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Toxicity and gut associated lymphoid tissue translocation of polymyxin B orally administered by alginate/chitosan microparticles in rats

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Abstract

Fluorescent calcium alginate/chitosan microparticles, prepared using a spray-drying technique followed by crosslinking reactions with calcium ions and chitosan, were assayed in-vivo for polymyxin B (PMB) oral toxicity, uptake by Peyer's patches and PMB oral absorption. A single PMB dose (300 mg kg^{-1}), loaded in microparticles or dissolved in water, was administered to rats by oral gavage under fasted and fed conditions. By monitoring incidence of mortality, animal behaviour, clinical signs and abnormality in several organs, PMB in water solution was found lethal at a dose lower than the LD50 (790 mg kg^{-1}) in the fasted state and toxic for the gastrointestinal tract in the fed state. However, no signs of acute toxicity at the level of the gastrointestinal tract were observed when animals were administered PMB loaded in microparticles under fasted and fed conditions. A lower PMB dose (125 mg kg^{-1}), loaded in microparticles or dissolved in water, was given to rats in a fed state to determine PMB levels in Peyer's patches, urine and serum as well as to detect the loaded microparticles inside Peyer's patches for three days after dosing. Abnormalities were observed at gut level only when PMB was dosed in a water solution. Detectable antibiotic levels in Peyer's patches and urine as well as more constant PMB serum concentrations were provided by dosing PMB loaded in microparticles. Therefore, the use of alginate/chitosan microparticles to target the lymphatic system could improve safety when administering PMB orally.

Introduction

Polymyxin B (PMB) is a cationic peptidic antibiotic used extensively for parenteral and topical treatment of Gram-negative infections, especially *Pseudomonas aeruginosa* (minimum inhibitory concentration (MIC) = 0.25–2.0 μg mL^{-1} (Jones et al 2005)). PMB has received renewed interest for the treatment of infections that are resistant to other antimicrobial agents. Multiresistant Gram-negative nosocomial organisms, particularly *P. aeruginosa* and *Acinetobacter* spp., have been monitored as having susceptibility to PMB (Gales et al 2001). However, PMB therapy is associated with considerable toxicity, mainly nephrotoxicity and neurotoxicity, even if recent studies have shown that the incidence of toxicity is less common and severe than previously reported.

Several attempts to generate less toxic derivatives have been made, leading to compounds having reduced antibacterial effect (Falagas & Kasiakou 2006). However, the oral route for PMB has not been considered owing to the pH value and proteolytic degradation in the gastrointestinal tract, as well as the negligible absorption through the intestinal epithelium, probably owing to its cationic charge.

Therefore, to achieve a PMB oral administration perspective, providing less toxicity as well as better patient compliance, calcium alginate/chitosan (CaA/CHT) microparticles were designed to target the gut-associated lymphoid tissue (GALT) by Peyer's patch uptake. Peyer's patch M cells, which are specialised cells staying over mucosa-associated lymphoid tissue (MALT), interspersed by enterocytes in the follicle-associated epithelium (FAE), can transport via endocytosis a variety of microparticulate matter, macromolecules and microorganisms (bacteria, viruses, protozoa) from the gut lumen to intra-epithelial lymphoid cells, and subsequently through the lymphatic system into the blood stream

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(Hussain et al 2001; Neutra & Kozlowski 2006). Therefore, drug delivery to the GALT by oral administration of microparticles could allow the absorption of drugs with poor permeability characteristics, protecting labile drugs from the gastrointestinal environment and avoiding the enzymatic degradation from enterocytes as well as first-pass metabolism. The developed CaA/CHT microparticles were evaluated in-vitro and ex-vivo by Coppi et al (2004, 2006). They showed a proper size for Peyer's patch uptake and lymphatic translocation, a suitable drug loading level, integrity and drug retaining ability in simulated gastrointestinal fluids, PMB microbiological activity preservation, and evidence of uptake by M cells in a rat model.

This study was undertaken to verify in-vivo PMB oral toxicity and absorption, as well as microparticle uptake by Peyer's patches, following a single oral dose of PMB loaded in fluorescent CaA/CHT microparticles to rats. The results of the experiments were analysed in comparison with those provided by the administration of PMB water solutions.

Materials and Methods

Materials

Polymyxin B sulfate (PMB) and chitosan (CHT) (low MW ≈ 70 000) were purchased from Fluka Chemie (Buchs, Switzerland). Sodium alginate (NaA) (MW ≈ 147 000, containing 62% mannuronic acid and 38% guluronic acid) was donated by Kelco International (Bagnolex Cedex, France). Fluorescein isothiocyanate (FITC) was from Sigma-Aldrich (Milan, Italy). All the other chemicals were of analytical grade. For the microbiological assay, tryptic-soy broth (TSB, Difco Laboratories, Detroit, MI) and *Escherichia coli* ATCC 25922 (10^5 CFU mL^{-1}) (polymyxin B MIC = 0.25–2.0 μg mL^{-1} (Jones et al 2005)), as a standard strain from American Type Cultures Collection (Manassas, VA), were used.

Microparticle preparation and in-vitro characterization

Microparticle preparation and in-vitro characterization were as described by Coppi et al (2006). Briefly, fluorescent calcium alginate/chitosan (CaA/CHT) microparticles were prepared by spray-drying a 0.5% water solution of NaA/PMB/FITC (3:1:0.01) and by crosslinking the microparticles with calcium chloride and chitosan. The obtained crosslinked microparticles were characterized for morphology and size, fluorescence, FITC release in simulated intestinal fluid, PMB content, PMB in-vitro release in simulated gastrointestinal fluids and microbiological activity preservation.

In-vivo study

The in-vivo study was performed on Sprague–Dawley male rats (approximately 2-months old, 200 ± 20 g) in accordance with procedures adhering to the European Community regulations controlling experiments in live animals (CEE Council 86/609). Moreover, the project was approved by the local ethical committee for the experimentation on animals. The animals were housed alone in metabolic cages with free access to water, maintained at constant room temperature (21°C), humidity (50–60%) and day length (12 h) for one week before the experiments.

PMB toxicity evaluation

To investigate the oral toxicity of PMB, single PMB doses (300, 150, 125 and 62.5 mg kg^{-1}) were administered in 2-mL water solutions by oral gavage to four groups of six rats (PMB-solution group) under fasted (for 24 h before the experiments) and fed conditions. To evaluate the effect of the microencapsulation on PMB oral toxicity, three groups of six rats were administered either a single 300 mg kg^{-1} PMB dose loaded in microparticles suspended in 2 mL water by a vortex shaker (ZX3, VELP, Usmate, Milan, Italy) (PMB-microparticle group), an unloaded microparticle water suspension (2 mL, containing the same microparticle amount dosed to the PMB-microparticle group; the alginate group), or 2 mL distilled water (the control group). The administration was performed by oral gavage to fasted and fed rats. All treated animals were monitored (lethality incidence, behaviour, clinical signs) for 24 h following dosing. The surviving animals were killed on day one by anaesthetic injection of 2 mL kg^{-1} ketamine (Ketavet, Parke Davis, Berlin, Germany, 50 mg mL^{-1}). Macroscopic abnormalities in several organs such as stomach, intestine, kidney and heart were observed morphologically by visual observation in-situ.

Microparticle uptake and PMB oral absorption

Microparticle uptake by Peyer's patches and PMB oral absorption was performed by administering in a single dose to three groups of rats, each composed of 18 rats, either PMB water solution (2 mL, containing 125 mg kg^{-1} PMB; PMB-solution group), PMB-loaded microparticles suspended in water by a vortex shaker (ZX3, VELP, Usmate, Milan, Italy) (2 mL, containing a microparticle amount corresponding to 125 mg kg^{-1} PMB; PMB-microparticle group), or 2 mL distilled water (the control group), respectively. Administration was by oral gavage to fed rats. The rats were maintained in metabolic cages to collect urine. At 24, 48 and 72 h after dosing, six animals from each group were killed by anaesthetic injection of 2 mL kg^{-1} Ketavet 50 (50 mg mL^{-1}). Blood was drawn directly from the heart and the intestines were excised. PMB levels were determined in urine and in serum from blood (by clotting of the blood sample at room temperature followed by centrifugation), by a microbiological 'agar well diffusion' method using *E. coli* as standard strain. Wells were filled with 100-μL samples and, after incubation at 37°C overnight, the size of the inhibition zones was measured and compared with standards constituted with PMB sodium citrate solutions in concentrations ranging from 0.125 to 1.00 μg/100 μL. The determinations were performed in triplicate.

PMB levels were also determined in a defined number of Peyer's patches (n = 15), corresponding to the total Peyer's patch number found in normal rats (Zhou et al 2006). These were isolated from the whole small intestine at 24 h after gavage, homogenized through a glass Potter homogenizer in 6%

sodium citrate water solution (5 mL) and analysed by microbiological assay as described above.

Moreover, Peyer's patches were isolated from the excised intestines, washed with saline, fixed in paraformaldehyde (3%, w/v) for 1 h, embedded in cryostatic medium, sliced into 12-μm thick sections at −20°C using a microtome cryostat (mod. HM 500 O, Microm International GmbH, Walldorf, Germany) and viewed by epifluorescence confocal laser scanning microscopy (mod. DM IRE2, Leica Microsystems Heidelberg GmbH) at 1.11 μm steps.

Statistical analysis

The effect of the microencapsulation on serum levels obtained at 24, 48 and 72 h after dosing was statistically analysed using the Kruskal–Wallis one-way non-parametric analysis of variance, with $P < 0.05$ taken as demonstrating significance.

Results and Discussion

Microparticle characteristics

As observed by Coppi et al (2004, 2006), the obtained CaA/CHT microparticles exhibited a good water dispersibility, a nearly spherical shape with a wrinkled surface (Figure 1) and a size ranging from 0.1 to 2.5 μm (mean size 0.78 ± 0.55), with approximately 75% of the population being less than 1 μm, which can be considered proper for lymphatic administration (Clark et al 2001). In fact, it seems that sizes less than 3 μm are preferable because particles in the range of 3–10 μm are retained in the Peyer's patches and do not migrate into the mesenteric lymph nodes. Also, too small particles can present difficulties with initiating phagocytosis (Florence 1997). Moreover, microparticles were still fluorescent following a 4-h release assay in simulated intestinal fluid, as observed by epifluorescence videomicroscopy (Figure 2). The antibiotic entrapped inside the microparticles (loading level 11.86 ± 0.70% w/w, encapsulation efficiency approximately 47%), which was found associated to the alginate chain by an electrostatic interaction and maintaining its microbiological activity, was released negligibly in simulated gastric fluid at pH 3.0 and gradually in simulated intestinal fluid. Therefore, the microparticles would be able to transport a substantial percentage of PMB to the absorption site.

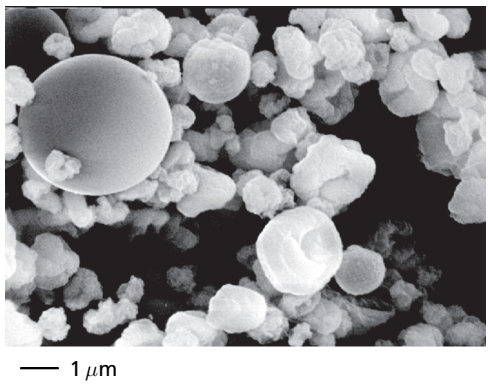

Figure 1 SEM micrograph of CaA/CHT microparticles.

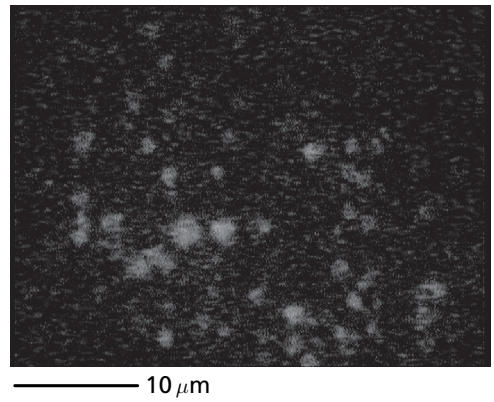

Figure 2 Epifluorescence microscopy image of CaA/CHT microparticles.

In-vivo study

The in-vivo study aimed to evaluate PMB acute oral toxicity, microparticle uptake by Peyer's patches, and PMB oral absorption from microparticles in comparison with PMB dosed in water solution.

Microparticles were given to rats in water suspensions which were prepared immediately before oral administration. The water medium did not provide microparticle structure modifications or extraction of the antibiotic associated to alginate by an electrostatic interaction, as demonstrated by Coppi et al (2004, 2006).

Toxicity evaluation

To exclude the possible side effects provided by the administration procedure as well as by the microparticle materials, water and unloaded microparticles were given under fasted and fed conditions to the control and alginate groups, respectively. No modifications in animal behaviour were observed, and at the post-mortem examination no clinical signs or organ abnormality were detected. These findings indicated the safety of the oral gavage method and the polymer materials.

To investigate PMB acute oral toxicity and to select the proper dose to be loaded into microparticles, single doses of PMB (300, 150, 125 and 62.5 mg kg^{-1}) in 2-mL water solution were administered. These four doses were less than the dose required to kill 50% (LD50) of test mice reported in the literature (790 mg kg^{-1}) (Material safety data sheet from Bedford Laboratories and Pfizer Inc.).

All animals treated with 300 mg kg^{-1} PMB in the fasted state died within 30 min. Post-mortem examination revealed a severe intestinal haemorrhage (Figure 3C), probably the cause of death. In a fed state all animals survived, but signs of suffering, such as dyspnoea or haemorrhagic zones surrounding the eyes, were observed. At 24 h after dosing, the animals were killed and stomachs larger than those from the control and alginate groups (Figure 4A, B and C), reasonably providing

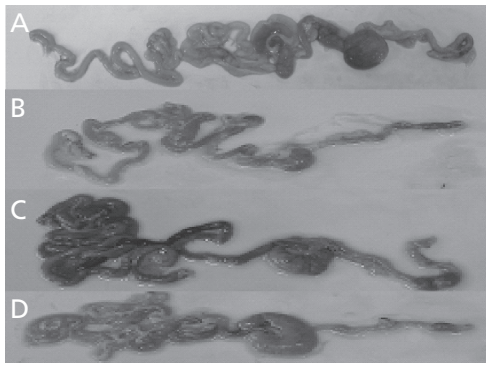

Figure 3 Intestines excised from the control group (A), the alginate group (B), PMB-solution group (C) and PMB-microparticle group (D) treated in the fasted condition.

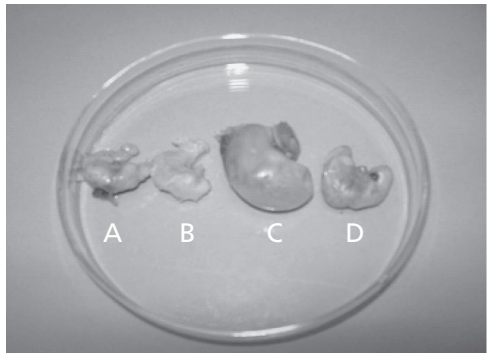

Figure 4 Stomachs excised from the control group (A), the alginate group (B), PMB-solution group (C) and PMB-microparticle group (D) treated in the fed condition.

symptoms of dyspnoea, were noticed. After dosing 150 or 125 mg kg^{-1} PMB in the fasted state, half of the animals died within 30 min. At day one after dosing, intestinal haemorrhage and enlarged stomachs were observed in the dead and surviving animals. In the fed condition, all animals survived but with evident symptoms of suffering. PMB 62.5 mg kg^{-1} in the fasted and fed state did not prove to be lethal nor were there clinical signs of suffering in any of the animals. This indicated that PMB oral administration was safe for rats at a dose lower than 100 mg kg^{-1}, and it was lethal at a significantly lower dose than the known LD50 for mice (Material safety data sheet from Bedford Laboratories and Pfizer Inc.), the amount causing the death of 50% of the rats being approximately 125–150 mg kg^{-1}.

To evaluate the effect of microencapsulation on PMB acute oral toxicity, a PMB toxic dose was selected for the microparticle administration. Thus, water suspensions containing microparticle amounts corresponding to a dose of 300 mg kg^{-1} PMB were given to rats (PMB-microparticle group) under fasted and fed conditions.

Contrary to the results obtained when dosing PMB in a water solution (PMB-solution group), all animals of the PMB-microparticle group survived, without appreciable signs of acute toxicity. When the organs were examined only a slightly enlarged caecum was detected, compared with those from the control and alginate groups (Figure 3D). The stomach appeared to be normal (Figure 4D). Therefore, microparticle administration significantly reduced the PMB acute toxicity at the level of the gastrointestinal tract, providing protection for the stomach and intestine.

Microparticle uptake and PMB oral absorption

To investigate microparticle uptake and PMB oral absorption, PMB was administered to feeding rats by dosing in a water solution (PMB-solution group) and in loaded microparticles (PMB-microparticle group) at a 125-mg kg^{-1} dose. The animals survived for at least three days, compared with rats dosed with water (control group). At 24, 48 and 72 h after gavage, six animals from each group were killed to detect any organ abnormality, microparticle uptake by Peyer's patches, and PMB levels in serum and urine. The transcytotic capacity of M cells was investigated by isolating the rat Peyer's patches and viewing Peyer's patch vertical sections through confocal fluorescent microscopy (Figure 5). Oval-shaped Peyer's patches were observed with the follicle-associated epithelium (FAE) exposed to the external environment of the gut lumen.

Unlike Peyer's patches from the control and PMB-solution groups, which did not provide any fluorescence, a marked dotty fluorescence inside the Peyer's patches along the FAE and in the germinative dome was observed from the PMB-microparticle group at 24, 48 and 72 h after dosing. This could be evidence of microparticle transport across M cells and their translocation into the subepithelial region. Moreover, clusters of microparticles were observed in the follicle germinal centre indicating their possible accumulation in macrophages (Ermak & Giannasca 1998).

To investigate the actual PMB uptake by Peyer's patches and GALT translocation following microparticle uptake, PMB levels were detected by microbiological assay in serum and in urine at 24, 48 and 72 h after gavage. PMB serum levels obtained from the PMB-solution and PMB-microparticle groups are listed in Table 1.

Although PMB is known to be an oral non-absorbable antibiotic, a serum level peak of approximately 0.2 μg mL^{-1} was found 48 h after gavage in the PMB-solution group, suggesting a poor and slow absorption process via the oral route. Following PMB microparticle administration, PMB serum

Table 1 Polymyxin B serum levels at 24, 48 and 72 h after administration of PMB (125 mg kg^{-1}) in a water solution (PMB-solution group) and loaded in microparticles (PMB-microparticle group) to rats

Rat group	PMB serum level (μg mL^{-1})
PMB-solution group at 24 h	0.066 ± 0.006
PMB-solution group at 48 h	0.199 ± 0.049
PMB-solution group at 72 h	0.047 ± 0.005
PMB-microparticle group at 24 h	0.100 ± 0.005
PMB-microparticle group at 48 h	0.083 ± 0.003
PMB-microparticle group at 72 h	0.084 ± 0.004

Data represent mean ± s.d. ($P < 0.05$).

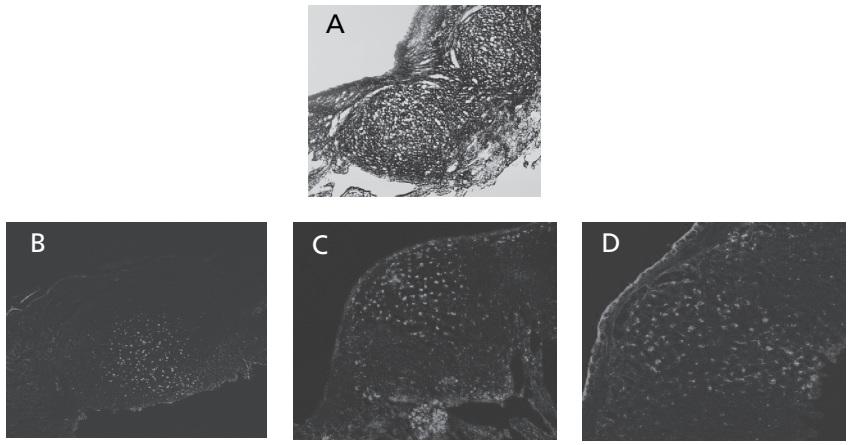

Figure 5 Confocal scanning laser microscopy images of Peyer's patches from the PMB-microparticle group: transmission image (A), epifluorescence images at 24 h (B), 48 h (C) and 72 h (D) after dosing.

levels of 0.08–0.1 μg mL^{-1} were provided at 24, 48 and 72 h. Therefore, serum levels of the antibiotic would be maintained over an extended period compared with those obtained by the PMB-solution group, the differences in the serum levels being significant as demonstrated by the Kruskal–Wallis test. Although antibiotic serum levels above the antibacterial activity in-vitro (MIC = 0.25–2 μg mL^{-1} (Jones et al 2005)) were not produced by dosing PMB loaded in microparticles, it is known that significant antibacterial effects can be obtained by sub-MIC concentrations (Rolinson 1977). Furthermore, peak levels in the blood below the in-vitro MIC were found in mice following administration of a median protective dose (Merrikin & Rolinson 1979). In fact, polymyxins have been noted to lose approximately 50% of their activity in the presence of serum, which may be caused by protein binding (Hermsen et al 2003), even if contemporary studies about PMB pharmacokinetics are lacking. Therefore, free PMB serum concentration levels could not provide a clear evaluation of absorption.

Although PMB is almost completely excreted by the kidneys, only trace amounts were found in the urine of the rats at 48 and 72 h after dosing of the antibiotic in both the water solution and loaded in microparticles. This finding could be attributed to an extensive tubular reabsorption according to the behaviour of colistin (polymyxin E) observed in rats (Hermsen et al 2003). Unlike the Peyer's patches from the control and PMB-solution groups, the Peyer's patches recovered at 24 h from the whole small intestines of the PMB-microparticle group revealed a microbiological activity, corresponding to approximately 1 μg PMB. Since PMB was not detected at 24 h after gavage in Peyer's patches isolated from the PMB-solution group, the absorption process of the antibiotic dosed in a water solution involved, presumably, a non-lymphoid mechanism across non-FAE enterocytes, whereas PMB loaded in microparticles was absorbed by a lymphoid transport across M cell FAE. By considering that the PMB level in Peyer's patches was higher than the PMB serum level and that the microparticles remained entrapped inside the Peyer's patches for at least three days, as shown by fluorescence confocal microscopy, microparticles could act as a 'reservoir' providing a PMB maintenance dose in the blood stream.

Conclusions

A single oral dose of calcium alginate/chitosan microparticles as a carrier for PMB to the lymphatic system demonstrated the suitability of the microparticles to protect the antibiotic in the gastrointestinal tract, decreasing its toxic effects. The toxicity of PMB, partly attributed to its diaminobutyric acid component (Falagas & Kasiakou 2006), could have been reduced also by the electrostatic interaction occurring between the amino group and the carboxylic group of alginate. The microparticle permanence inside the intestinal Peyer's patches provided prolonged antibiotic serum levels compared with the drug dosed in a water solution, leading to a reduction in dose frequency. Therefore, this microparticulate carrier, properly administered by capsules, could offer clear advantages in terms of reduced PMB toxicity and prolonged systemic antibacterial effects.

References

Clark, M. A., Jepson, M. A., Hirst, B. H. (2001) Exploiting M cells for drug and vaccine delivery. *Adv. Drug Dev. Rev*. **50**: 81–106

Coppi, G., Iannuccelli, V., Sala, N., Bondi, M. (2004) Alginate microparticles for Polymyxin B Peyer's patches uptake: microparticles for antibiotic oral administration. *J. Microencapsulation* **21**: 829–839

Coppi, G., Sala, N., Bondi, M., Sergi, S., Iannuccelli, V. (2006) *Ex-vivo* evaluation of alginate microparticles for Polymyxin B oral administration. *J. Drug Target*. **14**: 599–606

Ermak, T. H., Giannasca, P. J. (1998) Microparticle targeting to M cells. *Adv. Drug Deliv. Rev*. **34**: 261–283

Falagas, M. E., Kasiakou, S. K. (2006) Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Critical Care* **10**: R27

Florence, A. T. (1997) The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. *Pharm. Res*. **14**: 259–266

Gales, A. C., Reis, A. O., Jones, R. N. (2001) Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol*. **39**: 183–190

Hermsen, E. D., Sullivan, C. J., Rotschafer, J. C. (2003) Polymyxins: pharmacology, pharmacokinetics, pharmacodynamics, and clinical applications. *Infect. Dis. Clin. North Am*. **17**: 545–562

Hussain, N., Jaitley, V., Florence, A. T. (2001) Recent advances in the understanding of uptake microparticulates across the gastrointestinal lymphatics. *Adv. Drug Deliv. Rev*. **50**: 107–142

Jones, R. N., Anderegg, T. R., Swenson, J. M. (2005) Quality control guidelines for testing Gram-negative control strains with polymyxin B and colistin (polymyxin E) by standardized methods. *J. Clin. Microbiol*. **43**: 925–927

Merrikin, D., Rolinson, G. N. (1979) Antibiotic levels in experimentally infected mice in relation to therapeutic effect and antimicrobial activity *in vitro*. *J. Antimicrob. Chemother*. **5**: 423–429

Neutra, M. R., Kozlowski, P. A. (2006) Mucosal vaccines: the promise and the challenge. *Nature* **6**: 148–158

Rolinson, G. N. (1977) Subinhibitory concentrations of antibiotics. *J. Antimicrob. Chemother*. **3**: 111–113

Zhou, J., Xiao, C., Zhao, L., Jia, H., Zhao, N., Lu, C.,Yang, D., Tang, J. C.-O., Chan, A. S. C., Lu, A.-P. (2006) The effect of triptolide on CD4+ and CD8+ cells in Peyer's patches of SD rats with collagen induced arthritis. *Int. Immunopharmacol*. **6**: 198–203