BACTERIAL PATHOGENICITY

Characterisation and protective capacity of monoclonal antibodies elicited in mice against protein epitopes of antibiotic-exposed *Escherichia coli*

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The binding capacity and the protective activity of three monoclonal antibodies (MAbs) – ARM 1-4, ARM 1-7 and ARM 2-2 – obtained from spleen cells of mice immunised with *Escherichia coli* O6:K– pre-treated with sub-MIC of aztreonam were studied. The MAbs belonged to IgG₁ isotype and showed different reactivity toward protein epitopes of *E. coli* in an immunoblotting assay. ARM 1-4 recognised epitopes on molecules of 30 kDa and 40 kDa. ARM 1-7 identified an epitope of a molecule of 41 kDa, and ARM 2-2 recognised epitopes of molecules of 15 kDa and 41 kDa. In ELISA the MAbs cross-reacted with *E. coli* O7:K–, *E. coli* O111:B4 and *E. coli* O128:K– with different binding affinity. Furthermore, the MAbs showed complement-dependent bactericidal activity. The MAbs displayed different protective capacities when given to mice 90 min before lethal challenges with $2 \times \text{LD}_{50}$, $4 \times \text{LD}_{50}$ and $8 \times \text{LD}_{50}$ of *E. coli* strains. In all but one instance (ARM 1-4 versus *E. coli* O7:K–) it was not possible to correlate protective capacity with binding affinity of a MAb to a given bacterial cell. Therefore, the epitopes recognised by the MAb may be more closely associated with bacterial virulence than in binding to the bacterial cell.

Introduction

Gram-negative bacterial infections often progress to serious illness, septic shock and death [1, 2].

In bacterial resistance against host defence, an essential role is played by the outer structures of gram-negative bacteria. Lipopolysaccharides (LPS) are considered major antigenic and toxic components. Immunotherapy with antibodies against endotoxin improves survival in animal models [3–6] and several studies have indicated the importance of the immune response against outer-membrane proteins (OMPs) in protection against gram-negative bacteria [7–12].

In a previous study it was observed that MT 1-F, a monoclonal antibody (MAb) raised in mice after immunisation with antibiotic-treated *Escherichia coli* O6:K– recognised a protein epitope of 12 kDa and

conferred protection against lethal *E. coli* infections [13].

The present study was undertaken to analyse the protective activity of three murine MAbs, ARM 1-4, ARM 1-7 and ARM 2-2, from spleen cells obtained from mice immunised with antibiotic-treated *E. coli* O6:K–. The MAbs recognised protein structures of untreated *E. coli* O6:K– with different mol. wts, and were used in an animal model to study their activity against lethal challenge with homologous and heterologous strains of *E. coli*.

Materials and methods

Bacteria

E. coli O6:K– (ATCC 25922, Difco Laboratories) was used for immunisation of mice. *E. coli* O7:K– and *E. coli* O111:B4 were a kind gift of Professor J. Verhoef (Eijkman-Winkler Laboratory for Medical Microbiology, University Hospital of Utrecht, The Netherlands); *E. coli* O128 was kindly provided by Professor A. Caprioli, (Istituto Superiore Sanità, Rome).

Immunisation procedure

The immunisation procedure was described previously [14]. *E. coli* O6:K–, either untreated or grown overnight in the presence of $0.5 \times \text{MIC}$ of aztreonam (Bristol-Myers Squibb, Rome, Italy; $\text{MIC} = 0.078 \text{ mg/L}$) was killed in formalin 0.2%, washed three times in sterile saline and adjusted to an optical density (OD) equivalent to 10^9 cfu/ml. The bacterial suspension was injected intraperitoneally (i.p.) into female BALB/c mice according to the following scheme: day 1, 10^7 cfu/mouse; day 7, 2×10^7 cfu/mouse; days 14, 21, 35 and 56, 1×10^8 cfu/mouse. On day 42 of the immunisation procedure, 10 mice from each group were anaesthetised with diethyl ether and bled by retro-orbital puncture to collect serum.

Production and selection of hybridomas

Hybridomas were produced by a minor modification of the method of Fazekas De St Groth and Scheidegger [15]. Spleen cells from mice immunised with antibiotic-treated *E. coli* O6:K– were fused with mouse myeloma SP1 cells. The supernates of wells containing growing clones were tested by ELISA for antibodies directed against untreated *E. coli* O6:K–. The antibody-producing clones were subcloned by limiting dilution and, after growth, 3×10^6 cells were injected i.p. into normal mice, which had been primed 7 days before by i.p. injection of 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane; Sigma) [16]. The ascitic fluids obtained were collected aseptically and stored at -20°C . Antibody isotyping was performed with the Monoclonal Antibody Isotyping Kit I (HRP/ABTS, Pierce Chemical Co., Rockford, IL, USA) by a double immuno-diffusion technique. MABs from ascitic fluid were purified by protein G column chromatography (Immunopure plus IgG purification Kit; Pierce) [17].

ELISA

The binding of MABs to bacterial cells was determined by ELISA as described previously [18]. Viable, untreated *E. coli* O6:K–, *E. coli* O7:K–, *E. coli* O111:B4 and *E. coli* O128:K–, (10^7 cfu/ml in coating buffer solution) were used to coat the microtitration plates (Nunc, Nunc, Denmark). The reactivity of two-fold diluted MABs (starting concentration $5 \mu\text{g/ml}$) was measured in a microtitration plate reader (Easy Beam, STL-Lab Instrument, Innsbruck, Austria) at 492 nm with peroxidase-labelled goat anti-mouse IgG conjugate (BioRad, Laboratories Milan, Italy). Optical densities (OD) below 0.2 were considered negative. Endpoint titres in ELISA at OD 0.7 were calculated by linear regression with a spreadsheet computer programme and expressed as $\log_2$ dilution factor (DF).

Immunoblotting

Proteins of untreated *E. coli* O6:K– were resolved by PAGE under reducing conditions in slab gels [19]. The

material was transferred to nitrocellulose paper [20] and strips were incubated for 18 h at room temperature with the test MABs. The reactivities were detected by enhanced chemiluminescence (ECL, Amersham International Plc, Airlington Heights, IL, USA), as described previously [21].

In further experiments, proteins of untreated *E. coli* O6:K– (1 mg/ml) were digested with proteinase K ($2.5 \mu\text{g/ml}$) for 1 h, the material was transferred to nitrocellulose by electrophoresis, and the reactivities of the MABs were tested as described above.

Bactericidal assay

The bactericidal activity of MABs was measured as described previously [22]. A $100\text{-}\mu\text{l}$ sample of *E. coli* O6:K– (5×10^3 cfu/ml) was added to $100 \mu\text{l}$ of purified MAB (50 ng/ml) in the presence of $200 \mu\text{l}$ of guinea-pig serum as a complement source. After incubation for 45 min at 37°C in a water bath with shaking, $100 \mu\text{l}$ of the mixture was plated on blood agar. After incubation overnight at 37°C , the number of colonies was counted. Mouse SP1 myeloma-inducing ascitic fluid and guinea-pig serum alone were used as controls. Serum bactericidal activity of the test MABs was expressed as a relative percentage of the total amount of surviving bacteria incubated in buffer only.

In-vivo protection experiments

Female BALB/c mice (no. 450, Nossan, Correzzana, Italy) 6–8 weeks old, average weight 25 g, were used. The mice were housed in groups of 10 and provided with food (Standard pellets; Dottor Piccioni, Gessate, Italy) and water *ad libitum*. In passive protection experiments, doses of $2 \times \text{LD}_{50}$, $4 \times \text{LD}_{50}$ and $8 \times \text{LD}_{50}$ of live *E. coli* O6:K–, *E. coli* O7:K–, *E. coli* O111:B4 and *E. coli* O128:K– (the LD_{50} for these strains were 3×10^7 cfu/mouse, 2×10^8 cfu/mouse, 1×10^8 cfu/mouse, 1×10^8 cfu/mouse respectively) were injected into mice 90 min after i.p. injection of test MAB ($5 \mu\text{g/mouse}$). Ten female BALB/c mice given sterile saline instead of MAB served as a control group for each *E. coli* strain used. Mice were checked daily for 7 days and deaths were recorded [13].

All animal experiments were approved by the Institute's Ethical Committee. Care was taken to avoid any pain to the animals.

Statistical analysis

Data obtained after bacterial challenge were analysed by the χ^2 test. Data obtained in ELISA were expressed as the mean DF and the SD of three independent experiments done in duplicate and analysed by Student's *t* test; *p* values < 0.05 were considered significant.

Results

Characterisation of MAb

Around 100 hybrid clones were obtained by fusing splenocytes of mice immunised with antibiotic-treated *E. coli* O6:K- and SP1 plasmacytoma cells [13]. The clones considered in this report (ARM 1-4, ARM 1-7 and ARM 2-2) produced MAbs of IgG₁ isotype, as shown by immunodiffusion.

MAb specificity was analysed with a preparation of *E. coli* O6:K- separated by SDS-PAGE under reducing conditions and blotted on to a nitrocellulose membrane. MAb ARM 1-4 reacted with epitopes carried by molecules of 30 and 40 kDa; MAb ARM 1-7 recognised epitopes expressed by molecules of 41 kDa. MAb ARM 2-2 reacted with epitopes of molecules of 15 and 41 kDa (Fig. 1). In immunoblotting experiments, after enzymic digestion of the *E. coli* O6:K- antigenic preparation with proteinase K, none of the MAbs reacted with any epitopes (data not shown).

ELISA was used to show whether the epitopes recognised by the MAbs on the immunising strain *E. coli* O6:K- were exposed on the surface of other

E. coli strains. Each MAb bound to all the *E. coli* serotypes used, and the reactivity of every MAb did not vary significantly when tested against the different bacterial serotypes (Table 1). However, the reactivity of the MAbs to the same *E. coli* strain differed. ARM 1-4 bound to *E. coli* O6:K- to a significantly greater degree than did ARM 2-2 (12.2 ± 0.9 versus 7.0 ± 1.5 , $p < 0.001$), but not to a greater degree than ARM 1-7 (9.6 ± 2.8). No major differences were observed between ARM 1-7 and ARM 2-2 in binding to *E. coli* O7:K-. ARM 1-4 presented a significantly higher activity towards the latter serotype than ARM 1-7 (13.3 ± 2.5 versus 6.0 ± 1.5 , $p < 0.001$) and ARM 2-2 (13.3 ± 2.5 versus 6.8 ± 0.9 ; $p < 0.05$). When the activity towards *E. coli* O111:B4 was measured, ARM 1-4 presented a significantly higher binding capacity than ARM 2-2, but there were no differences between ARM 1-4 and ARM 1-7 nor between ARM 1-7 and ARM 2-2. All the MAbs reacted with serotype O128:K- in a similar manner (Table 1).

The bactericidal capacity of the MAbs against live *E. coli* O6:K- was tested with guinea-pig complement. These experiments showed that MAbs ARM 1-4 and ARM 1-7 had 40% bactericidal activity, whereas MAb ARM 2-2 showed an activity of 55%. Experiments without complement showed that, despite the presence of MAbs, the survival of *E. coli* O6:K- was $> 100\%$, indicating that killing was complement mediated.

Protection studies

The protective capacity of MAbs ARM 1-4, ARM 1-7, and ARM 2-2 was investigated in non-immunised animals. Groups of ten BALB/c mice were given the MAbs (5 µg/mouse) by i.p. injection and challenged 90 min later with 2, 4 or 8 × LD₅₀ of different live *E. coli* strains.

After observation for 7 days all the animals given ARM 1-4 and challenged with 2 × LD₅₀ *E. coli* O6K- and *E. coli* O111:B4 survived. When 2 × LD₅₀ of *E. coli* O7:K- and *E. coli* O128:K- were injected, the mortality was 50% and 60% respectively. With a challenge of 4 × LD₅₀, six mice survived after injection of *E. coli* O6K- and two mice after *E. coli* O128:K-; no protection was observed against *E. coli* O7:K- and *E. coli* O111:B4. When the inoculum was 8 × LD₅₀ all animals had died after 48 h (Fig. 2). A

Fig. 1. Immunoblot of SDS 10% gel ECL stained, showing the proteins labelled in *E. coli* O6:K- after reaction with: lane 1, MAb ARM 1-4; 2, MAb ARM 1-7; 3, MAb ARM 2-2. Mol. wt marker positions are indicated on the left of the figure.

Table 1. Reactivity of MAbs ARM 1-4, ARM 1-7 and ARM 2-2 towards different strains of *E. coli*

MAb	ELISA titres* towards serotype			
	O6:K-	O7:K-	O111:B4	O128:K-
ARM 1-4	12.2 SD 0.9	13.3 SD 2.5	12.0 SD 1.5	9.0 SD 2.5
ARM 1-7	9.6 SD 2.8	6.0 SD 1.5	9.0 SD 3.0	5.2 SD 2.0
ARM 2-2	7.0 SD 1.5	6.9 SD 0.5	6.9 SD 2.5	6.9 SD 1.5

*The values are expressed as log₂ dilution factor.

Fig. 2. Survival of BALB/c mice protected with ARM 1-4 and challenged with different lethal doses (2, 4 or $8 \times \text{LD}_{50}$) of: ▨, *E. coli* O6:K-; □, *E. coli* O7:K-; ▩, *E. coli* O111:B4; ▤, *E. coli* O128:K-; ■, control.

different pattern of survival was observed when MAb ARM 1-7 was used. Overall, all the mice given this MAb survived a challenge of $2 \times \text{LD}_{50}$, $4 \times \text{LD}_{50}$ and $8 \times \text{LD}_{50}$ of *E. coli* O6:K-. Furthermore, a protective effect against *E. coli* O111:B4 was observed when the mice were challenged with $2 \times \text{LD}_{50}$ (60% survival). The protection recorded after challenge with $2 \times \text{LD}_{50}$ of *E. coli* O7:K- and *E. coli* O128:K- was 30% and 20%, respectively, but no protection was shown against challenges of $4 \times \text{LD}_{50}$ and $8 \times \text{LD}_{50}$ (Fig. 3).

MAb ARM 2-2 protected mice challenged with *E. coli* O6:K- up to $8 \times \text{LD}_{50}$ (100% survived with challenges of 2 and $4 \times \text{LD}_{50}$, and 90% with $8 \times \text{LD}_{50}$). No protection was observed in mice challenged with *E. coli* O7:K-. However, ARM 2-2

improved the survival of mice challenged with $2 \times \text{LD}_{50}$ of *E. coli* O111:B4 and of *E. coli* O128:K- up to 50%. The survival of mice given $4 \times \text{LD}_{50}$ of *E. coli* O111:B4 was 30% and that of mice challenged with *E. coli* O128:K- was 50%, but no protection was observed with challenges of $8 \times \text{LD}_{50}$ of *E. coli* O111:B4 and *E. coli* O128:K- (Fig. 4).

Discussion

Sub-MICs of several antibiotics may alter the surface structure of members of the Enterobacteriaceae, influencing host-parasite relationships, such as serum bactericidal activity, opsonophagocytosis and complement consumption [14, 18, 23-26].

Fig. 3. Survival of BALB/c mice protected with ARM 1-7 and challenged with different lethal doses (2, 4 or $8 \times \text{LD}_{50}$) of: ▨, *E. coli* O6:K-; □, *E. coli* O7:K-; ▩, *E. coli* O111:B4; ▤, *E. coli* O128:K-; ■, control.

Fig. 4. Survival of BALB/c mice protected with ARM 2-2 and challenged with different lethal doses (2, 4 or 8 $\times$ LD50) of: ▨, live *E. coli* O6:K-; □, *E. coli* O7:K-; ▩, *E. coli* O111:B4; ░, *E. coli* O128:K-; ■, control.

A previous study demonstrated that the serum of mice immunised with *E. coli* O6:K- pre-exposed to sub-MIC of aztreonam (T-mice) contained antibodies towards protein epitopes that were not recognised by the serum of mice immunised with the untreated bacteria of the same strain (NT-mice). T-mice showed a higher degree of protection against lethal challenges with homologous and heterologous live bacteria than NT-mice. This protection did not seem to depend on quantitative differences in the antibody titre, but it was probably due to different kinds of antibody produced during the immunisation process [27].

In an attempt to identify the antibodies responsible for the protection, the splenocytes of T-mice were fused with SP1 plasmocytoma cells and those clones that produced antibodies recognising bacterial structures identified only by T mice pooled serum were selected. A recent study identified MAb MT 1F reacting with a protein epitope of a molecule of 12 kDa on the surface of the untreated *E. coli* O6:K-, which offered protection against lethal challenges of different serotype of *E. coli* [13].

In the present study three additional MAbs were characterised: ARM 1-4, ARM 1-7 and ARM 2-2, all of IgG₁ isotype. The MAbs reacted with different patterns of proteins on *E. coli* O6:K-. This reactivity was probably due to the expression of the same epitope on proteins of different mol. wt, or to the rearrangement of proteins following the denaturation process of the bacterial preparation used in immunoblotting studies. In these studies, sera drawn from T-mice as well as the MAbs presented different reactivity, as compared to sera from NT mice sera, especially to proteins of 15 and 40–41 kDa. It is possible that the antibiotic treatment of the bacterial preparation used in the immunisation procedure caused stress, with a re-arrangement of the bacterial

protein profiles revealing several new antigenic determinants [13, 28–30]. Therefore, the antigenic presentation to the immune system by treated bacteria could have been different from that of untreated bacteria, triggering the production of different antibodies.

As shown in ELISA, the MAbs reacted not only with the immunising bacterial strain, but also with the other *E. coli* serotypes. Furthermore, despite variation in the binding capacity of the MAbs when tested against the same *E. coli* serotype, the reactivity of the single MAb did not change significantly when tested against different serotypes. Therefore, it is possible that the epitopes recognised by the MAbs may be shared by other *E. coli* strains. This observation agrees with the results of earlier crossed immuno-electrophoretic studies, in which cross-reactivity among OMPs of Enterobacteriaceae was observed [31].

In the protection studies, MAbs ARM 1-7 and ARM 2-2 protected mice with live challenges of *E. coli* O6:K- up to 8 $\times$ LD50, while the protection offered by ARM 1-4 against the same bacterial challenge decreased at the higher bacterial loads. The MAbs presented a variable degree of cross-protection, which in all instances but one (mice protected with ARM 2-2 and challenged with *E. coli* O128:K-) was related to the bacterial load. These observations, combined with those of ELISA and immunoblotting experiments, present the hypothesis that protection may depend on the type of epitope bound by the MAb, more than on its binding capacity to either homologous or heterologous *E. coli* serotype. ARM 1-4, for instance, showed significantly greater binding to *E. coli* O6:K- in ELISA than ARM 2-2. However, the latter MAb gave protection up to a challenge of 8 $\times$ LD50 of *E. coli* O6:K- whereas ARM 1-4 did not. Furthermore, ARM 1-7 which bound to *E. coli* O7:K- significantly less

well than ARM 1-4, was as effective as the latter in protecting animals against a challenge of $2 \times \text{LD}_{50}$ of *E. coli* O7:K- ($p = 0.9271$, χ^2 test). ARM 2-2, which showed a binding capacity similar to that of ARM 1-7, completely failed to protect animals against lethal challenges with *E. coli* O7:K-, whereas ARM 1-7 provided some degree of protection at $2 \times \text{LD}_{50}$. It was possible to correlate enhanced binding with improved protection only in the case of MAb ARM 1-4 and ARM 2-2 against *E. coli* O7:K-.

At this stage, the epitopes which are more directly involved in evoking protective antibodies are unknown and further studies are needed to establish this.

The capacity of the MAb to improve serum opsonic activity in the passively immunised mice was probably involved in the mechanism of protection. This can be underscored by the fact that the three MAb displayed *in vitro* complement-dependent bactericidal activity against live *E. coli*. This observation parallels previous findings which showed how MAb MT 1F protected against challenge with live bacteria [13].

In conclusion, the data show the involvement of the bacterial outer-protein structures in evoking protective antibodies and provide evidence for the importance of the epitope to which the immunoglobulins bind.

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