SHORT ARTICLE

Effect of EDTA on the resistance of clinical isolates of *Acinetobacter baumannii* to the bactericidal activity of normal human serum

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Acinetobacter baumannii is an opportunist nosocomial pathogen of world-wide importance and produces severe infections in immunocompromised patients. However, the virulence factors contributing to its pathogenic properties are not well known. The effect of normal human serum against 18 clinical isolates of the most prevalent biotypes of *A. baumannii* in Chile was investigated. The effect of pre-treatment of the cells with ethylene diamine tetraacetic acid (EDTA) or bismuth subsalicylate (BSS), compounds known to decrease the amount of lipopolysaccharide (LPS) and bacterial capsular polysaccharide (CPS), respectively, in other gram-negative bacteria, was evaluated. Most isolates (16 of 18) showed resistance to normal human serum. Prior treatment with EDTA rendered nine of these isolates susceptible to serum, while seven isolates maintained their resistance. Pre-treatment with BSS did not modify the serum-resistant behaviour of the isolates. The results suggest that LPS might be involved in the resistance of *A. baumannii* to human serum whereas CPS does not seem to contribute to this property.

Introduction

In Chile, isolates of *Acinetobacter baumannii*, mainly of biotype 9, exhibit wide antibiotic resistance patterns and produce diverse nosocomial infections, especially among patients in intensive care units. In other countries, different biotypes prevail in hospitals [1,2]. Prevalence of certain biotypes suggests that those micro-organisms have special properties or factors. Work in this laboratory has demonstrated that nosocomial isolates of *A. baumannii* produce a smooth-type LPS (S-LPS), and this macromolecule might contribute to pathogenicity [3]. Furthermore, many strains of *Acinetobacter* are capsulate and the capsular polysaccharide (CPS) might be associated with protection of bacteria from phagocytosis and complement [2].

Gram-negative organisms that give rise to bacteraemia are more resistant to normal human serum and

phagocytosis than bacteria causing other types of infections [4,5]. A significant correlation between the degree of resistance of gram-negative bacteria isolated from septicaemia to the lytic activity of complement *in vitro* and their ability to invade and survive in human fluids has been reported [4]. The participation of some bacterial components, such as outer-membrane or surface proteins, CPS and LPS, in this property has been demonstrated [4,5] but little is known about host defence mechanisms against *Acinetobacter* spp. [2]. Preliminary results reported by Traub *et al.* [6] revealed that treatment of clinical isolates of *A. baumannii* and *Acinetobacter* genospecies 3 with defibrinated human blood, with and without specific antibodies, failed to completely kill 22 strains of these organisms.

As the mechanisms of serum resistance of *A. baumannii* are incompletely understood, the aim of the present study was to evaluate the influence of pre-treatment of the cells with EDTA or BSS on this property by using clinical isolates of different biotypes. These reagents are known to diminish the LPS and CPS content, respectively, in several gram-negative bacteria, including the non-fermentative species *Pseudomonas aeruginosa* [7,8].

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Materials and methods

Human serum

Sera were obtained from 10 clinically healthy individuals who had not received any antibacterial treatment in the previous 30 days. Blood samples were incubated at 37°C for 1 h and then at 4°C for a further hour. Sera were removed aseptically, pooled and held at -70°C until required. The pooled serum was used within 3 months of collection and its complement activity was evaluated by haemolysis before use.

Micro-organisms

Eighteen isolates from bacteraemic patients in different Chilean hospitals were included in the study. One strain of a serum-sensitive *Escherichia coli* (*E. coli* Garret, kindly provided by Dr Felipe Cabello, Valhalla, New York, USA) was used as susceptible control. Isolates of *A. baumannii* were identified and biotyped by standard microbiological methods.

Serum bactericidal activity

Bacterial cultures (100- μ l volumes) grown in Trypticase Soy Broth (Life Technologies, Gaithersburg, MD, USA) at 37°C, and containing *c.* 1×10^8 cfu/ml (OD₅₄₀ 0.17), were each mixed with 900- μ l volumes of serum, in duplicate. Immediately, 100 μ l were removed from each tube to determine the initial bacterial viable counts (t_0) and the mixtures were incubated at 37°C for 24 h. Further 100- μ l volumes were removed at 2, 3 and 24 h. For viable counting, the samples were diluted with 1 ml of saline and collected on a membrane filter (Millipore) of 0.22 μ m diameter pore size. The filters were placed on the surface of Müeller-Hinton agar plates (Life Technologies) and incubated as above. Bacterial death was considered to have been achieved when surviving cells represented $\leq 0.1\%$ of the initial inoculum [9]. The bacterial suspension was also assayed with heat-inactivated serum (56°C for 30 min).

Pre-treatment with EDTA and BSS

To examine the effect of EDTA and BSS on the serum resistance phenotype of *A. baumannii*, the cultures were grown in trypticase soy broth supplemented with either 10 mM EDTA (Sigma) or 2.0 mM BSS (Proctor & Gamble, Cincinnati, OH, USA), respectively, until reaching the inoculum used in the serum resistance assay (OD₅₄₀ 0.17). These concentrations of EDTA and BSS had been shown in previous assays to be subinhibitory for the isolates.

Results

The bactericidal effect of pooled normal human serum (90% v/v) on 18 strains of *A. baumannii* after

incubation for 3 h is shown in Table 1. At 1 h, only one of the strains (*A. baumannii* ACA-33, biotype 9) showed any killing by the serum (data not shown). Sixteen (88.9%) of these strains maintained their serum resistance even after they had been exposed to the pooled serum for 3 h, and only one other strain (strain 93-70, biotype 1) was killed by this time. Similar results were obtained 24 h after challenge and some growth of the resistant strains was evident during the experiment. However, when pooled normal human sera was used at 30% v/v in the assays, none of the strains showed killing after 3 h (data not shown). The control strain, *E. coli* Garret (O1), was serum-sensitive when either 90% or 30% serum was used. This strain of *E. coli* was killed ($<0.1\%$ survivors) in <15 min, whereas the serum-sensitive strain of *A. baumannii* (ACA-33) was found to be resistant for >30 min, but not at 1 h after exposure to serum (data not shown). When heat-inactivated (56°C for 30 min) serum was used instead of normal human serum, no loss of viability was observed among the strains of *A. baumannii* or with the control strain, suggesting that the antibacterial activity observed was due to complement activity.

EDTA and BSS were used to assess whether LPS or CPS participate in the serum resistance of *A. baumannii*, as these reagents are known to reduce significantly the content of LPS and CPS, respectively, in several gram-negative bacteria [7,8]. Pre-treatment of the strains with EDTA rendered nine isolates (HJJA-1, HS-10, HS-34 and ACA-34 of biotype 9; HJJA-25, HS-4 and HS-13 of biotype 8; HJJA-14 of biotype 17; and 95-52 of biotype 1) susceptible to serum killing, suggesting the participation of LPS in the serum resistance in *A. baumannii* (Table 1). The remaining seven strains retained their serum-resistant phenotype after EDTA treatment, probably because of the presence of different structures that may influence this property. Pre-treatment with BSS did not modify the response of any of the isolates to serum.

Discussion

Resistance to the bactericidal activity of serum is an important factor in bacterial virulence [4]. Results obtained in this study demonstrate that most clinical isolates of *A. baumannii* exhibit intrinsic resistance to normal human serum and that few susceptible strains are isolated. The fact that EDTA rendered isolates susceptible to serum killing suggests that LPS is involved in the resistance in some way, as shown with other gram-negative bacteria [4,7]. For example, a serum-resistant isolate of *E. coli* became serum-sensitive after incubation in 10 mM EDTA, a procedure known to release LPS [7], and this treatment resulted in enhanced and stable binding of C5b-9 to the organism. The O side chains of LPS are known to play an important role in the virulence of *P. aeruginosa* by conferring resistance to the bactericidal effect of

Table 1. Bactericidal activity of pooled normal human sera on isolates of *A. baumannii*

Strain no.	Biotype	Pre-treatment	Viable cells at 3 h*
HJJA-7; HJJA-9; HS-54; ACA-37; HJJA-10	9	None EDTA BSS	≥ 100 ≥ 100 ≥ 100
HJJA-1; HS-10; HS-34; ACA34	9	None EDTA BSS	≥ 100 ≤ 0.1 ≤ 100
ACA-33	9	None EDTA BSS	< 0.1 < 0.1 < 0.1
HJJA-8	8	None EDTA BSS	> 100 > 100 > 100
HJJA-25; HS-4; HS-13	8	None EDTA BSS	≥ 100 ≤ 0.1 ≥ 100
ACA-7	18	None EDTA BSS	> 100 > 100 > 100
HJJA-14	17	None EDTA BSS	> 100 < 0.1 > 100
95-52	1	None EDTA BSS	> 100 < 0.1 > 100
93-70	5	None EDTA BSS	< 0.1 < 0.1 < 0.1
<i>E. coli</i> Garret		None EDTA BSS	< 0.1 < 0.1 < 0.1

*Calculated as a percentage of the viable cell number at t_0 , immediately after mixing the bacteria with the serum.

complement [10]. The O groups endow the long polysaccharide chain with strong hydrophilicity, an important property in both evading phagocytosis and resisting the activity of complement.

Jankowski *et al.* [11] found that 12% of clinical isolates of *A. calcoaceticus* var. *anitratus* (now *A. baumannii*) and 84% of *A. lwoffii* were susceptible to the complement-mediated (alternatively or classically activated) bactericidal activity of human serum. The authors employed an inoculum of 10^5 cfu/ml and categorised as susceptible those strains showing $>50\%$ killing in 3 h. In the present investigation, inocula of 10^7 cfu/ml were used, the incubation period was extended to 24 h and the criterion for killing was stricter. Nonetheless, the results were similar in that 11.1% of the *A. baumannii* strains were susceptible to human serum.

It is difficult to compare the results of the present study with those obtained by Traub *et al.* [6], as these authors used defibrinated blood. It is known that, under these conditions, several antibacterial mechanisms, such as the complement lytic system, antibodies, phagocytes and associated low mol. wt antibacterial peptides (like defensins) and also iron deprivation might be operative.

It is known that the treatment of mucoid gram-negative

bacteria with BSS removes between 50 and $>90\%$ of the CPS, depending on the bacterial species [8]. This treatment has been used successfully in *A. baumannii* and *P. aeruginosa* for isolating plasmids. Thus, the lack of effect of BSS on the serum resistance of *A. baumannii* suggests that CPS does not participate in this property, as has been demonstrated for other bacteria [5]. The effect of other bacterial components, such as outer-membrane proteins, in the resistance to serum killing cannot be ruled out.

The results of the present study suggest that the LPS of *A. baumannii* strains isolated from bacteraemic patients might be important in allowing the strains to survive in the blood and contributing to the pathogenesis of this species.

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