Immune response to *Acinetobacter calcoaceticus* infection in man

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Summary. After growth in an iron-depleted chemically-defined medium *Acinetobacter calcoaceticus* expressed four high mol. wt outer-membrane proteins (OMPs) which were repressed under iron supplementation or in a complex laboratory medium. Immunoblotting with serum from a septicaemic patient infected with *A. calcoaceticus* revealed antibody binding to these iron-repressible OMPs, indicating that they were expressed *in vivo*, and also to the 42- and 18-Kda OMPs. Although the antibody response to the OMPs did not vary significantly during convalescence, the response to the O-polysaccharide component of lipopolysaccharide decreased significantly. However, antibodies in serum from patients with *A. calcoaceticus* wound infections reacted with the iron repressible OMPs and a 54-Kda antigen suggesting a difference in immune recognition between local and systemic infection.

Introduction

Acinetobacter strains are often found in soil and freshwater samples, and commonly constitute part of the normal human flora.¹ Although many clinical isolates reflect colonisation rather than infection, an increasing number of reports^{1–7} suggest that *Acinetobacter* spp. have become important nosocomial pathogens. Patient groups most susceptible to infection are predominantly those immunocompromised through trauma, major surgery, burns or malignancies.¹ The lower respiratory tract is a common site of infection, particularly in patients with tracheostomies and endotracheal tubes.³ A rapid increase in resistance of *A. calcoaceticus*, and in particular of the biovar *anitratus*, to many antibacterial drugs has been noted.² However, despite increasing evidence that *Acinetobacter* infections are difficult to treat, there have been few reports of the host immune response to them. Brade and Galanos⁸ identified a lipopolysaccharide (LPS) antigen in a passive haemolysis assay. Antibodies raised in rabbits to it could be removed only partially by adsorption with enterobacterial lipid A, indicating the presence of an additional antigen. Antibodies to this antigen were present in different uninfected animals, including man. It has been reported that *A. calcoaceticus* can enhance the virulence of other bacteria in mixed infections, perhaps by slime-induced inhibition of neutrophils.⁹ However, the mechanisms of virulence of *Acinetobacter* *in vivo* are only poorly understood.

The ability to adapt to, and multiply in, the tissues

of the host is a pre-requisite for pathogenicity.¹⁰ Although the nature of the host environment is ill-defined, it is widely recognised that for many bacterial species, in the absence of specific uptake systems, the amount of freely available iron is too small to sustain growth.¹¹ An iron-restricted environment induces several phenotypic changes, including changes in the composition of the outer membrane (OM) of bacteria growing *in vivo*.¹²

We have shown recently that a clinical isolate of *A. calcoaceticus* produced the iron-chelator 2,3-dihydroxybenzoic acid (DHBA) when grown *in vitro* in an iron-depleted chemically-defined medium.¹³ Disk-diffusion studies with Fe-DHBA chelates showed that this compound could overcome 2,2-dipyridyl-induced growth inhibition, suggesting that it was functioning as a siderophore. A preliminary examination of Sarkosyl-insoluble OM fractions revealed several proteins of 77–87 Kda, which were repressed under iron-replete growth conditions.

In this report we characterise further the phenotypic changes in the OM in response to growth in iron-depleted media and examine the human immune response to both protein (OMP) and LPS components. We report the presence of IgG antibodies against the iron-regulated outer-membrane proteins (IROMPs) and the other major OMPs.

Materials and methods

Bacterial strains and growth conditions

A. calcoaceticus strains 1–11 from blood and 1–12, 1–13, 1–14, 1–15 and 1–16 from wounds were isolated

from infected patients at the Queen Elizabeth Hospital and the Accident Hospital, Birmingham. Strains were routinely maintained on nutrient-agar slopes at 4°C and cultured in either a modified iron-depleted chemically-defined medium (CDM-Fe)¹⁴ (pH 7.4) consisting of: 40 mM glucose; 0.62 mM KCl; 0.5 mM NaCl; 3.2 mM K₂HPO₄; 40 mM (NH₄)₂SO₄; 0.4 mM MgSO₄; 50 mM 3-(N-morpholino) propane sulphonic acid; and arginine, aspartic acid, proline and glutamic acid each at 100 µg/ml; or in chelex-treated tryptone soy broth (TSB-Fe).¹⁵ Iron-replete bacteria were those grown in TSB-Fe or CDM-Fe supplemented with 1 µM FeSO₄ (TSB+Fe or CDM+Fe). Bacteria were grown in TSB+Fe, TSB-Fe, CDM+Fe and CDM-Fe to early stationary phase (E₄₇₀ 0.9) in an orbital shaking incubator at 37°C, harvested by centrifugation at 10 000 *g* for 10 min and washed once with saline.

Preparation of outer membranes

The washed bacterial pellet was resuspended in 20 ml of distilled water and broken by 10 60-s pulses of sonication in an ice bath, with 60-s intervals for cooling. Unbroken cells were removed by centrifugation at 10 000 *g* for 10 min. Sarkosyl (sodium N-lauryl sarcosinate, Sigma) was added to the supernate to a final concentration of 2% w/v. The mixture was incubated for 1 h at room temperature and then centrifuged at 100 000 *g* for 1 h.¹⁶ The OM pellets were washed twice by resuspension in distilled water and centrifugation at 100 000 *g* for 1 h and were stored at -20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

OM preparations were electrophoresed according to the method of Lugtenberg *et al.*¹⁷ as modified by Anwar *et al.*¹⁸ on acrylamide 12% w/v gels. Each lane was loaded with c. 2.5 µg of protein. For visualisation of LPS antigenic sites, OMs were heated at 100°C for 10 min in denaturing buffer containing SDS 2.5% w/v and 2-mercaptoethanol 2% v/v, cooled and then incubated with proteinase K (1 mg/ml, Sigma) for 60 min at 60°C before electrophoresis. Separated OMPs were either stained with Coomassie Blue R-250 0.1% w/v in methanol:acetic acid:water 50:10:40 or used for immunoblotting. Separated LPS was used for immunoblotting only.

Immunoblotting

OMPs or LPS separated on polyacrylamide gels were transferred to nitrocellulose (NC) paper and the antigens were visualised by a modification of the method of Towbin *et al.*¹⁹ The NC paper was incubated at room temperature in Tris-buffered saline with Tween (TBS-Tween) for 1 h to saturate non-specific binding sites and then incubated overnight at 4°C with serum diluted 1 in 50 in TBS-Tween. The

NC paper was washed thoroughly with TBS and incubated for 2 h at 37°C with protein A-alkaline phosphatase (Sigma) 0.5 µg/ml in TBS-Tween. After incubation, the NC paper was thoroughly washed again. The antibody binding sites were visualised with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and nitro-blue tetrazolium (NBT, Sigma) in accordance with the manufacturer's instructions. As a control, replicate NC transfers were stained with amido black 1% w/v in methanol 10% w/v and acetic acid 7% v/v to show qualitative transfer of all proteins from the acrylamide gel to the NC paper; the NC replicates were used to identify the protein bands.

Serum

Blood was taken by venepuncture from patients infected with *A. calcoaceticus*. It was allowed to clot at 37°C for 2 h. After centrifugation at 2000 *g* for 10 min, the serum was collected and stored at -20°C.

Results

Effect of iron-depleted growth on *A. calcoaceticus* OMPs

The SDS-PAGE OMP profiles of *A. calcoaceticus* strain 1-11 grown in TSB+Fe (lane 1), TSB-Fe (lane 2), CDM+Fe (lane 3) and CDM-Fe (lane 4) are shown in fig. 1. The synthesis of four OMPs of 77, 78, 84 and 87 Kda was strongly induced in the OM of cells grown in CDM-Fe (lane 4). This did not occur to any significant extent in cells grown in either CDM+

Fig. 1. SDS-PAGE of OMPs of *A. calcoaceticus* strain 1-11 grown in TSB+Fe (lane 1), TSB-Fe (2), CDM+Fe (3) and CDM-Fe (4). OMPs were stained with Coomassie Blue. Numbers refer to mol. wts (Kda).

Fe (lane 3) or TSB + Fe (lane 1). These four proteins were also detected in the OM of cells grown in TSB - Fe (lane 2), although the 84-Kda OMP was not strongly expressed. Similar experiments with magnesium-limited cultures did not induce the expression of these four proteins (results not shown). These data indicate that expression of the four OMPs is not the result of poor growth in a defined medium, but rather that it arose from the specific depletion of iron. Iron depletion had no major effect on the expression of the other major OMPs of 54, 42, 35, 26 and 18 Kda. Similar OMP profiles were found with the other five clinical isolates of *A. calcoaceticus*.

Antibody response to *A. calcoaceticus* OM components

The antigens of *A. calcoaceticus* recognised by IgG antibodies in the serum of the patient 10 days after diagnosis of septicaemia are shown in fig. 2. IgG antibodies strongly recognised the 78- and 87-Kda IROMPs in *A. calcoaceticus* grown in conditions of iron depletion (lanes 2 and 4) but these antigens were undetectable or only barely detectable in iron replete cells (lanes 1 and 3). Of the lower mol. wt OMPs, only the 42- and 18-Kda proteins reacted strongly with IgG antibodies.

Strip immunoblotting was performed to measure any changes in antibody response during post-infection convalescence. Serum samples were taken up to

Fig. 2. Immunoblot of the OMPs in fig. 1 (lanes 1-4 as described) probed with serum from patient 1-11, obtained 10 days after diagnosis of septicaemia. Reactions were visualised with alkaline phosphatase-conjugated *S. aureus* protein A. Numbers refer to mol. wts (Kda).

54 days after diagnosis of septicaemia. The IgG antibody response with time to OMPs of *A. calcoaceticus* strain 1-11 grown in CDM - Fe is shown in fig. 3A. No major differences were seen although an increased response with time to a 100-Kda OMP was noted. The IgG anti-LPS activity against the same OM preparation, but after proteinase K digestion, is shown in fig. 3B. Strong responses to both the O-polysaccharide and core/lipid A LPS components were noted at diagnosis. These were greatly diminished during recovery from infection, particularly in the sample taken 54 days after diagnosis.

An immunoblot of the other five clinical isolates of *A. calcoaceticus* from patients with local wound infections is shown in fig. 4. The isolates were grown in CDM - Fe and the NC paper containing the electrophoresed OMPs was subsequently incubated with serum from one of the patients (1-12) taken 10 days after diagnosis of infection. The 87-Kda IROMP from all five isolates together with the 54-Kda OMP were strongly recognised by IgG antibodies. The 42-Kda OMP was detected only weakly. Essentially

Fig. 3. Strip immunoblot of OMPs (A) and LPS (B) of *A. calcoaceticus* strain 1-11 cultivated in CDM - Fe and probed with serum from patient 1-11, obtained at various time intervals after diagnosis of infection. Reactions were visualised with alkaline phosphatase-conjugated *S. aureus* protein A. Numbers refer to mol. wts (Kda).

Fig. 4. Immunoblot of OMPs of clinical isolates of *A. calcoaceticus* from local wounds of patient 1-12 (lane 1), 1-13 (2), 1-14 (3), 1-15 (4) and 1-16 (5) grown in CDM-Fe and probed with serum from patient 1-14 obtained 10 days after onset of infection. Numbers refer to mol. wts (Kda).

similar results were noted with replicate immunoblots probed with sera from the other patients.

Discussion

Information concerning both the pathogenic mechanisms of *A. calcoaceticus* and the host immune response to infection is scant. Immunoblotting techniques are used widely to determine antibody responses to cellular components, particularly the OM of gram-negative bacteria. However, in circumstances where sufficient bacteria cannot be recovered *in situ* from infections, the validity of this method is critically dependent on how the organisms are grown *in vitro*. Indeed, such results may be misleading in light of the well known phenotypic plasticity of the bacterial envelope.¹² The results of this investigation indicated clearly that *A. calcoaceticus* responded to iron-depleted growth conditions by inducing the expression of 77-87-Kda OMPs which were repressed under iron replete conditions. Although a new finding for *A. calcoaceticus*, it was not surprising. A large number of different bacteria have been shown to adapt to iron-restricted conditions by synthesising one or more high mol. wt IROMPs.¹¹ These proteins are thought to function as receptors to facilitate the internalisation of Fe-siderophore complexes, and this has been proven for certain bacteria, notably *E. coli*.²⁰ In a previous report¹³ we showed that *A. calcoaceticus* produced 2,3-dihydroxybenzoic acid (DHBA) when grown under iron-depleted conditions and this functioned as a siderophore. These data led us to suggest that one or more of the high mol. wt IROMPs might be a receptor

for this siderophore. At this stage it is not clear whether *A. calcoaceticus* can utilise siderophore molecules only produced by itself or whether it can acquire iron from exogenously supplied siderophores. Such a situation would be analogous to *E. coli* using the fungal siderophore ferrichrome via the 78-Kda *Fhu* IROMP receptor.²¹ Interestingly, it has been shown recently that *E. coli* is able to take up Fe-DHBA via *Fiu* and *Cir* receptors, and less efficiently via the *FepA* receptor, indicating a lack of absolute receptor specificity in this species.²² The demonstration of siderophore production in *A. calcoaceticus* provides an interesting contrast with that of the other members of the Neisseriaceae that are capable of removing iron from host binding proteins by a siderophore-independent mechanism.²³

With the exception of the high mol. wt IROMPs identified in this study, the lower mol. wt OMPs were of a similar size to those of *Acinetobacter* SP. FO-1 and *A. calcoaceticus* ATCC 23055²⁴ and *A. calcoaceticus* 69V.²⁵ The cell envelope profiles of the six clinical isolates in this study did not exhibit the marked heterogeneity reported by Dijkshoorn *et al.*²⁶ in their study of 78 clinical isolates. The OMP profiles appear similar to the "pattern D" established by these workers. The similarity of OMP profiles with those published previously confirms the usefulness of sarkosyl extraction to prepare *A. calcoaceticus* OMs. In agreement with Nishimura *et al.*²⁴ we found that the major 42-Kda OMP was heat modifiable, as was the 26-Kda OMP. Both proteins appeared in their fully modified form after treatment at 100°C for 10 min. None of the IROMPs was heat modifiable. We did not observe with the clinical isolates in this study heat modification of the 18-Kda OMP in contrast to that in *A. calcoaceticus* 69V.²⁵ This OMP also interacts with LPS, although the affinity is strain-dependent.²⁵ Assessment of the non-covalent association with peptidoglycan (PG) was made by measuring the effect of temperature on the solubilisation of OMs in the presence of SDS. Of the OMPs identified in this report, only the 42-Kda OMP was associated with PG. This feature suggests that it may function as a transmembrane pore protein.

Immunoblotting with the convalescent patient's sera revealed the presence of IgG antibodies directed against the IROMPs and showed that the 87-Kda IROMP was only partially repressed after growth in TSB+Fe, whereas the 77-Kda IROMP was fully repressed. These data indicate that *A. calcoaceticus* expresses these IROMPs *in vivo* in septicaemia and local wound infections. This finding of iron-restricted growth *in vivo* is a common feature of many gram-negative bacterial infections.^{18,27-33} However, this ability to adapt to and overcome the severely iron-restricted conditions of the human host must not be overlooked as an important determinant of virulence and disease pathogenesis for *A. calcoaceticus*.

Our immunoblotting studies did not indicate any significant differences in the antibody response to

OMPs during convalescence. The particularly strong response found with the "pre-immune" serum sample taken at diagnosis of septicaemia can be interpreted in two ways. Firstly, the patient may have been exposed to *A. calcoaceticus* prior to diagnosis of septicaemia, by, for example, a sub-clinical local infection, and therefore have already started to generate an immune response, or, secondly, the organism may have been part of the normal human flora. When pooled serum was taken from 20 healthy subjects and used in immunoblotting studies, a weak IgG antibody response to the major OMPs was noted, probably reflecting the colonisation of a proportion of the population.¹ The IgG anti-LPS response decreased during the convalescent period. Interestingly, our immunoblotting studies revealed the presence of O-polysaccharide bands in OMs after digestion with proteinase K. This finding of smooth-type LPS is in contrast to the R-like LPS isolated from strain *Acinetobacter* NCTC 10305.³⁴ It is not clear whether the structure of LPS varies between strains or depends on the method of preparation. It is possible that the immunoblotting technique affords a greater limit of detection than silver staining.

During this study an interesting difference between the immune response to a local infection and septicaemia became apparent. The IROMPs were detected in both cases. However, in septicaemia, only the 42- and 18-Kda OMPs were detected, whereas with a local

infection, the 54-Kda OMP was the major antigen and the 42- and 18-Kda OMPs were only weakly detectable. Caution must be exercised in interpreting the data in this study from only one septicaemic patient. However, it may prove possible to distinguish colonisation or local infection from septicaemia on the basis of the immune response. Furthermore, the immunoblotting study of serum from one locally-infected patient against the OMs from five wound isolates indicates that the major OMPs are immunologically cross-reactive.

Our finding that *A. calcoaceticus* expresses several IROMPs when grown under iron-depleted conditions *in vitro* and that these IROMPs are recognised by IgG antibodies in convalescent sera from infected patients, suggests that they are expressed *in vivo*. Of all growth requirements known, iron is probably the most thoroughly studied. Further work is required to determine whether other components, yet to be identified, such as extracellular virulence factor production are iron-regulated and what are the functions of the anti-IROMP antibodies. Such studies should improve our knowledge of this important nosocomial pathogen.

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