1990

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Citation/Publisher Attribution
Dudley, M. N., Blaser, J., Gilbert, D., & Zinner, S. H. (1990). Significance of "Extravascular" Protein Binding for Antimicrobial Pharmacodynamics in an In Vitro Capillary Model of Infection. *Antimicrob. Agents Chemother., 34*(1), 98-101. doi: 10.1128/AAC.34.1.98
Available at: http://dx.doi.org/10.1128/AAC.34.1.98

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1990, p. 98–101
0066-4804/90/010098-04$02.00/0

Vol. 34, No. 1

Significance of "Extravascular" Protein Binding for Antimicrobial Pharmacodynamics in an In Vitro Capillary Model of Infection

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Received 21 April 1989/Accepted 7 October 1989

The effect of protein binding in an "extravascular" space on antimicrobial pharmacodynamics was studied in an in vitro capillary model of infection. Simulated 500-mg oral doses of dicloxacillin (~96% bound) or cephalexin (<5% bound) were administered every 6 h for four doses. A 10-fold-higher dose of dicloxacillin was also studied to determine the effect of drug concentration on the reduction of bacterial killing in the presence of protein. *Staphylococcus aureus* ATCC 25923 was inoculated into peripheral chambers filled with either Mueller-Hinton broth or Mueller-Hinton broth plus 25% human serum. Serial samples for bacterial counts were collected over 24 h. The presence of serum in the chambers significantly reduced bacterial killing by dicloxacillin but not by cephalexin during the first 6 h (two-way analysis of variance, $F = 6.04$, $P < 0.05$) but not at 24 h. Reduction of dicloxacillin activity in serum-containing chambers persisted with the higher dose. These data suggest that despite attaining higher total drug concentrations in protein-containing extravascular spaces with highly bound drugs, protein binding reduces bactericidal activity during the early stages of treatment in this model.

The clinical significance of the reduction of antibacterial activity associated with the binding of drugs to human proteins remains controversial. Despite considerable discussion, only a few examples in humans (8, 10, 25) and in animal models of infection (18, 20) have demonstrated the significance of high plasma protein binding on treatment outcome.

Studies in animals (6, 19, 20), in vitro models (21), and humans (16, 22, 25) have shown that high serum protein binding significantly affects the kinetics of extravascular drug distribution. High binding to serum proteins (e.g., albumin) in the intravascular space tends to reduce the amount of drug available for diffusion into extravascular spaces (22, 25). However, a considerable amount of serum protein also is distributed to the extravascular space; for example, approximately 60% of the total exchangeable albumin in the body is found in the extravascular space, but at lower concentrations than in the intravascular space (17). These proteins can bind a drug and result in higher concentrations of total (i.e., bound plus unbound) drug than those observed in protein-free spaces (2, 9, 11, 19, 21).

There are few data on the effect of protein binding in an infected extravascular space on antibacterial pharmacodynamics following multiple simulated human doses. Therefore, the pharmacodynamics of simulated doses of a highly protein-bound drug (dicloxacillin) was compared with that of a drug with negligible protein binding (cephalexin) in an in vitro model of infection. The results show that during the early stages of treatment, high protein binding significantly retards the antimicrobial effects of dicloxacillin.

MATERIALS AND METHODS

Drugs. Cephalexin and dicloxacillin analytical-grade powders were supplied by Eli Lilly & Co., Indianapolis, Ind. Stock solutions were prepared in appropriate amounts of sterile, distilled, deionized water, which was further diluted in Mueller-Hinton broth (MHB) for dose administration.

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Bacteria. *Staphylococcus aureus* ATCC 25923 was used as the test strain. Susceptibility testing was done by the method of Stratton and Reller (24), with the exception that serum-supplemented medium (MHB-SER) was 75% MHB and 25% human serum. The MIC and MBC for dicloxacillin against this strain in MHB were 0.25 μg/ml; in MHB-SER, they were 2 and 8 μg/ml, respectively. The MIC and MBC for cephalexin against this strain were 4 and 8 μg/ml, respectively, in both MHB and MHB-SER.

Protein binding. The binding of dicloxacillin in MHB-SER was measured with an Amicon MPS-1 unit with YMT membranes (Amicon Corp., Danvers, Mass.). Dicloxacillin was prepared in MHB-SER, and approximately 1 ml was added to ultrafiltration units. Samples were centrifuged for 20 min at 1,000 × *g* (swinging bucket) at 37°C. The ultrafiltrate was collected and assayed for dicloxacillin content by agar well diffusion assay using *Bacillus subtilis*.

In vitro capillary model of infection. A two-compartment in vitro model was modified to simulate first-order oral absorption with peak concentrations occurring 1 h after a dose (3, 4). The total volume of each peripheral ("extravascular") compartment was 10 ml. The dilution rate was adjusted to obtain a half-life of 1 h in the central compartment for both drugs. The human pharmacokinetics of free (i.e., non-protein-bound) concentrations obtained with 500-mg doses of cephalexin or dicloxacillin given every 6 h was simulated in the central compartment of the model (Table 1) (9, 14, 15). To determine the effect of the dose on the pharmacodynamic properties of the more highly protein-bound drug, dicloxacillin, a 10-fold-higher dose (D 10) also was studied.

The effect of extravascular protein binding on pharmacodynamics was studied by filling peripheral chambers with either 100% MHB or MHB-SER. A single pool of heat-inactivated serum collected from healthy human volunteers was used. Pilot studies showed that the protein binding of dicloxacillin (concentrations of 10 μg/ml and lower) averaged 95.5% in MHB-SER and was not detectable in MHB. Binding of cephalexin to proteins in these media was as-

TABLE 1. Predicted pharmacokinetic conditions at equilibrium for cephalexin and two dicloxacillin regimens in model experiments

Drug and dose regimen	Free drug				Total drug in peripheral chambers with 25% serum	
	In central compartment		In all peripheral chambers			
	Peak concn (μg/ml)	% of dosing interval with drug concn >MIC	Cavg[a] (μg/ml)	AUC[b] (μg · h/ml)	Cavg (μg/ml)	AUC (μg · h/ml)
Cephalexin (C 20[c])	20	70	9.7	58	9.7	58
Dicloxacillin						
D 1[c]	1	62	0.5	2.9	11.1	64.4
D 10	10	100	4.8	29	107	644

[a] Cavg, average drug concentration derived from area under concentration-time curve divided by dosing interval.
[b] Area under the drug concentration versus time curve during a 6-h dosage interval at equilibrium.
[c] See legends to Fig. 1 and 2.

sumed to be negligible (less than 5%) (9). Protein concentrations in peripheral chambers containing MHB-SER were compared at 0 and either 24 or 30 h after the start of an experiment by using a refractometer (Schuco, Williston Park, N.Y.).

Bacteria from previously frozen inocula of approximately 4×10^7 CFU/ml were thawed, diluted with an equal part of fresh MHB, and incubated at 37°C for 90 min to bring organisms into the log-linear growth phase. A sample of this mixture (0.3 ml) was injected into peripheral chambers containing either MHB or MHB-SER; organisms were grown in the chambers for approximately 1 h prior to the administration of the first drug dose in order to obtain a final inoculum of ca. 5×10^5 to 10×10^5 CFU/ml. Samples (ca. 0.3 ml) were collected from peripheral chambers, and bacterial counts (CFU per milliliter) were determined by plating serial 10-fold saline dilutions of the sample on drug-free Mueller-Hinton agar. The limit of detection of bacterial growth was 10 CFU/ml.

Statistics. The significance of changes in the pharmacodynamic properties of cephalexin and dicloxacillin with serum supplementation (i.e., protein binding) or an increased dose of dicloxacillin was determined by using two-factor analysis of variance (23).

RESULTS

Figure 1 depicts the control growth of bacteria in both MHB and MHB-SER and the pharmacodynamic properties of four simulated 500-mg doses of cephalexin in chambers with or without serum. Growth of unexposed organisms was similar in both media. The first doses of cephalexin resulted in an approximately 90% reduction of the initial inoculum during the first 6 h; the reduction in CFU per milliliter was similar in MHB and MHB-SER. Three subsequent doses resulted in no significant reduction in CFU per milliliter for the remainder of the study.

In contrast to the effect of cephalexin, the inhibitory effect of dicloxacillin on bacterial growth was delayed in chambers containing MHB-SER (Fig. 2). Analysis of bacterial counts obtained at 6 h by two-factor analysis of variance (cephalexin versus dicloxacillin and MHB versus MHB-SER) demonstrated that the reduction of the antibacterial effect of dicloxacillin by serum was statistically significant compared with that for cephalexin ($F = 6.04$, 1 df; $P < 0.05$); however, this difference was not statistically significant at 24 h ($F = 5.14$, 1 df; $P > 0.05$).

To determine if the reduction in dicloxacillin pharmacodynamics in serum-supplemented media could be offset by higher drug concentrations, a 10-fold-higher dose was studied (Fig. 2, D 10). In MHB-containing chambers, the higher dose resulted in an increase in bacterial killing; however, at 6 h, a reduction in antimicrobial activity was still noted in chambers with MHB-SER and was not altered by dose size ($F = 0.67$, 1 df; $P > 0.05$ in the two-factor analysis of variance for low dose versus high dose and MHB versus MHB-SER). After 6 h, bacterial counts declined at similar rates with both dose levels in MHB and MHB-SER chambers.

There was no change in the MICs for dicloxacillin in bacteria recovered at 30 h following exposure to the high- or low-dose regimens. Protein concentrations at 24 and 30 h in peripheral chambers containing MHB-SER varied from their baseline value on average ($n = 2$) by 7.5 to 16% (range, 5.3 to 21%).

DISCUSSION

Reduced activity of highly bound antimicrobial agents in vitro in the presence of serum proteins has led to speculation that these agents would have reduced activity in vivo (8, 9). Merrikin et al. evaluated several isoxazolyl penicillins with

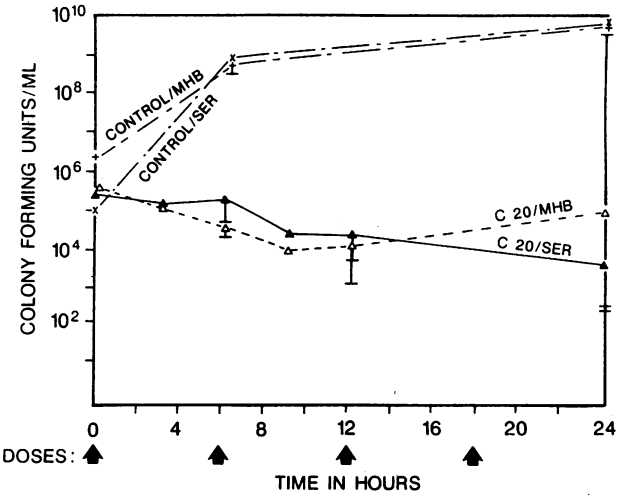

FIG. 1. Effect of extravascular protein binding on antibacterial effect of cephalexin against *S. aureus* ATCC 25923 in a two-compartment in vitro capillary model. Simulated oral doses were administered every 6 h (arrows). The presence of serum (C 20/SER) did not alter the antibacterial effect of 500-mg doses of cephalexin in serum-free chambers (C 20/MHB). Control growth of untreated bacteria was similar in MHB- and MHB-SER-containing chambers. Datum points indicate the mean and 1 standard deviation.

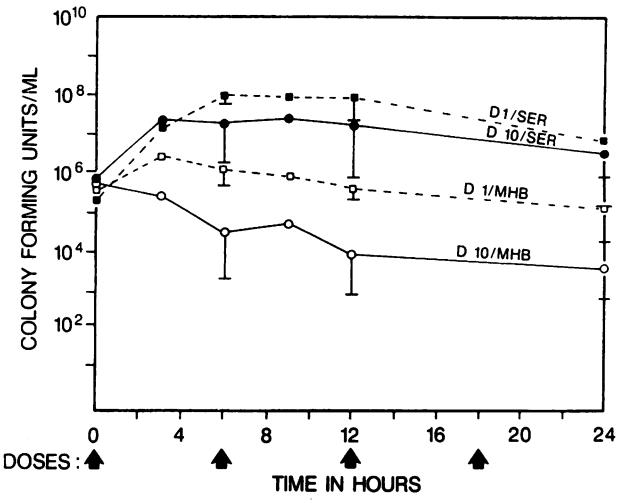

FIG. 2. Effect of extravascular protein binding on antibacterial effect of dicloxacillin against *S. aureus* ATCC 25923 in a two-compartment in vitro capillary model. Simulated oral doses were given every 6 h (arrows). Dosage regimens provided peak concentrations of 1 μg/ml (500-mg dose [D 1]) and 10 μg/ml (D 10). Chambers with serum (SER) showed a reduced effect of dicloxacillin compared with serum-free chambers. Datum points indicate the mean and 1 standard deviation.

similar MICs for a strain of *S. aureus* but various degrees of plasma protein binding in experimentally induced peritonitis in mice (18). Their study demonstrated that the curative dose for 50% of mice for each agent increased with progressive protein binding, indicating the need for large doses to obtain adequate free-drug concentrations. Chambers et al. reported the failure of single daily doses of cefonicid, a highly protein-bound cephalosporin, in intravenous drug abusers with tricuspid valve endocarditis due to *S. aureus* (7); subsequent studies showed that high, saturable serum protein binding accelerates the decline of unbound-drug concentrations, resulting in prolonged periods of subtherapeutic drug concentrations with single daily dosing (10). Others have implicated high protein binding in the poor response of a variety of infections treated with both high- and low-dose regimens of cefoperazone, ceftriaxone, fusidic acid, and teicoplanin (8, 12, 25).

While these earlier studies primarily focused on protein binding in the intravascular space, we chose to study the effect of protein binding in a simulated infected tissue site. The presence of serum in simulated extravascular spaces did not alter the antibacterial effect of cephalexin; this is consistent with the low protein binding of this agent. In contrast, the pharmacodynamic properties of dicloxacillin were significantly reduced in serum-supplemented chambers; this reduction persisted despite the use of a 10-fold-higher dose. This latter observation is remarkable since the higher-dose regimen (D 10) was designed to result in ratios of the drug concentration to the MIC in serum-containing chambers similar to those obtained with the lower-dose regimen for serum-free chambers (i.e., for D 10 the central peak was 10 μg/ml and the MIC in MHB-SER was 2 μg/m [ratio, 5], while for the 500-mg dose the central peak was 1 μg/ml and the MIC in MHB-SER was 0.25 μg/ml [ratio, 4]).

Antagonism to dicloxacillin activity by serum was most pronounced during the first dosage interval. These observations are consistent with a high binding capacity for the drug in the peripheral compartment coupled with a slow drug transfer from the central compartment. In this model, the onset of antibacterial effects was delayed because of a delay in the accumulation of adequate free-drug concentrations at the site of infection (6, 11, 19); this occurs because a large proportion of dicloxacillin diffusing to the site of infection is immediately bound to serum proteins. These data suggest that the onset of the antibacterial effects of highly bound antibiotics is delayed in the presence of binding proteins at the site of infection. A delay in the onset of antibacterial effects might be most important in the setting of surgical prophylaxis, in which the protective effect is greatest when therapeutic drug concentrations are present in extravascular tissues at the time of bacterial inoculation (1, 5). However, the large amount of drug ultimately accumulating in peripheral protein-containing extravascular spaces results in a relatively more protracted antibacterial effect following the suspension of treatment, since drug concentrations tend to decline more slowly under these conditions (6, 11, 13).

ACKNOWLEDGMENTS

The technical assistance of Martin Kuepker is acknowledged.
This work was supported in part by a grant from Eli Lilly & Co.

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