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Plasma protein binding may reduce antimicrobial activity by preventing intra-bacterial uptake of antibiotics, for example clindamycin

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Objectives: Although plasma protein binding (PPB) is accepted to be an essential factor in reducing antimicrobial activity, little is known about the underlying mechanisms. One possibility includes impaired penetration of an antimicrobial into bacterial cells in the presence of PPB. As a prerequisite for testing this hypothesis an optimized medium displaying high protein binding without impairing bacterial growth had to be identified for our model compound clindamycin.

Methods: Determination of PPB, bacterial growth and antimicrobial killing was performed in Mueller—Hinton broth (MHB) containing various amounts of human albumin or serum. [^{3}H]clindamycin was used to investigate clindamycin penetration into *Staphylococcus aureus*.

Results: Of all investigated media only $MHB_{50\%serum}$ and $MHB_{70\%serum}$ achieved protein binding comparable to pure serum. In contrast, $MHB_{20\%serum}$ and most media containing only albumin demonstrated considerably lower protein binding. Pure serum resulted in bacterial growth inhibition compared with MHB while $MHB_{16\%albumin}$ and $MHB_{50\%serum}$ did not result in significant differences in bacterial count after 24 h. However, in both $MHB_{16\%albumin}$ and $MHB_{50\%serum}$ the antimicrobial activity of clindamycin was reduced by >2 $\log_{10}$ cfu/mL compared with pure MHB. The radioactive signal after administration of [^{3}H]clindamycin to *S. aureus* was significantly decreased in pure serum as well as in $MHB_{16\%albumin}$ and $MHB_{50\%serum}$, while no significant difference was observed for $MHB_{4\%albumin}$ and $MHB_{20\%serum}$.

Conclusions: Reduction of the intracellular radioactive signal in the presence of serum proteins correlated both with the degree of protein binding and reduction of antimicrobial activity supporting the hypothesis of impairment of activity by PPB by reducing intra-bacterial antimicrobial concentrations.

Keywords: albumin, serum, pharmacodynamics, lincosamides

Introduction

The importance of plasma protein binding (PPB) on the pharmacokinetics of antimicrobial agents is well documented, but for some antibiotics no consensus about the relevance of protein binding for their pharmacodynamic action has been reached.[1,2] This can be ascribed to two facts: first, there is no standardization of *in vitro* pharmacodynamic models investigating the impact of PPB of antimicrobials; and second, little is known about the mode of action by which PPB impacts bacterial killing.[3]

In an attempt to better understand the mechanisms by which PPB impacts antimicrobial activity, we set out to describe correlations between degree of protein binding, impairment of antimicrobial activity and penetration of the antibiotic into the bacterial cell.

Clindamycin, an antimicrobial with high protein binding and an intracellular target, was chosen as a model compound.[4] Since lincosamides are commonly used for treatment of infections caused by staphylococci, *Staphylococcus aureus* was used as a representative strain.[5]

Materials and methods

Media

Mueller–Hinton broth (MHB; Merck, Germany) was mixed with human albumin (Baxter, Austria) to 4% ($MHB_{4\%albumin}$), 8%, 12% and 16% final albumin content or with pooled human serum (Sigma–Aldrich, Germany) to 20% ($MHB_{20\%serum}$), 50% and 70% serum content.

Determination of protein binding

The protein binding of clindamycin (Sigma–Aldrich, Germany) was determined using the ultrafiltration method in the above-described media at three different concentrations of clindamycin (10, 1 and 0.1 mg/L) as previously described.[1] Each experiment was performed in triplicate. Samples were analysed in duplicate by means of mass spectrometry using a Dionex UltiMate 3000 RSLC system (Dionex Corp., USA) coupled to a Bruker HCT trap system (Bruker Daltonics Inc., USA).

In vitro susceptibility tests

MIC values for *S. aureus* (ATCC 29213) were determined by the 2-fold serial microdilution method according to CLSI criteria.[6]

Bacterial growth and time–kill curves

Bacterial growth curves were performed in pure MHB, pure serum and $MHB_{16\%albumin}$ or $MHB_{50\%serum}$ as previously described.[1] Bacterial killing curves were performed in analogy with growth curves except that antibiotic was added at a concentration equal to the MIC. Each experiment was performed in triplicate.

Chemical analysis

We analysed the composition of each medium at the routine laboratory of the General Hospital of Vienna to determine concentrations of sodium, potassium, chloride, calcium and magnesium. Total protein and albumin concentrations as well as pH before and after 24 h of incubation with bacteria were determined.

Bacterial uptake of [^{3}H]clindamycin

The uptake of radiolabelled [^{3}H]clindamycin (Hartmann-Analytic, Germany) into *S. aureus* was tested in MHB, pure serum, $MHB_{16\%albumin}$ and $MHB_{50\%serum}$ containing 10^{10} cfu/mL bacteria. After 15 min of incubation at 37°C, [^{3}H]clindamycin and unlabelled clindamycin were added to a final concentration of 50 nCi/mL and 0.5 mg/L (representing the MIC for the used strain), respectively. After incubation for 30 min, this mixture was centrifuged at 4°C and the pellet was resuspended in ice-cold sodium chloride. After this procedure was repeated, an ultima gold solution, which is a high flashpoint liquid scintillation counter cocktail (PerkinElmer, USA), was added and the radioactivity was measured in a scintillation counter (WALLAC 1410; Finland). Each experiment was performed six times.

Results

Protein binding

Protein binding was independent of the concentration of clindamycin in the range 0.1–10 mg/L and rose with increasing concentration of albumin or serum. The non-specific binding of the ultrafiltration method of MHB was 8.4%. In accordance with previously reported values, the protein binding in pure serum observed in our study was 93 ± 4%.[4] Only $MHB_{50\%serum}$ and $MHB_{70\%serum}$ achieved levels of protein binding comparable to that of pure serum with levels of 86 ± 2% and 87 ± 3%, respectively. In contrast, $MHB_{20\%serum}$ and most media containing only albumin demonstrated considerably lower protein binding. $MHB_{16\%albumin}$ demonstrated the highest level of protein binding (64 ± 4%) of the investigated media containing only albumin.

In vitro susceptibility tests

Compared with pure MHB the MIC of clindamycin for *S. aureus* ATCC 29213 (0.25 mg/L) did not change in $MHB_{4\%albumin}$ or $MHB_{20\%serum}$. In contrast, in MHB containing 8%, 12% and 16% albumin and $MHB_{50\%serum}$ the MIC was 2-fold higher and in $MHB_{70\%serum}$ the MIC was 4-fold higher. The highest increase was observed in pure serum with an MIC of 2 mg/L. However, MICs might not be sensitive enough to detect small changes, therefore results from kill curves were considered more appropriate.

Bacterial growth and time–kill curves

Pure serum resulted in considerable growth inhibition compared with MHB (Figure 1a). In contrast $MHB_{16\%albumin}$ or $MHB_{50\%serum}$ did not result in a significant difference in bacterial count after

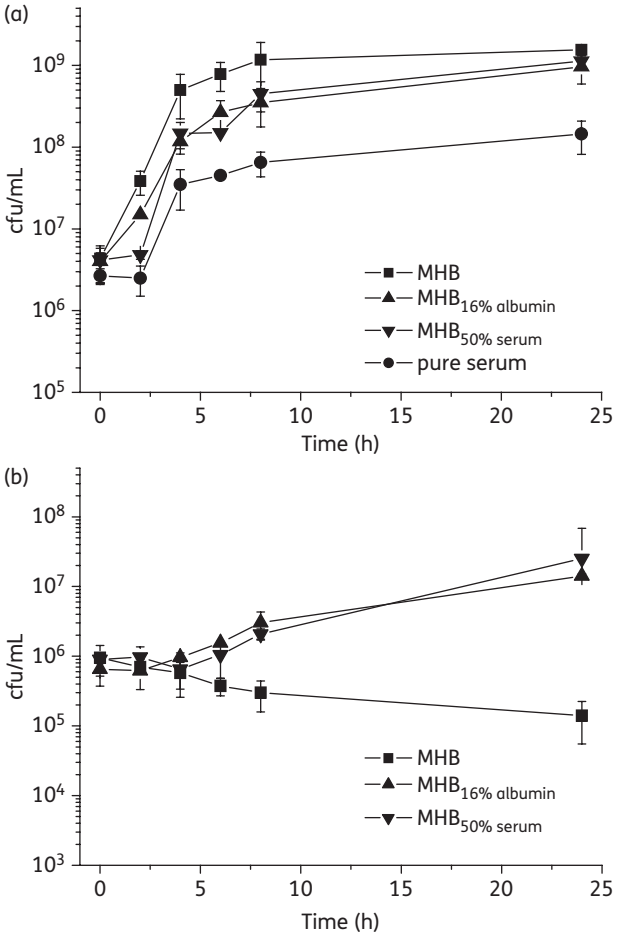

Figure 1. (a) Bacterial growth curves of *S. aureus* ATCC 29213 in pure MHB (squares), pure serum (circles), MHB containing 16% albumin (up triangles) and MHB containing 50% serum (down triangles). Each experiment was performed in triplicate; data are presented as means ± SD. (b) Bacterial time–kill profiles of clindamycin at concentrations equal to the MIC were performed in pure MHB (squares) and MHB containing 16% albumin (up triangles) or 50% serum (down triangles). Each experiment was performed in triplicate; data are presented as means ± SD.

24 h, although bacterial growth during the first hours seemed slightly decelerated. Since comparable bacterial growth is a prerequisite for investigation of the impact of protein binding on bacterial killing,[3] $MHB_{16\%albumin}$ and $MHB_{50\%serum}$ were chosen for bacterial time–kill experiments.

In the presence of albumin or serum the antimicrobial activity of clindamycin was significantly reduced and resulted in differences of $>2\ log_{10}$ cfu/mL for both protein-containing media compared with pure MHB (Figure 1b). No difference in antimicrobial activity was detectable between $MHB_{16\%albumin}$ and $MHB_{50\%serum}$.

Chemical analysis

For purchased pure serum the albumin concentration (37 g/L) was in the reference range for healthy adults (34–48 g/L) while total protein levels were slightly lower than expected (59 g/L, reference range 66–83 g/L). Measured albumin levels in MHB reflected anticipated albumin concentrations. Cation-adjusted MHB showed almost identical calcium values compared with pure serum (2.3 versus 2.4 mmol/L), while calcium concentration decreased with increasing albumin concentration. The pH in all media was 6.9 ± 0.1, with slightly lower values in media containing only albumin than in MHB containing serum.

Although after 24 h of incubation with bacteria media showed a tendency for decreased protein concentrations, this decrease did not reach the level of significance. In contrast, pH decreased significantly after 24 h for both bacterial growth and time–kill curves with values of 6.2 ± 0.4 and 6.6 ± 0.1, respectively.

Bacterial uptake of [^{3}H]clindamycin

Radioactivity was significantly reduced in pure serum as well as in $MHB_{16\%albumin}$ and in $MHB_{50\%serum}$, while no significant difference was observed for $MHB_{4\%albumin}$ and $MHB_{20\%serum}$ (Figure 2).

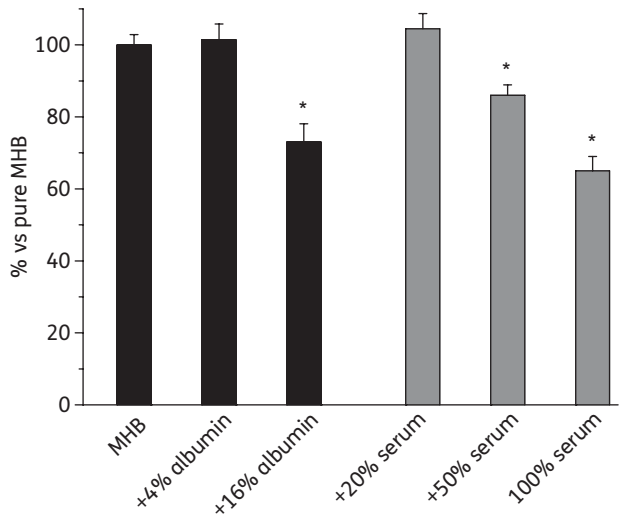

Figure 2. Percentage of uptake of [^{3}H]clindamycin in media containing various amounts of albumin and serum compared with pure MHB. Each experiment was performed six times; data are presented as means ± SD. *Significant difference compared with pure MHB ($P<0.05$).

Discussion

In order to investigate mechanisms behind the impairment of antimicrobial activity by protein binding we aimed to associate protein binding, modification of antimicrobial activity and uptake of antibiotic into bacterial cells in a three-step approach.

First, a medium allowing for optimal bacterial growth but displaying protein binding for clindamycin comparable to that of pure human serum had to be identified. $MHB_{50\%serum}$ (protein binding 86%) and $MHB_{16\%albumin}$ (64%) were used in the next step, which was designed to investigate solely the effect of protein binding on antimicrobial activity by means of time–kill curves after growth-modulating effects of the media had been excluded in Step 1. As expected, in the presence of albumin or serum at appropriate levels the antimicrobial activity of clindamycin was significantly reduced, which resulted in differences of $>2\ log_{10}$ cfu/mL compared with pure MHB. Indeed this reduction of activity corresponded to the calculated free fraction of clindamycin in the respective media (data not shown). Hence, the combined results from Steps 1 and 2 indicate that $MHB_{50\%serum}$ is the ideal medium to allow for the quantification of the effect of PPB on the antimicrobial activity of clindamycin and that $MHB_{16\%albumin}$, which displayed slightly lower values of protein binding than pure serum, might be an alternative.

The third step aimed to correlate results obtained from the determination of protein binding and time–kill curves with uptake of [^{3}H]clindamycin into bacterial cells. Indeed radioactivity was significantly decreased in pure serum as well as in $MHB_{16\%albumin}$ and $MHB_{50\%serum}$, while no significant difference was observed for $MHB_{4\%albumin}$ and $MHB_{20\%serum}$, supporting the hypothesis that protein binding reduces the activity of clindamycin by preventing its penetration into the bacterial cell.

Clindamycin binds primarily to α1-acid glycoprotein, explaining why high levels of protein binding could only be achieved by albumin concentrations exceeding physiological concentrations 4-fold.[4,7] In addition, human albumin undergoes several well-established proton-induced conformational changes, one of these structural conformations being pH and calcium dependent.[8,9] To investigate possible factors influencing the binding ability of albumin and α1-acid glycoprotein in our media, we investigated protein content, pH and certain ions at the time of inoculation and throughout the experiments.[10] pH decreased significantly after 24 h for bacterial growth and time–kill curves with values of 6.2 ± 0.4 and 6.6 ± 0.1, respectively; thus it cannot be ruled out that binding capacity somewhat decreased throughout time–kill curves.

In conclusion, $MHB_{50\%serum}$ was identified as the optimal medium for investigation of pharmacodynamics of clindamycin with regard to protein binding. For the first time reduction of intra-bacterial concentrations of an antimicrobial in the presence of human serum proteins was demonstrated and could be correlated with protein binding and reduction of antimicrobial activity. Whether this model will hold true for other classes of antimicrobials should be investigated in further studies.

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Transparency declarations

None to declare.

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