Identification and Typing of Bacteria

Biovar-specific epitopes of the urease enzyme of Ureaplasma urealyticum

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The importance of Ureaplasma urealyticum as a pathogen in premature neonates and patients with a profound defect in humoral immunity has, over the last few years, become well recognised. U. urealyticum is unique amongst the Mycoplasmataceae for its use of urea metabolism as an essential source of energy. The urease enzyme responsible for this is, therefore, of prime importance and any variability in expression of this enzyme may play a role in virulence of the organism. U. urealyticum is divided into 14 serovars comprising two biovars - the parvo-biovar and T960-biovar. In this study monoclonal antibodies (MAbs) were produced against the urease enzyme. Two distinct epitopes of the 72-kDa α-subunit were recognised by three different MAbs. Under denaturing conditions both epitopes were shown to be specific for the parvo-biovar.

Introduction

Ureaplasma urealyticum colonises the urogenital tract of 40–80% of sexually active females [1]. Males infected with U. urealyticum present with urethritis and, in individuals with hypogammaglobulinaemia, infection may progress to a septic arthritis [2]. In premature neonates, particularly those with a birth-weight <1500 g, U. urealyticum has been shown to cause pneumonia, disseminated disease and meningitis and has been described as a cause of death in such patients [3]. Furthermore, U. urealyticum is causally associated with premature birth and with spontaneous abortion [4]. A relationship between colonisation of the urogenital tract (UGT) in pregnant women and premature delivery has not been demonstrated, but there is a probable link between ureaplasma amnionitis and premature labour [5]. In published studies, no correlation between U. urealyticum serovar and disease has been shown [4] and it has been suggested that virulence is related to antigen variation within a serovar rather than to differences between the serovars [6]. However, the typing of U. urealyticum serovars is not standardised; it is technically difficult and, particularly in the case of serovars 11 and 13, it is unreliable. Furthermore, clinical isolates may show multiple serovar specificities due to cross-reactivity [7].

The 14 serovars are divided into two biovars on the basis of DNA-DNA homology [8], restriction endonuclease DNA digestion [9] and sensitivity to manganese salts [10]. The parvo-biovar contains the serovars 1, 3, 6 and 14 and the T960-biovar the remaining 10 serovars. It has been proposed that the parvo-biovar be designated a separate species - Ureaplasma parvum [11]. A simple method to identify the parvo-biovar would thus be useful. In this study monoclonal antibodies (MAbs) to the urease enzyme of U. urealyticum were sought to recognise distinct epitopes specific for the parvo-biovar.

Materials and methods

Organisms and media

U. urealyticum serovars 7 (ATCC 27819), 8 (ATCC 27618), 9 (ATCC 33175) and 14 (ATCC 33697) were obtained from the American Type Culture Collection (Rockville, MD, USA), serovars 10 (Western CX3), 11 (JSL-K2-CX3), 12 (JSL-U24-CX3) and 13 (JSL-U38-CX3) were kindly supplied by Professor J. Robertson, University of Edmonton, Alberta, Canada and serovars 1 (M54/92), 2 (23 T II), 3 (23 Sero III), 4 (354 T IV), 5 (Sero IV) and 6 (Pirillo T IV) were obtained from the Central Public Health Laboratories, 61 Colindale Avenue, London. Ureaplasms were grown in Shepard's 10C broth [12] or in a serum-poor (1%) medium according to Davis et al. [13].
Monoclonal antibodies

Six week old, male BALB/c mice were immunised by intra-peritoneal injection of a suspension of complete Freund's adjuvant (CFA) and *U urealyticum* serovar 14 protein. The *U urealyticum* protein was prepared as detailed below and 75 μg protein were used for each immunisation. The mice were immunised a total of four times at 3-week intervals, CFA being replaced after the first immunisation by incomplete Freund's adjuvant. Blood from the immunised mice was obtained from the retro-orbital venous plexus to determine the serum antibody titre. All procedures performed on the animals conformed with the federal German animal protection regulations. Three days after the final boost the mouse was killed, the spleen was removed and the spleen cells were harvested and washed in Dulbecco's modified Eagle's medium (DMEM). These cells were then fused by a standard PEG fusion protocol with the myeloma cell line X63-Ag8 653. An ELISA (see below) with *U urealyticum* protein as the solid phase antigen was used to screen the hybridoma supernatants for ureaplasma-specific antibody. All hybridomas producing ureaplasma-specific antibody were cloned twice and further characterised. Particular care was taken to exclude cross-reactivity with horse serum. For the production of MAbs, the hybridomas were grown to exhaustion in DMEM containing fetal calf serum. For the production of MAbs, the hybridomas were grown to exhaustion in DMEM containing fetal calf serum 5%. MAbs were purified with protein G sepharose beads (Pharmacia) and labelled with biotin (Sigma).

Preparation of ureaplasma proteins

Sediment of *U urealyticum* cultures in logarithmic growth phase was collected by centrifugation (20,000 g for 35 min, 4°C) washed twice in phosphate-buffered saline (PBS) and resuspended in PBS to a final concentration of 1 mg/ml. The protein concentration was determined by the method of Bradford [14].

SDS-PAGE

Proteins (30 μg/lane) in the ureaplasma sediment were heated for 5 min at 95°C with Laemmlı buffer containing β-mercaptoethanol 5% v/v and separated electrophoretically on polyacrylamide 7.5% gels [15]. The proteins were then either stained with Coomassie Brilliant Blue dye or transferred to nitrocellulose for immunostaining [16].

Immunoblots

After transfer of the electrophoretically separated proteins to nitrocellulose, the membranes were incubated with skimmed milk 5% solution, washed in PBS and then incubated with MAb. Bound MAb was detected with peroxidase-labelled goat anti-mouse antibody (Dianova) and developed with 4-chloro-1-naphthol. To determine whether the antigens recognised were localised in the membrane or cytoplasm, freshly harvested ureaplasma cells were lysed osmotically and the membranes were sedimented by ultracentrifugation at 34,000 g [17]. The proteins of the membrane fraction (sediment) and the cytoplasmic fraction (supernate) were separated by SDS-PAGE. The separated proteins were then transferred to nitrocellulose and immunostained with the MAb.

Antigen purification and identification

The 72-kDa antigen recognised by the three MAbs (BG10, DB7 and VB10) was purified by SDS-PAGE. Briefly, *U urealyticum* serovar 14 sediment was electrophoresed on preparative polyacrylamide gels and the respective protein bands (72 kDa) were cut out, re-electrophoresed and transferred to a nylon membrane (Immobilon®). The resulting bands were marked by staining with amido black and the N-terminal amino acid sequence was determined by the Edman method [18].

ELISA

An ELISA with ureaplasma protein as the solid phase antigen was used to screen the hybridoma supernatants and to determine the cross-reactivity of the MAb with ureaplasma serovars other than serovar 14 or with species of the *Mycoplasma* genus. Protein (0.5 μg/well) was bound overnight at 4°C to 96-well microtitration plates (Nunc). After washing the wells with PBS (without detergent) the wells were blocked with skimmed milk 1% solution. The antibody-containing solution was incubated for 1 h with agitation at room temperature, followed by a further wash step and incubation with peroxidase-labelled goat anti-mouse antibody. Bound antibody was detected by a colour change of the substrate orthophenylenediamine (OPD) read at 492 nm with an Immunoreader NJ 2000 (Nunc).

Competition ELISA

The optimal dilution of biotinylated MAb and solid phase ureaplasma protein concentration (at which solid phase antigen was limiting) was determined by a checker-board titration. The ureaplasma protein solid phase concentration was found to be optimal at 0.25 μg/well at a MAb concentration of 0.058 mg/ml (BG10), 0.27 mg/ml (VB10) and 0.025 mg/ml (DB7). Antigen was bound overnight at 4°C to the wells of 96-well microtitration plates (Nunc). The wells were then washed with PBS and blocked with skimmed milk 1% solution. Purified unlabelled MAb was serially diluted in PBS and added in equal volume to the biotinylated MAb. This was then incubated with the solid phase antigen for 2 h. After washing, the bound biotinylated MAb was detected with peroxidase-labelled streptavidin and the colour change of the substrate OPD was measured at 492 nm with an Immunoreader NJ 2000.
Results

Hybridomas and MAb

Seven stable hybridomas producing ureaplasma-specific antibody were cloned. The four MAb used in the experiments reported here were VB10, BG10, DB7 and GB2. MAb DB7 was an IgM antibody and the other three were IgG1.

SDS-PAGE and immunoblots

Immunoblots of the separated membrane and cytoplasmic fraction proteins showed clearly that the antigen recognised by MAbs VB10, DB7 and BG10 belonged to the cytoplasmic fraction. A typical result obtained with MAb BG10 is shown in Fig. 1. MAb GB2, which recognises a membrane protein, demonstrates the effective separation of the membrane and cytoplasmic proteins.

Fig. 2 shows a Coomassie Brilliant Blue-stained SDS-PAGE of the 14 U. urealyticum serovars and an immunoblot probed with MAb VB10. Despite the similar quantity of protein in each lane, the immunoblot demonstrates an intense 72-kDa band in serovars 1, 3, 6 and 14 (the serovars of the parvo-biovar). A slight degree of cross-reactivity is seen in serovars 8, 9 and 11, for which the bands are both less intensely stained and narrower. The results for MAbs VB10 and DB7 were identical to those shown in Fig. 2 for MAb VB10. Interestingly, the binding pattern of the MAb to the 14 serovars in ELISA based on non-denatured antigen did not demonstrate this biovar specificity and some (but not all) of the serovars of the T960-biovar produced equally high reactivity as those of the parvo-biovar (Table 1).

Urease α-subunit identification

Fifteen amino acids of the 72-kDa protein N-terminal end were identified by the Edman process and a homology search was performed with the computer program PC/Gene® 6.5 and the CD PROT 24 protein sequence databank (Mountainview, CA, USA). Complete homology was found with the α-subunit of the urease enzyme of U. urealyticum [19]. The amino-acid sequence of the 72-kDa protein N-terminal was compared with the known sequence of the urease α-subunit in Fig. 3.

Competition ELISA

The three MAbs (BG10, DB7 and VB10) all bound to the α-subunit of the urease enzyme. To determine if they recognised different epitopes, labelled MAb was used in a competition ELISA with non-labelled MAb. Fig. 4a shows the results obtained when biotinylated MAb VB10 was incubated with serially diluted, unlabelled BG10 or DB7. The results demonstrate that MAb BG10 competed with the labelled MAb VB10 for binding to a common epitope. The possibility that the two antibodies recognised two distinct epitopes located close to each other and thus antibody binding to one epitope resulted in steric inhibition of antibody binding to the second epitope cannot be excluded. MAb DB7 did not compete with biotinylated MAb VB10 for binding to its epitope and thus recognised a distinct epitope. This was confirmed by a further experiment in...
Fig. 2. Coomassie Brilliant Blue-stained SDS-PAGE (A) and immunoblot probed with MAb VB10 (B) of ureaplasmal serovars. SDS-PAGE was performed on 7.5% w/v gels and 15 μg of ureaplasmal proteins were electrophoresed in each lane. In the Coomassie-stained gel the left-hand lane shows the high mol wt marker.

Table 1. Reactivity of MAbs in ELISA with non-denatured U. urealyticum antigen

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Extinction: 0-0.09 = -; 0.1-0.49 = +; 0.5-0.99 = 2+; 1.0-1.49 = 3+; >1.49 = 4+.

U. u

| P70 | M F K I S R K N Y S D L X G I |

Fig. 3. Alignment of the N-terminal amino-acid sequence of the purified 72-kDa protein with the protein sequence of the urease enzyme of U. urealyticum in the CD PROT 24 protein sequence databank. Homology is found with the first 15 amino acids. U. u: first 20 amino acids of the U. urealyticum urease α-subunit sequence [19]; P70: N-terminal amino-acid sequence as determined by the Edman method.

which biotinylated MAb DB7 was incubated in competition with unlabelled MAbs VB10 and BG10. The labelled MAb DB7 was not prevented from binding to its antigen by either MAb (Fig. 4b). As a positive control in these experiments, the biotinylated MAb was incubated in competition with its own unlabelled form, i.e., in Fig. 4a, the biotinylated MAb VB10 competed for its epitope with unlabelled MAb VB10 and in Fig. 4b labelled MAb DB7 competed with unlabelled MAb DB7. To exclude the possibility that
Fig. 4. Competition ELISA between biotinylated MAb and serially diluted unlabelled MAb. Ureaplasma antigen was the solid phase antigen (0.25 μg/well) and the starting concentration for MAb GB10 (▼) was 0.58 mg/ml and for DB7 (▲) was 0.25 mg/ml. a, Biotinylated MAb VB10 competing for its epitope with serially diluted unlabelled MAb GB10 but not with unlabelled MAb DB7; b, confirms that biotinylated MAb DB7 did not compete with MAb VB10 and furthermore shows that labelled MAb DB7 did not compete with unlabelled MAb BG10. Bound biotinylated MAb was detected by peroxidase-labelled streptavidin. As positive controls (■), biotinylated MAb were incubated with unlabelled MAb VB10 (a) and unlabelled DB7 (b) and as a negative control (♦) with MAb GB2 which recognises an unrelated membrane antigen.

antibody binding to an epitope not present in the urease molecule might also inhibit binding of the MAb under investigation, a MAb specific for a surface protein was used as a negative control (GB2).

Discussion

Little is known about the serovar- or biovar-specific antigens of _U. urealyticum_. The serovar-specific antigens appear to be on or in the surface membrane and they may be either lipid or proteinaceous [20]. Two-dimensional gel electrophoresis has shown conflicting results with serovar-specific proteins, biovar-specific proteins and proteins common to all serovars [21]. Watson _et al._ [22] have described a 71-kDa variable antigen which has both serovar-specific as well as cross-reactive epitopes. This antigen is also surface localised. Thirkell _et al._ [23] identified a 96-kDa surface protein which was specific for serovar 8. This paper describes an intracellular cytoplasmic antigen, the urease α-subunit, which has at least two distinct epitopes, the primary structures of which are parvo-biovar specific.

The urease enzyme is a metallo-enzyme containing nickel in its core. It consists of three subunits, α, β and γ with an Mr of 72, 14 and 11 kDa, respectively. The β- and γ-subunits appear not to be immunogenic and no antibodies have been described that recognise them. Several epitopes of the α-subunit have been described [24]. The MAbs described in this paper recognised two epitopes of the α-subunit, as demonstrated by competition ELISA. The interesting feature of these epitopes is their biovar-specific nature under denaturing conditions. Thirkell _et al._ described a MAb against urease that showed a similar phenomenon in which the serovars 3, 6 and 14 were not recognised under denaturing conditions [25]. This indicates that the primary structure of the urease α-subunit has both parvo- and T960-biovar specific sequences.

This is perhaps not surprising, given the different urease gene structure between the two biovars. Neyrolles _et al._ [26] found that the urease genes of serovar 1, as a representative of the parvo-biovar, contained four additional genes, ureE, ureF, ureG and ureH, compared to those of serovar 8. These genes putatively code for accessory proteins which play a role in the synthesis of the nickel metallo-centre. Given that these structures are likely to be hidden from antibody in the tertiary folded state, it may be hypothesised that they contain the epitopes described in this paper. In addition, Neyrolles _et al._ found polymorphism in the promoters of the operons for these genes, suggesting a possible difference in the level of expression between the biovars. The proposition that urease expression may not be constitutive, as previously thought, is supported to some degree by the difference in the quantity of urease α-subunit seen in Fig. 2. In serovars 8, 9 and 10, the signal intensity is weak, indicating possible cross-reactivity of the antibodies. However, the bands were also markedly narrower, which would indicate that less protein was present. Neyrolles _et al._ [26] also showed the presence of ureD, a gene suggested to have a regulatory function on the urease operon, in serovar 1 (parvo-biovar) and not in serovar 8 (T960-biovar), and this would perhaps support the suggestion that the parvo-biovar can upregulate its urease expression. This paper describes two epitopes of an intracellular protein identified to be the α-subunit of the urease enzyme that are, under denaturing conditions, specific for the parvo-biovar. These findings support previous reports of a difference in urease between the biovars at a genetic level. The existence of biovar-specific epitopes at a primary (denatured) level has been shown only
for the T960-biovar [25] and to our knowledge these are the first MAbs shown to recognise parvo-biovar-specific epitopes. These epitopes may help in resolving possible differences in virulence between the two biovars and furthermore in easily identifying the proposed new ureaplasma species U. parvum [11].

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References