Journal of Clinical Infectious Diseases & Practice

Dalhoff and Schubert, J Clin Infect Dis Pract 2016, 1:2
DOI: 10.4172/2476-213X.1000111

Research Article OMICS International

The Impact of Protein Binding on Antibacterial Activities of Antibiotics is more than Predicted by Considering its Numerical Value Alone: Effects of Serum Proteins on Activities of Poorly Penetrating Agents and Inhibition of ß-Lactamase Activity-Part II

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Received date: August 30, 2016, **Accepted date:** September 22, 2016, **Published date:** September 27, 2016

Abstract

Objectives: Protein binding decreases antibacterial activities as the free fraction only crosses membranes thus reaching intracellular targets. However, serum components may increase antibacterial activities. Therefore, the effect of serum proteins on activities of ß-lactams and macrolides was examined.

Methods: Strains with defined resistance genotypes were selected; MRSA, ermB-, mefA-, gyrA Ser81-Phe-mutants of *S. pneumoniae*, and TEM-1 or TEM-3 ß-lactamase producing *E. coli* were used. Ten antibiotics known to penetrate into bacteria either well or poorly and/or known to be labile or stable to inactivation by ß-lactamases were used. Strains were incubated in Brain Heart Infusion Broth (BHI), BHI +50% heat inactivated human serum or active serum, or 45 g/L albumin. MICs were determined and Kill-kinetics was recorded following exposure to constant or fluctuating drug concentrations. Kill constants and areas under the bacterial kill curves were calculated.

Results: Albumin and inactive serum increased MICs and reduced kill rates of the agents studied in conformity with their protein binding. However, active serum increased the activities of such agents known to penetrate poorly into strains with permeation barriers. In addition, active as well as inactive serum restored the activities of ß-lactams against ß-lactamase producing strains due to enzyme inhibition.

Conclusions: Serum proteins permeabilized bacteria and inhibited ß-lactamase activity. The impact of serum proteins on antibacterial activities against specific drug-bug associations is more than predicted by considering the numerical value of protein binding alone.

Keywords: Permeabilization; ß-lactamase inhibition; Macrolides; Penicillins; Cephalosporins

Introduction

Activities of antibacterials could be directly correlated with the extent of their protein binding causing a proportionate increase of MICs and a decrease of bactericidal activities [1-4]. However, synergistic effects of human serum on antibacterial *in vitro* activities of tetracyclines, β-lactams, aminoglycosides, macrolides, glycopeptides, and fluoroquinolones have been described [5-20]. Likewise, rat- or rabbit sera enhanced the activity of vancomycin against enterococci and staphylococci [21,22]. It is important to note that these data were generated in the absence of any cellular immunity and that the sera used were either deprived from complement factors and/or that serum-resistant indicator strains have been used. Thus, serum antibiotic synergistic effects were independent from the immune system. However, the mechanism(s) underlying the serum antibiotic synergistic effects have never been assessed. These phenomena may be due an increased uptake of the antibacterial agents studied as Enterococci are intrinsically tolerant to the bactericidal action of ß-lactams, aminoglycosides and glycopeptides due an inefficient uptake which can be overcome by combinations of these agents with cell wall active agents [5-7,23,24]. Likewise, macrolides are poorly taken up, if at all, by Gram-negative bacteria [24-26]. Moreover, ß-lactams, tetracyclines, and fluoroquinolones pass the outer membrane of Enterobacteriaceae and non-fermentrs having been used as indicator arganisms in the serum antibiotic synergism studies [14-16,20] through porins [27] and are extruded by efflux pumps [28-30]. Any decrease in the ability or rate of entry and an increased export, respectively, of these compounds can lead to resistance [23,24]. In theory, augmented intracellular accumulation [31,32] and/or efflux pump inhibition could restore the susceptibility of bacteria to antibiotics; however, efflux pump inhibitors have not yet progressed into clinical use [33,34]. Furthermore, human serum interacted synergistically with ß-lactams against ß-lactamase producing Gram-negative bacteria only but not against ß-lactamase negative strains due to ß-lactamase inhibition [11,12,35].

These data suggest that serum antibiotic synergisms were recorded in particular under those experimental conditions under which either the agents were taken up poorly or their activities were reduced because of enzymic inactivation. Therefore, the aim of this study was to expose Gram-positive and Gram-negative bacteria with geno- and/or phenotypically well-defined resistance mechanisms or bacteria known to be intrinsically macrolide-resistant to selected antibacterials;

pairs of agents were studied known to cross bacterial membranes either well or poorly and being good or poor substrates for ß-lactamases, respectively.

Materials and Methods

The methods used in this study are identical to those described in the companion manuscript [36]. Briefly, blood was sampled from twelve healthy volunteers immediately prior to commencement of each experimental series and pooled. Serum was split into two parts; one part was used without any further processing (active serum) while the other part was heated at 56°C for 30 minutes (inactive serum) and used immediately thereafter. In general, serum was used fresh immediately after preparation and was never stored.

Strains, antibacterial agents, and media

The following American Type Culture Collection (ATCC) wild type strains have been used throughout this study: *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 13597, and *H. influenzae* ATCC 33391. Strains with defined resistance mechanisms were: *S. aureus* ATCC 33593 (mecA=methicillin resistance determinant encoding the low-affinity penicillin-binding protein 2a), *S. pneumoniae* 6297 (clinical isolate harboring ermB=erythromycin ribosomal methylase class B); *S. pneumoniae* 13597 (clinical isolate harboring mefA=macrolide efflux A); *S. pneumoniae* 19397 (gyrA Ser81-Phe=laboratory generated gyrase A mutant with a serine to phenylalanine exchange in position 81); *E. coli* C165 (TEM 1 ß-lactamase producing clinical isolate hydrolyzing penicillins and narrow spectrum cephalosporins, such as cephalothin or cefazolin, but not cephalosporins with an oxyimino side chain, such as cefotaxime, ceftazidime, ceftriaxone, or cefepime); *E. coli* UL10 (TEM 3 ß-lactamase displaying the ESBL phenotype). Strains have been adapted to growth in serum or body fluids in *in vivo* infection models and were maintained on blood agar plates. *E. coli* ATCC 11775 used as an external control for MIC testing and time-kill experiments.

Brain Heart Infusion Broth (BHI; Becton Dickinson Diagnostics, Heidelberg, Germany), BHI + 50% each of heat inactivated human serum or active human serum, and 45g/L human albumin (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) was used. The final pH of the media was adjusted to 7.2. Agents studied are summarized in Table 1. The antibacterials were obtained from commercial sources. Antibacterial agents were dissolved and stock solutions were prepared according to manufacturer's instructions and stored in aliquots at -20°C. The antibiotic solutions were thawed and diluted in the appropriate media immediately prior to experiments.

MIC testing and time-kill experiments

In general, all the equipment used was siliconized to avoid adsorption of ß-lactamases and/or of agents studied to surfaces of glass- or plastic ware. MICs were determined according to CLSI guidelines [37]; time-kill curves were generated by exposure to multiples (1-,4-,8-,16-, and 32-times) of the MICs recorded in the corresponding media to ensure that bioequivalent concentrations were used. Sampling, processing of samples, quatitation of viable counts as well as drug concentration-assays has been described in detail in the companion manuscript [36]. In general, growth controls in the corresponding drug free media, MIC determinations or time-kill assays were run in parallel under the four experimental conditions studied. Bacteriostatic and bactericidal activities were evaluated in duplicate on separate occasions; if data varied, the higher values are reported in the tables. Kill-rates (k [h], antibiotic exposed cultures) were calculated in analogy to growth rates (µ [h], drug-free cultures) for the log-linear phase of declining CFUs [38]; as the maximal bactericidal activities of all the agents studied under any of the experimental conditions was achieved at eight times the MICs, data summarized in Table 1 represent the kill rates at this concentration.

Agent (PB)/strain	Geno-/ phenotype	BHI			BHI + i.a. serum			BHI + a. serum			BHI + albumin		
Amoxicillin (30)		MIC	k	µ	MIC	k	µ	MIC	k	µ	MIC	k	µ
S.a. ATCC 29213	wt	0.12	-3.25	2.42	0.12	-3.12	2.43	0.06	-3.56	2.42	0.25	-3.18	2.43
S.a. ATCC 33593	mecA	64	n.d	2.08	64	n.d	2.1	64	n.d	2.1	64	n.d	2.09
S.pn. ATCC 6303	wt	0.015	-1.48	0.85	0.015	-1.46	0.83	0.006	-2.68	0.85	0.015	-1.52	0.85
S.pn. 6297	ermB	2	-2.58	0.84	4	-2.25	0.84	2	-2.82	0.88	2	-2.63	0.85
S.pn. 13597	mefA	2	-1.25	0.84	2	-1.16	0.83	0.5	-2.14	0.86	2	-1.2	0.85
S.pn. 19397*	gyrA Ser81-Phe	2	-0.84	0.83	2	-0.12	0.84	2	-1.78	0.85	2	-0.16	0.84
E. coli ATCC 11775	wt	4	-3.82	3.66	4	-3.73	3.65	2	-4.09	3.66	4	-3.52	3.65
E. coli C165	TEM 1	>128	3.69	3.71	8	-3.45	3.7	4	-3.68	3.74	>128	3.38	3.72
E. coli UL10	TEM 3	>128	3.68	3.68	8	-3.32	3.69	8	-3.54	3.71	>128	3.28	3.69
H.i. ATCC 33391	wt	0.25	-3.44	0.66	0.25	-3.31	0.67	0.25	-3.64	0.67	0.5	-3.24	0.66
Cefuroxime (40)													

S.a. ATCC 29213	wt	2	-2.62	2.42	2	-2.31	2.43	2	-2.68	2.42	4	-2.18	2.43
S.a. ATCC 33593	mecA	32	n.d	2.08	64	n.d	2.1	64	n.d	2.1	64	n.d	2.09
S.pn. ATCC 6303	wt	0.03	-1.34	0.85	0.03	-1.3	0.83	0.015	-2.42	0.85	0.03	-1.28	0.85
S.pn. 6297	ermB	4	-1.77	0.84	4	-1.56	0.84	4	-2.71	0.88	4	-1.68	0.85
S.pn. 13597	mefA	8	-0.78	0.84	8	-0.44	0.83	8	-0.76	0.86	16	-0.31	0.85
S.pn. 19397*	gyrA Ser81-Phe	8	1.33	0.83	8	1.45	0.84	8	1.42	0.85	8	1.25	0.84
E. coli ATCC 11775	wt	4	-2.44	3.66	8	-2.18	3.65	2	-2.68	3.66	4	-2.22	3.65
E. coli C165	TEM 1	8	-2.22	3.71	8	-2.24	3.7	8	-2.62	3.74	8	-2.19	3.72
E. coli UL10	TEM 3	>128	3.65	3.68	16	-1.98	3.69	8	-2.43	3.71	>128	3.67	3.69
H.i. ATCC 33391	wt	1	-2.83	0.66	1	-2.78	0.67	0.5	-2.95	0.67	1	-2.69	0.66
Cefotaxime (40)													
S.a. ATCC 29213	wt	1	-0.28	2.42	1	-0.26	2.43	1	-0.3	2.42	1	-0.29	2.43
S.a. ATCC 33593	mecA	>64	n.d	2.08	>64	n.d	2.1	>64	n.d	2.1	>64	n.d	2.09
S.pn. ATCC 6303	wt	0.03	-1.92	0.85	0.03	-1.88	0.83	0.03	-1.98	0.85	0.03	-1.86	0.85
S.pn. 6297	ermB	0.12	-1.52	0.84	0.25	-1.5	0.84	0.12	-1.59	0.88	0.5	-1.5	0.85
S.pn. 13597	mefA	0.12	-0.88	0.84	0.12	-1.26	0.83	0.12	-1.32	0.86	0.12	-1.3	0.85
S.pn. 19397*	gyrA Ser81-Phe	0.06	-2.44	0.83	0.12	-0.86	0.84	0.06	-0.9	0.85	0.12	-0.88	0.84
E. coli ATCC 11775	wt	0.06	-2.44	3.66	0.12	-2.39	3.65	0.06	-2.47	3.66	0.06	-2.45	3.65
E. coli C165	TEM 1	0.12	-2.48	3.71	0.12	-2.51	3.7	0.12	-2.52	3.74	0.12	-2.46	3.72
E. coli UL10	TEM 3	32	-2.32	3.68	8	-2.4	3.69	0.5	-2.43	3.71	32	-2.31	3.69
H.i. ATCC 33391	wt	<0.06	-2.88	0.66	<0.06	-2.9	0.67	<0.06	-2.92	0.67	<0.06	-2.9	0.66
Ceftriaxone (60-95)													
S.a. ATCC 29213	wt	2	-0.2	2.42	16	-0.06	2.43	8	-0.23	2.42	16	-0.08	2.43
S.a. ATCC 33593	mecA	32	n.d	2.08	>64	n.d	2.1	>64	n.d	2.1	>64	n.d	2.09
S.pn. ATCC 6303	wt	<0.06	-1.22	0.85	>64	n.d	0.83	0.5	-0.18	0.85	>64	n.d	0.85
S.pn. 6297	ermB	0.25	-1.14	0.84	1	-0.24	0.84	1	-0.89	0.88	1	-0.16	0.85
S.pn. 13597	mefA	2	-0.98	0.84	8	-0.32	0.83	4	-0.87	0.86	8	-0.24	0.85
S.pn. 19397*	gyrA Ser81-Phe	1	-0.76	0.83	>64	n.d.	0.84	32	-0.74	0.85	>64	n.d.	0.84
E. coli ATCC 11775	wt	<0.06	-2.32	3.66	0.25	-1.08	3.65	0.12	-2.39	3.66	0.25	-0.93	3.65
E. coli C165	TEM 1	0.12	-2.28	3.71	1	-1.86	3.7	0.12	-2.29	3.74	1	-1.78	3.72
E. coli UL10	TEM 3	16	-2.26	3.68	64	-1.94	3.69	0.25	-2.28	3.71	16	-2.07	3.69
H.i. ATCC 33391	wt	<0.06	-2.49	0.66	0.25	-1.66	0.67	0.12	-2.57	0.67	0.25	-1.34	0.66
Imipenem (20)													

S.a. ATCC 29213	wt	0.015	-2.23	2.42	0.015	-2.28	2.43	<0.015	-2.21	2.42	0.015	-2.22	2.43
S.a. ATCC 33593	mecA	32	n.d	2.08	32	n.d	2.1	32	n.d	2.1	32	n.d	2.09
S.pn. ATCC 6303	wt	0.06	-1.67	0.85	0.06	-1.67	0.83	0.06	-1.72	0.85	0.06	-1.65	0.85
S.pn. 6297	ermB	0.25	-1.23	0.84	0.25	-1.2	0.84	0.25	-1.22	0.88	0.25	-1.2	0.85
S.pn. 13597	mefA	0.5	-1.14	0.84	0.5	-1.09	0.83	0.5	-1.16	0.86	0.5	-1.1	0.85
S.pn. 19397*	gyrA Ser81-Phe	0.5	-0.43	0.83	0.5	-0.4	0.84	0.25	-0.41	0.85	1	-0.39	0.84
E. coli ATCC 11775	wt	0.06	-2.56	3.66	0.06	-2.51	3.65	0.06	-2.53	3.66	0.12	-2.55	3.65
E. coli C165	TEM 1	0.5	-2.48	3.71	0.5	-2.5	3.7	0.5	-2.51	3.74	0.5	-2.49	3.72
E. coli UL10	TEM 3	0.5	-2.46	3.68	0.5	-2.46	3.69	0.5	-2.49	3.71	0.5	-2.47	3.69
H.i. ATCC 33391	wt	0.5	-3.44	0.66	0.5	-3.36	0.67	0.5	-3.42	0.67	0.5	-3.41	0.66
Faropenem (95)													
S.a. ATCC 29213	wt	0.12	-2.73	2.42	0.5	-2.56	2.43	0.06	-3.42	2.41	0.5	-2.24	2.43
S.a. ATCC 33593	mecA	64	n.d	2.08	>128	n.d	2.1	128	n.d	2.11	>128	n.d	2.09
S.pn. ATCC 6303	wt	<0.06	-1.66	0.85	0.25	-1.42	0.83	0.12	-2.42	0.82	0.5	-1.58	0.85
S.pn. 6297	ermB	2	-2.83	0.84	16	-2.12	0.84	8	-2.72	0.82	16	-2.08	0.85
S.pn. 13597	mefA	0.5	-1.75	0.84	4	-1.27	0.83	4	-2.62	0.83	8	-1.07	0.85
S.pn. 19397*	gyrA Ser81-Phe	0.5	-2.73	0.83	4	-2.23	0.84	4	-2.75	0.83	8	-2.1	0.84
E. coli ATCC 11775	wt	0.12	-2.99	3.66	0.5	-2.18	3.65	0.06	-3.27	3.64	0.5	-2.28	3.65
E. coli C165	TEM 1	0.5	-2.68	3.71	1	-2.65	3.7	0.5	-3.32	3.74	2	-2.22	3.72
E. coli UL10	TEM 3	0.5	-2.69	3.68	1	-2.68	3.69	0.5	-3.4	3.71	2	-2.24	3.69
H.i. ATCC 33391	wt	0.25	-1.68	0.66	1	-1.34	0.67	0.06	-2.23	0.67	2	-1.13	0.66
Erythromycin (75)													
S.a. ATCC 29213	wt	0.5	0.98	2.38	1	0.92	2.41	0.12	0.99	2.41	1	0.93	2.42
S.a. ATCC 33593	mecA	1	0.63	2.17	2	0.6	2.11	1	0.65	2.1	2	0.6	2.1
S.pn. ATCC 6303	wt	0.06	0.12	0.95	0.12	0.03	0.82	0.06	0.1	0.83	0.25	0.03	0.84
S.pn. 6297	ermB	>64	n.d	0.92	>64	n.d	0.82	64	n.d	0.82	>64	n.d	0.83
S.pn. 13597	mefA	8	0.03	0.9	64	n.d	0.83	4	0.08	0.84	8	n.d	0.83
S.pn. 19397*	gyrA Ser81-Phe	>128	n.d	0.91	>128	n.d	0.83	16	0.02	0.84	>128	n.d	0.83
E. coli ATCC 11775	wt	128	n.d	3.62	>128	n.d	3.64	16	1.98	3.66	>128	n.d	3.63
H.i. ATCC 33391	wt	16	0.18	0.65	32	n.d	0.66	8	0.75	0.67	32	n.d	0.64
Roxithromycin (85)													
S.a. ATCC 29213	wt	1	1.07	2.38	4	0.26	2.41	2	0.88	2.41	2	0.17	2.42
S.a. ATCC 33593	mecA	2	1.89	2.17	16	2.33	2.11	8	2.54	2.1	8	2.38	2.1

S.pn. ATCC 6303	wt	0.12	1.46	0.95	1	0.03	0.82	0.5	0.84	0.83	1	0.22	0.84
S.pn. 6297	ermB	>128	n.d	0.92	>128	n.d	0.82	128	n.d.	0.82	>128	n.d	0.83
S.pn. 13597	mefA	32	1.33	0.9	>128	n.d	0.83	16	1.29	0.84	>128	n.d	0.83
S.pn. 19397*	gyrA Ser81-Phe	>128	n.d	0.91	>128	n.d	0.83	64	n.d	0.84	>128	n.d	0.83
E. coli ATCC 11775	wt	>128	n.d	3.62	>128	n.d	3.64	64	n.d	3.66	>128	n.d	3.63
H.i. ATCC 33391	wt	8	1.47	0.65	32	n.d	0.66	8	0.26	0.67	16	n.d	0.64
Clarithromycin (40)													
S.a. ATCC 29213	wt	0.12	1.28	2.38	0.5	1.2	2.41	0.25	1.58	2.41	0.5	1.2	2.42
S.a. ATCC 33593	mecA	0.25	1.8	2.17	1	2.4	2.11	1	2.2	2.1	1	2.5	2.1
S.pn. ATCC 6303	wt	0.03	0.5	0.95	0.12	0.42	0.82	0.12	2.05	0.83	0.12	0.56	0.84
S.pn. 6297	ermB	16	2.83	0.92	128	n.d	0.82	64	n.d	0.82	32	n.d	0.83
S.pn. 13597	mefA	0.5	0.53	0.9	2	0.48	0.83	1	3.04	0.84	1	0.62	0.83
S.pn. 19397*	gyrA Ser81-Phe	64	n.d	0.91	>128	n.d	0.83	64	n.d	0.84	>128	n.d	0.83
E. coli ATCC 11775	wt	8	0.18	3.62	16	0.03	3.64	0.5	0.25**	3.66	16	0.02	3.63
H.i. ATCC 33391	wt	16	0.12	0.65	8	0.05	0.66	4	0.67**	0.67	16	0.1	0.64
Azithromycin (12-50)													
S.a. ATCC 29213	wt	1	0.32	2.38	1	0.3	2.41	0.12	1.34	2.41	1	0.3	2.42
S.a. ATCC 33593	mecA	0.5	0.22	2.17	1	0.18	2.11	0.12	0.2	2.1	1	0.16	2.1
S.pn. ATCC 6303	wt	0.12	1.63	0.95	0.25	1.59	0.82	<0.06	3.62	0.83	0.25	1.67	0.84
S.pn. 6297	ermB	128	n.d	0.92	>128	n.d	0.82	64	n.d	0.82	>128	n.d	0.83
S.pn. 13597	mefA	8	0.45	0.9	64	2.19	0.83	1	0.98	0.84	32	2.3	0.83
S.pn. 19397*	gyrA Ser81-Phe	128	n.d	0.91	>128	n.d	0.83	64	n.d	0.84	>128	n.d	0.83
E. coli ATCC 11775	wt	8	2.19	3.62	32	2.28	3.64	0.5	2.66**	3.66	16	2.37	3.63
H.i. ATCC 33391	wt	1	0.31	0.65	1	0.3	0.66	0.25	1.36**	0.67	1	0.29	0.64

*: penicillin- and macrolide resistant phenotype; S.a: *S. aureus*; S.pn: *S. pneumoniae*; H.i: *H. influenzae*; wt: Wild Type; n.d: Not Done; **: transiently bactericidal for 2 h to 3 h followed by regrowth in parallel to drug-free controls

Table 1: Antibacterial activities, expressed as minimal inhibitory concentrations (MIC, mg/L), kill constants (k, h, negative figures indicate reduction of viable counts, positive figures indicate growth, recorded in drug-exposed cultures) and growth rates (μ, h, recorded in drug-free controls) of the agents studied (values in parenthesis indicate protein binding) against Gram-positive and Gram-negative indicator strains with defined resistance-genotypes incubated in Brain-Heart Infusion Broth (BHI), BHI + inactive (i.a.) serum, BHI + active (a.) serum or BHI + albumin.

Beta-latamase assay

β-lactamase activity was determined by the chromogenic cephalosporin nitrocefin- (Oxoid, Basingstoke, UK) method using a known β-lactamase positive strain as control. Samples were centrifuged at 15,000 g for 15 minutes. The lower limit of detectability was 0.1 units per mg protein. β-lactamase activity was determined in the supernatant as well as in the sonic extracts of the pellet. ß-lactamase activity is expressed relative to control assay, i.e. growth in the corresponding medium in the absence of sera.

The experiments were performed in six parts, i.e. 1st MIC-testing, 2nd and 3rd time-kill experiments with ß-lactams and macrolides, respectively, as well as 4th to 6th repetitions of each of these experiments on three separate occasions, so that always the same group of volunteers donated blood on six occasions. Biochemical and

hematological analysis of the eight pooled serum samples revealed that all parameters were within the normal range on every occasion.

Results

Controls

All the strains tested grew well throughout the entire incubation period under any of the experimental conditions studied. Growth rates were highly comparable under the different conditions studied (Table 1). The activity of ciprofloxacin against *E. coli* ATCC 11775 used as an external control in the course of MIC- and time-kill assays was always within the limits of reproducibility as were the drug concentrations quantitated through the experiments. Drug concentrations quantitated prior to commencement and at the end of the time kill experiments corresponded to >95% to the weighed amount of substance – except ß-lactam concentrations in the presence of TEM-1 or TEM-3 ß-lactamase producing strains.

Impact of serum proteins on bacteriostatic and bactericidal activities

The rationale for the selection of the drug/bug-associations studied was, that first, the general effects of serum on bacteriostatic and bactericidal activities of the agents against the indicator strains tested was studied. Second, the hypothesis that serum factors may affect the fluidity of bacterial membranes thus increasing antibiotic uptake or decreasing efflux of antibiotics was addressed by studying the activities of macrolides penetrating poorly into Gram-negative bacteria or against Gram-positive bacteria harboring the membrane spanning MefA efflux pump as compared to the activities of macrolides against the ribosomal target mutant ErmB or the gyrase mutant both of which should remain unaffected. Furthermore, pairs of either effectively of poorly penetrating ß-lactams were included into the comparison: cefotaxime and ceftriaxone both penentrating effectively, whereas faropenem is taken up poorly in contrast to imipenem. Third, the hypothesis that serum antibodies may inhibit enzymic activities of ß-lactamases was examined by studying the activities of ß-lactams with differing ß-lactamase stabilities against *E. coli* strains producing either TEM-1 or TEM-3 ß-lactamases with different substrate profiles.

Ad 1: Impact of different serum preparations on MICs and kill rates

Albumin and inactive serum increased MICs and reduced kill rates of highly protein bound agents studied in conformity with their protein binding whereas moderately or minimally bound agents remained almost unaffected (Table 1). It is apparent that serum proteins had a differential effect on minimally to moderately bound cefotaxime as well as imipenem on the one hand, and highly bound ceftriaxone and faropenem on the other hand. The four agents were highly active in the absence of serum, but inactive serum, albumin, and a bit variably active serum, too, reduced the activities of ceftriaxone and faropenem against all the Gram-positive and Gram-negative wild-type strains, leaving cefotaxime and imipenem almost unaffected. There was a trend that albumin reduced the activities of macrolides less markedly than inactive serum. This effect becomes obvious by comparing the impact of albumin on the activities of faropenem and roxithromycin, respectively. Both agents are highly protein bound, but albumin reduces the activity of faropenem more markedly than the activity of roxithromycin.

Ad 2: Impact of different serum preparations on the activities of poorly penetrating agents

The activities of macrolides were increased in the mefA- but not the ermB- *S. pneumoniae* mutant. Furthermore, MICs of clarithromycin and azithromycin being poorly active or inactive against Gram-negatives were significantly reduced in the presence of active serum and their kill rates increased correspondingly. Cefotaxone, ceftriaxone, respectively, as well as imipenem were in conformity with their different protein binding rates equally active under the experimental conditions studied, whereas faropenem penetrating poorly into *H. influenzae* gained activity in the presence of active serum as compared to inactive serum or albumin (MICs for faropenem 0.06 mg/L versus 1 and 2 mg/L, respectively).

Ad 3: Impact of different serum preparations on ß-lactamase activities

Active serum restored the activities of all the ß-lactams tested in accordance with their ß-lactamase stability against the two TEM-type ß-lactamase producing *E. coli* strains. This phenomenon becomes obvious by comparing the restorative effect of active serum on the activity of amoxicillin being instable against TEM-1 and TEM-3 ß-lactamases against both strains producing these ß-lactamases. The activities of the other ß-lactams being stable against TEM-1 ß-lactamase were not affected by serum proteins; however, their activities against the TEM-3 producer were restored by active serum. Inactive serum had a similar but weaker effect as active serum on the activities of the ß-lactams against the TEM-1 and TEM-3 producers. Quantitation of ß-lactamase activities in those assays containing active and inactive serum, respectively, as compared to growth in BHI without any supplementations revealed that ß-lactamase activities were below the limit of detectability in the presence of active serum and ranged from 12% to 23% in the presence of inactive serum (data not shown).

Both, the impact of serum proteins on ß-lactamase producing bacteria as well as on mefA mutants were independent from protein binding.

Discussion

Data generated in this study confirm that only the free fraction of antibacterial agents was active against the Gram-positive and Gram-negative strains tested. They also demonstrate that other phenomena independent from protein binding add to the activities of ß-lactams and macrolides; this serum-antibiotic synergy was independent from any impact of the immune system as all strains grew well in the presence of active or inactive serum; furthermore, components of the cellular immune system were absent, so that the differences in bactericidal activities observed in active serum and inactive serum or albumin, respectively. As compared to unsupplemented medium were not due to different growth rates or the impact of complement. The two essential observations of this study were first, that active serum increased the activities of such agents known to penetrate poorly into strains with permeation barriers. Second, active as well as inactivated serum restored the activities of ß-lactams against ß-lactamase producing strains and active serum restored the activities of macrolides against mefA but not ermB mutants.

Association between structure dependent penetration of antibacterials and serum antibiotic synergism

Cefotaxime and ceftriaxone are zwitterions which facilitates penetration through outer membranes of Gram-negative bacteria. Both compounds are characterized by very similar microbiological and pharmacodynamic properties [39-41] but different protein binding. Therefore, serum proteins affected the activity of the highly protein bound ceftriaxone only, and active serum did not restore its activity as ceftriaxone penetrates well into bacteria. Although carbapenems are considered to penetrate into bacteria efficiently [42], imipenem and faropenem differ from each other in their C-2 side chain [43]. The uncharged substituent at the C-2 position of faropenem as compared to a charged substituent of imipenem reduces penetration of faropenem into Gram-negatives resulting in a retarded bactericidal activity in particular against *H. influenzae* despite its high affinity to penicillin binding proteins in Gram-negatives [44]. Proteins of the inactivated serum preparation and human serum albumin reduced the activity of highly protein bound faropenem but not the activity of minimally bound imipenem; active serum restored the bactericidal activity of faropenem against *H. influenzae* and *E. coli*.

Likewise, Gram-negative bacteria are inherently resistant to hydrophobic antibiotics such as macrolides which can neither diffuse effectively through hydrophobic pathways across the outer membrane nor interact electrostically with the LPS despite their mono- or dicationic structure [25,45,46]. Active serum decreased MICs of clarithromycin and azithromycin. These results are well in agreement with previously published data which were generated by using active sera, too [8,9]. Furthermore, active serum restored the activities of macrolides against mefA mutants. Others described that cell culture media and biological fluids acted synergistically with clarithromycin and azithromycin increasing their activities against *P. aeruginosa* [47]. This synergism was due to an alteration of outer membrane fluidity followed by an increased accumulation and a reduced efflux of the agents [47]. In analogy, active serum may hypothetically have affected the activity of MefA consecutive to a modification of membrane fluidity, thus contributing to an explanation of the data generated in this study why active serum increased the activities of macrolides against membrane spanning MefA- pump mechanism but not against the ErmB- methylase target mutant.

Supportive evidence for serum protein mediated membrane-permeabilization

The hypothesis that components present in active serum may have augmented activities of poorly penetrating antibacterials due to membrane-permeabilization is supported by previous studies having applied physicochemical, biochemical, and microbiological methods to investigate the impact of serum proteins on the integrity and/or fluidity of bacterial membranes. It has been demonstrated that IgG increased the uptake of poorly penetrating ß-lactams into Gram-negatives [48] which is consistent with the finding of this study that poorly penetrating agents gained activity in the presence of active serum. IgG was found to disintegrate the outer membrane of Gram-negative bacteria releasing high amounts of periplasmic markers into the medium. Marker release was achieved with IgG in the absence of humoral or cellular immune response thus indicating that membrane permeabilzation was not a consequence of immune reactions. By using the Langmuir-Schaeffer technique as a measure for biophysical interactions of agents with phopholipids it was demonstrated that the film area normalized per phospholipid molecule versus the resultant compressional force for the phopholipids was significantly modified in the presence of IgG thus indicating that IgG inserted into lipid acyl chains. An interaction of IgG with liposomal membranes has been proven, too [48-51]. As electrostatic and hydrophobic forces drive the antigen-antibody binding it is important to note that the hinge region of IgG is rich in positively charged amino acids which are exposed to the binding sites by a "clicked open" process. The shape of IgG is altered to provide more flexibility to the hinge region; the angle between the two Fab regions of an IgG molecule may extend up to 180°, so that the positively charged amino acids in the hinge region interact freely with negatively charged lipids [51-53]. Poorly penetrating agents diffuse into bacteria with greater ease as a consequence of these physicochemical interactions [48]. Although model membranes being representative for Gram-negative bacteria have been examined, analogous phenotypical findings have been generated for Gram-positive bacteria, too [17,48]. As complement inactivation at 56°C should leave the immunefunction of the IgG molecule intact it could be expected that not only active serum, but also heat inactivated serum should reduce MICs of poorly penetrating agents which, however, has not been observed under the experimental conditions applied in this study. This phenomenon may likely be due to the fact that immunogobulins are thermally unstable and change their secondary structure upon heating, so that the structure of the membrane interacting hinge region is affected at 56°C more strongly than the Fab fragment which is denaturated at 61°C [54,55]. Permeabilization and sensitization of a variety of Gram-negative and Gram-positive wild-type strains could not only be proven under *in vitro* conditions but in experimental animals as well [11,12]. This latter finding may likely be clinically relevant in as far as strains growing *in vivo* under hostile conditions are much more impermeable than their counterparts growing *in vitro* in a cosy environment.

Serum protein mediated ß-lactamase inactivation

The second finding of this study was that active as well as inactivated serum restored the activities of ß-lactams against ß-lactamase producing strains which was paralleled by an inhibition of the enzymic ß-lactamase activity. It has been described previously that commercially available IgG preparations contained antibodies against ß-lactamases inactivating the enzymic activities of various ß-lactamases produced by *in vitro* as well as *in vivo* grown bacteria [35]. It has also been demonstrated that sera collected either from age-matched healthy volunteers, or from patients suffering from acute urinary tract infections, or from cystic fibrosis patients suffering from chronic infections, respectively, contained either low, or medium and high titers of antibodies against ß-lactamases [56]; this finding has been confirmed recently in CF-patients [57,58] and in healthy volunteers [59]. As ß-lactamase production could be de-repressed with a variety of naturally occuring structural analoguous to the ß-lactam core [60-62], antibodies are produced by the healthy, non infected host, too. Irrespective of whether sera may originate from healthy or infected individuals, antibodies against ß-lactamases may likely be omnipresent, so that the findings generated in previous as well as in this actual study may translate into the clinical arena.

The serum antibiotic synergistic effects being likely due to membrane permeabilization-, inhibition of efflux pumps, and ß-lactamase-inhibition against specific drug/bug-associations could not have been predicted based on conventional susceptibility testing or PK/PD studies. Thus, the impact of serum proteins on antibacterial activities against specific drug-bug associations is more than predicted by considering the numerical value of protein binding alone.

Limitations

Serum-antibiotic synergistic effects observed in this study may likely be due to membrane permeabilizing activities of IgG and/or other serum proteins. This hypothesis is in agreement with the findings that serum-antibiotic synergisms were found in those studies in which unprocessed serum has been used without any storage [8-11,13,17]. However, others [8,15,19,20] used serum-ultrafiltrate being devoid of peptides. Serum-antibiotic synergisms were obtained with sera prepared by either method, so that not only serum proteins, but also low molecular weight unknown factors may have contributed to the synergistic effects. The open question which serum protein/-s either independently or in cooperation may have caused the synergistic effects should be addressed by using physicochemical, biochemical and microbiological methods. Furthermore, the hypothesis has been raised that such permeabilizing effects may have caused an increased influx of otherwise poorly penetrating antibacterials. An augmented uptake of poorly penetrating agents and/or a decreased pump-mediated efflux has been demonstrated by others [47,48], however, intracellular drug concentrations in the absence or presence of serum proteins have not been analyzed in the course of this study. Therefore, it may be likely, but it remains unproven at the current point in time, that serum-antibiotic synergisms are due to serum protein mediated modulations of bacterial membrane fluidities followed by an increased uptake of the agents studied.

Conclusions

Serum proteins permeabilized bacteria thus increasing the activities of poorly penetrating antibacterial agents and inhibited ß-lactamase activity. The impact of serum proteins on antibacterial activities against specific drug-bug associations is more than predicted by considering the numerical value of protein binding alone.

Acknowledgement

The continued support of our collaborators who donated blood throughout the entire study period is gratefully acknowledged; this study would not have been possible without their dedicated assistance. These data have been presented in part at the 55th Interscience Conference on Antimicrobial Agents and Chemotherapy, 2015, San Diego, CA, USA, Abstract No. A 487.

Funding

None to declare

Author Contributions

A.D. and S.S. have contributed equally to the design and execution of the work described as well as to the analysis and interpretation of the data generated and have drafted and revised the manuscript.

Transparency Declaration

None to declare

Ethics

Not applicable

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