam ring of a penicillin or a cephalosporin. The effect of these enzymes was first observed by *Abraham* and *Chain* (1940), immediately after the first report on the clinical use of benzylpenicillin (*Chain* et al., 1940). For an adequate antibiotic therapy it is important to know the resistance of a bacterial species against ß-lactam antibiotics. Similarly, the resistance of the bacterial flora of an individual may be of importance in this respect. At present many ß-lactamases from a large number of different strains of bacteria have been purified and characterized (*Bush*, 1989). The gastrointestinal tract is the largest source of bacteria in humans. Some 400 distinct species of bacteria can normally be isolated from the faeces (*Holdeman* et al.,1976). Some of these bacteria may produce ß-lactamases and release them into the intestinal contents, thereby interfering with the activity of antibiotics that reach the lower intestinal tract. In the present study, the effect of faecal enzyme preparations on 4 antibiotics was studied i.e. benzylpenicillin (penicillin G), cefotaxime, aztreonam and carumonam.

Benzylpenicillin, the first discovered penicillin, is effective *in vitro* against Gram-positive cocci and some Gram-negative bacteria. It is also active against a number of anaerobic microorganisms.

Cefotaxime is a third-generation cephalosporin with a high intrinsic activity and a broad spectrum (*Heymes* et al., 1977; *Drasar* et al., 1978; *Neu* et al., 1979). Its spectrum of activity includes, in addition to *Haemophilus influenzae* and the Enterobacteriaceae, some *Pseudomonas aeruginosa* and *Bacteroides fragilis* strains.

Aztreonam (SQ 26,776) is a synthetic monobactam that is minimally (<1%) absorbed after oral administration (*Swabb* et al., 1983). Its poor intestinal absorption and the selective activity against Gram-negative bacilli (*Sykes* et al., 1982) render this drug particularly suitable for selective decontamination (*de Vries-Hospers* et al., 1984) of immunocompromised patients.

Carumonam (Ro 17-2301; AMA-1080) is also a synthetic monobactam. *In vitro* it has been shown to be active against many, predominantly Gram-negative, aerobic rods, primarily members of the families Enterobacteriaceae, Neisseriaceae; *Haemophilus* spp., and *Pseudomonas aeruginosa.* It is not active or only weakly active against Gram-positive and anaerobic bacteria. It originates from sulfazecin, an N-sulfonated monocyclic ß-lactam antibiotic first discovered in the culture broth of *Pseudomonas acidophila* sp. nov. (*Imada* et al., 1985). Modification of this compound resulted in carumonam, an antibiotic with a high antibacterial activity (*Kishimoto* et al., 1983).

These 4 antibiotics were incubated with faecal enzyme preparations from

12 healthy human volunteers and the remaining amount of antibiotic was quantitated by reversed-phase high-performance liquid chromatography (HPLC) .

MATERIALS AND METHODS

Faecal samples

Faecal samples were collected from 12 healthy adult volunteers (5 females, 7 males). During a period of 24 months samples were collected at months 0, 6, 14 and 24. The volunteers did not receive antimicrobial drugs, at least not two weeks prior to the collection of the faecal samples. From volunteer 9, faecal samples were also collected during a shorter period of time (2 weeks). Faecal samples were stored at -20°C.

Faecal enzyme preparations

Faecal enzyme preparations were prepared as described by *Welling* et al. (1987). 0.5 g of faeces was suspended and homogenized in 1.5 ml of demineralized water containing 0.1% (w/v) Triton X-100. The suspensions were centrifuged for 10 min (9000 x g). The supernatants were centrifuged for 60 min at 100,000 x g (50Ti rotor, Beckman L5-65 ultracentrifuge). The supernatants were dialyzed against phosphate-buffered saline, pH 7.2. The retentate was used as enzyme preparation.

Antimicrobial agents

Benzylpenicillin (sodium salt) was from Gist-Brocades NV, Delft, The Netherlands. Cefotaxime (sodium salt) was from Roussel B.V., Hoevelaken, The Netherlands. Aztreonam (SQ 26,776) and its inactive open ring form (SQ 26,992) were a gift from the Squibb Institute for Medical Research, Princeton, NJ, USA. Carumonam (Ro 17-2301/010) was a gift from Hoffmann-La Roche BV, Mijdrecht, The Netherlands.

Enzymatic inactivation

An amount of 25 μl of antibiotic (1 mg/ml phosphate-buffered saline, pH 7.2 [PBS]) was incubated for 20 h at 37°C with 200 μl faecal enzyme preparation. As controls were incubated, 25 μl PBS and 200 μl enzyme preparation, to account for the background from the faecal enzyme preparation and 25 μl antibiotic solution (1 mg/ml PBS) and 200 μl PBS to account for any nonenzymatic degradation of the antibiotic. The incubation was terminated by putting the samples on ice. Aliquots of 50 μl of the incubation mixture were analyzed in duplicate by HPLC. Peak heights were proportional to concentration and the percentage inactivation was calculated by considering the peak height obtained after HPLC of the incubation mixture with antibiotic and without faecal enzyme preparation as 0% enzymatic inactivation.

High-performance liquid chromatography

The chromatography system consisted of a Waters M 6000A pump, a Rheodyne 7125 injector and a Pye-Unicam LC-UV detector. The reversed-phase (C18) column used for all HPLC assays was a Nucleosil 10 C-18 column (250 x 4.6 mm) from Nacherey-Nagel, Düren, Germany) equipped with a guard column containing the same material.

Elution conditions were as follows: the column was eluted at a flow-rate of 1.5 ml/min for benzylpenicillin with 15 mM sodium phosphate pH 7.2 and methanol in a ratio of 70 : 30 (v/v). The absorbance was monitored at 214 nm;

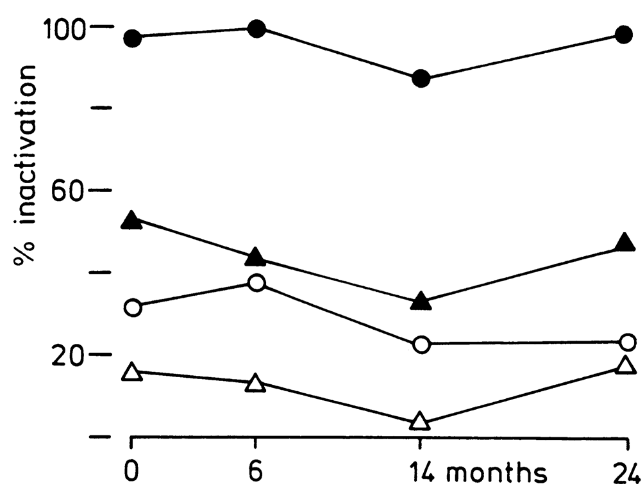

Figure 1: Percentage inactivation of cefotaxime (●), benzylpenicillin (▲), aztreonam (○), carumonam (△) after 20 h incubation at 37°C by faecal enzyme preparations from 12 healthy human volunteers at month 0, 6, 14, 24.

cefotaxime with 7 mM phosphoric acid and methanol in a ratio 60 : 40 (v/v). The absorbance was monitored at 254 nm; aztreonam and carumonam with 5 mM tetrabutylammoniumhydrogensulphate (adjusted to pH 3.0 with 1 M K_2HPO_4) and methanol in a ratio of 70 : 30 (v/v). The absorbance was monitored at 293 nm.

RESULTS

Inactivation of benzylpenicillin, cefotaxime, aztreonam and carumonam was investigated with an HPLC-assay. The principle of this assay is that the antibiotic is incubated with a faecal enzyme preparation. In addition appropriate control samples are similarly treated and the disappearance of the antibiotic can be monitored by reversed-phase HPLC. In order to obtain information on the variability of the inactivation of the antibiotics by faecal enzyme preparations, it was studied over a period of 24 months at month 0, 6, 14 and 24. The average percentage inactivation after 20 h of incubation at 37°C determined with faecal enzyme preparations from 12 volunteers, ranged from 97 to 13%. Cefotaxime was inactivated for 97% (median value 98%), benzylpenicillin for 44% (median value 30%), aztreonam for 28% (median value 19%) and carumonam for 13% (median value 11%). In Figure 1 the average values of inactivation at month 0, 6, 14 and 24 are shown. This figure also shows that in this group of volunteers the extent of inactivation of a particular antibiotic is relatively constant. This may be entirely different within an individual (see Figure 2 as an example). Cefotaxime inactivation remains at a high relatively constant level. Carumonam is hardly inactivated, but differences in inactivation from 37% (month 0) to 4% (month 14) may occur. The variability in inactivation of benzylpenicillin and aztreonam is larger. The faecal enzyme preparation of this volunteer inactivated benzylpenicillin for 100% at month 0 and 14, but

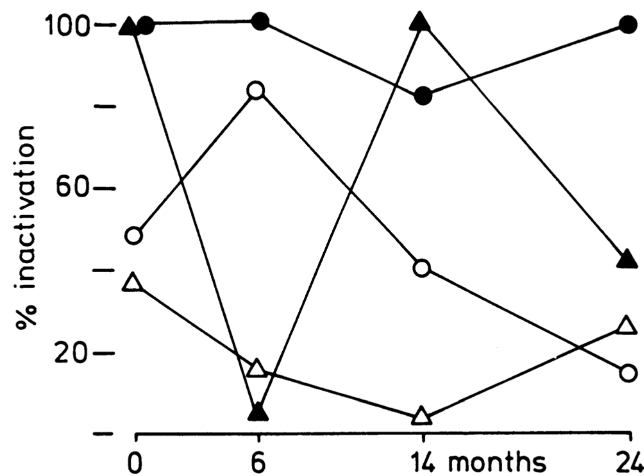

Figure 2: Percentage inactivation of cefotaxime (●), benzylpenicillin (▲), aztreonam (○), carumonam (△) after 20 h incubation at 37°C by faecal enzyme preparations from one volunteer at month 0, 6, 14, 24.

for only 5% at month 6 and at month 24, 42% inactivation was measured. Aztreonam inactivation ranged from 84% at month 6 to 15% at month 24. Similar variability in inactivation was observed with the other faecal enzyme preparations except for those which hardly inactivated a particular antibiotic at all. This prompted us to investigate this inactivation also during a shorter period of time. Faecal enzyme preparations from 3 volunteers were used to determine the inactivation of aztreonam over a period of 14 days (*Welling* and *Groen*, 1989). Figure 3 shows the inactivation of aztreonam by the faecal enzyme preparations of one volunteer. For example at day 9 we found hardly any inactivation (7%) and then a rapid increase in inactivation to 69% at day 12. The inactivation of aztreonam by faecal enzymes from 2 other volunteers showed fluctuations from 0 to 15% (day 1 and 3, respectively) and 43 to 22% (day 7 and 9, respectively). The results of the long and the short study period show that inactivation cannot be predicted.

DISCUSSION

Our first study on the effect of faecal enzymes on antibiotics was initiated by the results of *de Vries-Hospers* et al. (1984). Aztreonam was orally administered to 10 volunteers in order to eliminate selectively the potentially pathogenic Gram-negative bacteria. During this selective decontamination with aztreonam faecal counts of Gram-negative bacilli decreased in most volunteers. In the faeces of two volunteers in whom Gram-negative bacilli persisted, aztreonam was not detectable. *Ehret* et al. (1987), who conducted a similar study with 8 volunteers, found 64 to 876 mg of aztreonam/kg in the faeces of 6 volunteers, while the remaining two had low levels or no aztreonam at all in the faeces after oral administration of 300 mg/day. These results indicated that aztreonam can be inactivated by faecal material. We showed that this inactiva-

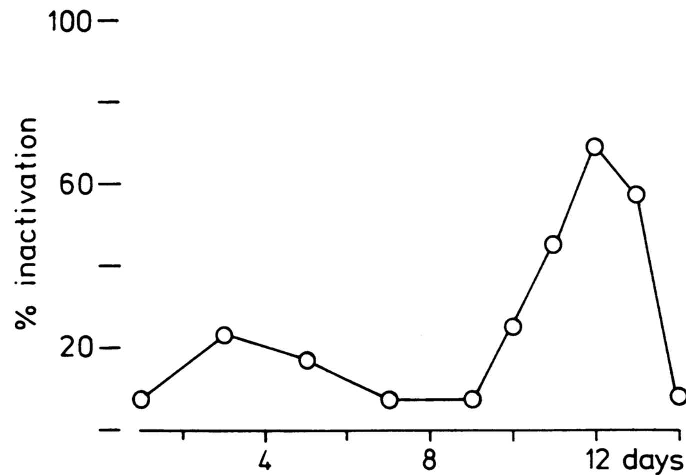

Figure 3: Percentage inactivation of aztreonam (❍) after 20 h incubation at 37°C by faecal enzyme preparations from one volunteer over 14 days.

tion is most probably due to ß-lactamase activity and that this enzyme activity could be inhibited by clavulanic acid (*Welling* et al., 1987) .

Although *Swabb* et al. (1983) regard degradation unlikely, others (*Livermore* and *Williams*, 1981; *Phillips* et al., 1981) have also reported on inactivation of aztreonam by ß-lactamases. In a next study (*Welling* and *Groen*, 1989) individual differences in inactivation of aztreonam were monitored over a longer period of time (24 months, at month 0, 6 and 24) and during 14 days. Considerable inter- and intra-individual differences were found. The conclusion from that study was that aztreonam inactivation cannot be predicted and that it may be worthwhile to determine prior and during selective decontamination with aztreonam the extent of inactivation.

This type of inactivation by faecal ß-lactamases will most likely not be limited to one ß-lactam antibiotic but probably is a widespread phenomenon. This prompted us to study the effect of faecal enzyme preparations on a number of other ß-lactam antibiotics. Surprisingly, the third generation cephalosporin cefotaxime was most rapidly inactivated by all faecal enzyme preparations. This could be due to ß-lactamases produced by the anaerobic component of the bacterial flora. The 4 antibiotics were inactivated to a different extent but when the inactivation percentages of the 12 volunteers are averaged, at a fairly constant level at 0, 6, 14 and 24 months (see Figure 1). This suggests a stability of the bacterial flora which is only meaningful when this group of volunteers is considered as a population. Examination of the inactivation of each antibiotic by individual enzyme preparations at month 0, 6, 14 and 24 provides an entirely different picture (see Figure 2). For example, benzylpenicillin may be inactivated for 100% at one particular sampling time (month 0) but not at all at another sampling time (month 6). The same is true for shorter intervals (Figure 3). At one particular day aztreonam was hardly inactivated (day 9) and a few days later (day 12) it was inactivated for 69% by a faecal enzyme preparation of the same subject.

Examination of the individual inactivation data also reveals that when carumonam is inactivated for more than 20% by a particular faecal enzyme prepara-

tion, penicillin is also inactivated (for 20% or more) by the same enzyme preparation from 8 out of 8 volunteers and aztreonam (for 20% or more) by those from 7 out of 8 volunteers. This type of inactivation could be due to different enzymes or to one enzyme with different substrate affinities since the three antibiotics generally were not inactivated to the same degree. Although this pattern of inactivation was most frequently found, we also observed 84% inactivation of aztreonam while benzylpenicillin was hardly inactivated (5%).

Several authors have reported on penicillin and cephalosporin resistance in *Bacteroides* sp. (*Anderson* and *Sykes*, 1973; *Britz* and *Wilkinson*, 1978; *Del Bene* and *Farrar*, 1973; *Garrod*, 1955; *Olsson* et al., 1976; *Pinkus* et al., 1968; *Richmond* and *Sykes*, 1973). Quantitatively, the *Bacteroides fragilis*-group is predominant in the human faecal flora (*Meijer-Severs* and *van Santen*, 1986). They may represent therefore together with other anaerobic species in the intestine, a large potential source of antibiotic-inactivating enzymes. *Meijer-Severs* and *van Santen* (1986) found considerable interindividual differences in *Bacteroides* cultural counts (between 8.83 and 10.24) although the total anaerobic cultural counts showed only one log difference. According to *Simon* and *Gorbach* (1984), the composition of the bacterial flora is relatively stable in single subjects over longer periods of time, while the metabolic activity measured by determination of bacterial enzymatic activity may show marked changes. We have found that the enzymatic activity may be considerably different inter- and intra-individually and that it may change with time. This could be due to dietary modulations of the composition of the bacterial flora. This variable bacterial population in the colon may produce a number of different ß-lactamases or, depending on the composition of the flora, variations in the concentration of one type of enzyme.

The practical consequences of these findings are different for different antibiotics. When the antibiotic is intended for selective decontamination of the intestinal tract (aztreonam and carumonam) it may be worthwhile to know to which extent it will presumably be inactivated by the faecal enzymes prior and during selective decontamination. When the antibiotic has a broad spectrum and is not intended for selective decontamination (benzylpenicillin, cefotaxime) a fraction of the antibiotic may reach the lower intestinal tract through the biliary canal after parenteral administration (benzylpenicillin, cefotaxime). It is epidemiologically important when the antibiotic is then inactivated by bacterial enzymes. Then disturbance of the bacterial flora is prevented and therewith a barrier is maintained against colonization (colonization resistance, *van der Waaij*, 1982) by potentially pathogenic microorganisms from the environment.

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