MICROBIAL PATHOGENESIS

Protective features of monoclonal antibodies to *Escherichia coli* during experimental infection of mice with homologous and heterologous serotypes of *E. coli*

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Murine monoclonal antibodies (MAbs) MT1F and ARM1-4, recognising proteins on the surface of untreated *Escherichia coli* O6:K⁻, protected 100% of mice challenged intraperitoneally with 2 × LD₅₀ of the same strain. MAb MT1F protected 70% of animals challenged with 2 × LD₅₀ of *E. coli* O111:B4, whereas ARM1-4 gave complete protection. Lower survival was observed in mice given either MAb and challenged with *E. coli* O128:K⁻, with values ranging from 30 to 42%. However, the protection afforded against *E. coli* O111:B4 and *E. coli* O128:K⁻ was significantly improved when the mice were pre-treated with a mixture of the two MAbs. Control mice, pre-treated with unrelated ascitic fluid and challenged with any of the *E. coli* serotypes, showed 100% mortality and organ histological lesions resembling those of the early stages of septic shock. The mice had high levels of circulating endotoxin and tumour necrosis factor- α (TNF- α) at 90 min after challenge. In contrast, mice treated with MAbs and surviving the infection displayed moderate histological lesions, enhanced bacterial clearance and lower serum levels of TNF- α , despite circulating endotoxin levels that were higher than in the control group. Protection by the MAbs was probably due to the prevention of the bacterial spread to organs and of the cascade of events leading to septic shock. This occurred in spite of the presence of high levels of circulating endotoxin.

Introduction

Escherichia coli is one of the most common aetiological agents of hospital-acquired infection, causing about one-third of gram-negative bacteraemias [1, 2]. Treatment of these clinical events remains difficult despite the use of potent antibiotics, and many strains are becoming resistant to antibiotic therapy [3]. Therefore, interest in the possibility of immunotherapy for such infections has emerged [4–6]. However, the complexity and the high degree of antigenic variability of the outer structures of gram-negative bacteria are major problems in finding common target epitopes. In an attempt to identify protective antigens shared by different serotypes [7, 8], monoclonal antibodies

(MAbs), directed against epitopes on lipopolysaccharide (LPS), in particular against the core region of lipid A, have been reported to be effective in preventing fatal infections with gram-negative bacilli [9–13]. However, despite the attractive simplicity of this approach, it is still controversial and data regarding the existence of such cross-reactive antibodies and the nature of the target epitopes are conflicting [14, 15]. A different approach was based on the observation that antibodies to outer-membrane proteins (OMPs) were found to be protective in experimentally-induced infections [16–21].

In the search for bacterial epitopes with a potentially wider cross-protection, an earlier study demonstrated that mice immunised with *E. coli* O6:K⁻ pre-exposed to a subminimal inhibitory concentration of a β -lactam antibiotic showed enhanced protection against homologous and heterologous bacterial lethal challenges compared with mice immunised with untreated bacteria

[22]. The enhanced protection was transferred to normal mice by sera from mice immunised with antibiotic-treated bacteria, thus suggesting a key role for antibodies. In further studies, several MABs were produced from the spleens of BALB/c mice immunised with *E. coli* O6:K⁻ pre-exposed to antibiotic [19, 20]. The MABs identified protein epitopes on the surface of the untreated *E. coli*, were able to activate complement and gave protection against lethal challenge with homologous and, to a certain degree, against heterologous *E. coli* serotypes [19, 20]. The present study was done to gain further insights into the mechanism of protection conferred by these MABs.

Materials and methods

Bacteria

E. coli O6:K⁻ (ATCC 25922; Difco Laboratories Detroit, MI, USA), *E. coli* O111:B4 (kind gift of Professor J. Verhoef Eijkman, Winkler Laboratory for Medical Microbiology, University Hospital of Utrecht, The Netherlands) and *E. coli* O128:K⁻ (kindly provided by Professor A. Caprioli, Istituto Superiore di Sanità, Rome, Italy) were used.

Animals

Female BALB/c mice (Nossan, Correzzana, Italy) 6–8 weeks old and with an average weight of 25 g were used. The mice were housed in groups of five and provided with food and water *ad libitum*. The Institute's Ethical Committee approved all animal experiments. Care was taken to avoid any pain to the animals.

MABs

Murine MABs MT1F and ARM1-4 were produced and purified by protein G chromatography as reported previously [19, 20]. To elicit the MABs, the mice had been immunised with *E. coli* O6:K⁻ grown overnight in the presence of $0.5 \times \text{MIC}$ of aztreonam (Bristol-Myers Squibb, Rome, Italy) and then killed with formalin [22]. Both MABs were of the IgG1 isotype. MT1F reacted with a polypeptide of 12 kDa [19] and ARM1-4 with polypeptides of 30 and 40 kDa [20] in the untreated *E. coli* O6:K⁻.

Protection studies

Passive protection experiments were performed in triplicate in groups of 10 mice. Animals were inoculated intraperitoneally (i.p.) with 1 ml containing MT1F (5 µg/mouse), ARM1-4 (5 µg/mouse) or a mixture of MT1F (2.5 µg/mouse) and ARM1-4 (2.5 µg/mouse). After 90 min, $2 \times \text{LD}_{50}$ of live *E. coli* O6:K⁻, *E. coli* O111:B4 or *E. coli* O128:K⁻ (the LD_{50} for these strains was 3×10^7 , 1×10^8 and 1×10^8 cfu/mouse, respectively) were injected i.p. As a control group for each challenge strain, five female

BALB/c mice received 1 ml of ascitic fluid induced by murine myeloma SP1 cells, instead of the test MAB. The absence of toxicity of the MABs at the doses used was determined in groups of five mice. Mice were checked daily for 10 days and deaths were recorded. Results were expressed as the mean percentage survival and SD of three independent experiments.

Bacterial clearance

At 1, 3 and 5 h after challenge with $2 \times \text{LD}_{50}$ of *E. coli* O6:K⁻, five mice from each group (controls and MAB-treated mice) were anaesthetised with diethyl ether and killed by cervical dislocation. After aseptic removal, livers and lungs were weighed, each was placed in 50 ml of sterile saline, and homogenised under a vented hood. Ten-fold serial dilutions of the homogenates were made and 10 µl of each dilution were plated on MacConkey agar. The plates were incubated for 18 h at 37°C and then the colonies were counted. Results are expressed as cfu/mg of homogenised tissue and are the means and SD of three independent bacterial counts of each organ sample.

Serum collection

At 90 min after challenge, five mice from each group were anaesthetised and bled from the retro-orbital plexus. Sterile precautions were taken throughout the procedures. The sera harvested from each group were pooled and stored in 0.1-ml volumes at -70°C until analysed.

Endotoxin determination

The endotoxin contamination of the MAB preparations, the unrelated ascitic fluid and the media used during the experiments, as well as the endotoxin levels in sera drawn after challenge of the mice and diluted 1 in 10, was determined in triplicate by the chromogenic Limulus amoebocyte lysate (LAL) test (QCL-1000, Bio-Whittaker, Walkersville, MD, USA) according to the manufacturer's instructions.

TNF-α determination

TNF-α levels were measured in sera by the cytolysis produced in actinomycin D-sensitised fibroblast cell line L-929 [23]. Results were expressed in U/ml. One unit of TNF-α bioactivity was expressed as the titre that produced 50% cytolysis, based upon two-fold dilutions of serum samples. This endpoint corresponded with 35 SD 8 pg/ml of a recombinant human TNF-α standard (Endogen, Boston, MA, USA). All positive samples were incubated with rabbit anti-mouse TNF-α polyclonal antiserum (IP-400; Genzyme, Boston, MA, USA) and tested for inhibition of TNF-α bioactivity in L-929 cells, to confirm true positivity.

Histology

Histological examination of two mice from each group and randomly selected from those that died after bacterial challenge, along with two mice that survived the infection was done within 24 h of bacterial injection. The latter were anaesthetised with diethyl ether and killed by cervical dislocation 24 h after challenge. Furthermore, two mice taken randomly from the survivors were killed after 10 days. The brains, lungs, livers and spleens were removed and perfused with formaldehyde 10% in phosphate buffer (pH 7.8), dehydrated and processed in paraffin. Sections were cut at 5 μ m and stained with haematoxylin and eosin (H&E) or Giemsa.

Statistical analysis

Data obtained after bacterial challenge were expressed as the mean percentage survival and SD for three independent experiments each with groups of 10 mice. Survival of different groups was compared by the χ^2 test. Multiple linear regression and non-parametric analysis were used to determine the correlation between serum TNF- α and mortality in each set of mice; p values <0.05 were considered to be significant.

Results

Protection studies

All the control mice pre-treated with ascitic fluid and challenged with $2 \times \text{LD}_{50}$ of the different *E. coli*

strains died within 24 h of challenge. The known protective capacity of MT1F and ARM1-4 was verified by passive transfer experiments. Both MABs gave 100% protection against lethal challenge with *E. coli* O6:K⁻ (Fig. 1). When the animals were inoculated with $2 \times \text{LD}_{50}$ of *E. coli* O111:B4, the survival ranged from 70% to 100%, and ARM1-4 showed a protective activity significantly greater ($p < 0.05$) than that of MT1F. Survival of animals challenged with *E. coli* O128:K⁻ ranged from 30% to 42%, and did not differ significantly between the two MABs.

When the mice were treated with the two MABs together and challenged with *E. coli* O6:K⁻, their survival approached 100% and no significant differences were observed between the groups treated with the single MAB and with the mixture (Fig. 1). However, when the animals given the MAB mixture were challenged with *E. coli* O111:B4, their mean survival approached 90%, and the protective capacity compared with MT1F was significantly ($p < 0.05$) improved. Furthermore, in mice inoculated with the mixture and subsequently challenged with *E. coli* O128:K⁻, mean survival was 63% and protection was significantly ($p < 0.001$) higher than with either MT1F or ARM1-4 alone.

Bacterial clearance

To verify whether the MABs were able to prevent bacterial spread to the organs after i.p. injection, colony counts in lung and liver homogenates were obtained from controls and from mice protected with the MABs

Fig. 1. Survival of mice pre-treated with MT1F, ARM1-4 or with a mixture of MT1F and ARM1-4 (A1) and challenged with $2 \times \text{LD}_{50}$ of *E. coli* O6:K⁻ (□), *E. coli* O111:B4 (▨) or *E. coli* O128:K⁻ (▩). Data are expressed as the mean percentage survival and SD from three independent experiments. * $p < 0.05$ ARM1-4 versus MT1F; ** $p < 0.05$ A1 versus MT1F; *** $p < 0.001$ A1 versus both MT1F and ARM1-4.

alone or in combination. Live *E. coli* O6:K⁻ was recovered mostly in the liver while fewer bacteria grew from lung homogenates of either control or MAb-treated mice. In control animals, the bacterial counts in both lung and liver samples increased with time (Fig. 2). In contrast, animals that had received MABs either alone or in combination showed, in all instances, bacterial counts significantly lower than those of control mice ($p < 0.001$, Student's *t* test). The numbers of bacteria recovered from organ homogenates of MAB-treated mice changed little with time, indicating

that the MABs controlled bacterial spread to the organs after i.p. challenge.

Endotoxin measurements

The endotoxin contamination of the MAB preparations, ascitic fluid and the media used throughout the study was < 32 pg/ml. Control and MAB-treated mice had serum endotoxin levels of 0.2 ± 0.05 and 0.24 ± 0.03 ng/ml, respectively, before bacterial challenge. At 90 min after challenge with *E. coli* O6:K⁻, control

Fig. 2. Bacterial counts in (a) liver and (b) lung homogenates at 1, 3 and 5 h after challenge with $2 \times \text{LD}_{50}$ of *E. coli* O6:K⁻: control mice (□); mice protected with MT1F (▨), ARM1-4 (▤) and the MAB mixture (■). Data (means, SD) are expressed as cfu/mg of homogenised tissue.

mice had values of 1.7 SD 0.5 ng/ml whereas in mice pre-treated with MT1F, ARM1-4 and with the MAb mixture, endotoxin levels were significantly enhanced (5.2 SD 0.3, 4.8 SD 0.6 and 5.6 SD 0.8 ng/ml, respectively; $p < 0.001$ MAb-treated versus control mice). No significant differences in the serum endotoxin content were observed between each set of MAb-treated mice.

Serum TNF- α

To find out the correlation, if any, between survival and systemic levels of TNF- α , serum levels of the cytokine were measured 90 min after challenge with *E. coli* O6:K⁻. In control and MAb-treated mice, the levels of TNF- α ranged from 1.3 SD 1.2 U/ml to 6.4 SD 1.2 U/ml before bacterial challenge. After challenge, high levels of the cytokine were observed in control mice (158 SD 36 U/ml), whereas in mice treated with either MAb or with the mixture, TNF- α levels were significantly lower (29.3 SD 7.2 U/ml for MT1F, 18.8 SD 2.8 U/ml for ARM1-4 and 23.4 SD 6.4 U/ml for the MAb mixture respectively; $p < 0.001$, Student's *t* test, MAb-treated versus control mice). Serum levels of TNF- α correlated significantly with mortality ($r = 0.95$, Bravais Person test), with high levels of cytokine associated with a high mortality rate.

Histology

Organs from control mice that died within 24 h of challenge displayed features of systemic damage. The

lungs showed diffuse atelectasis with marked thickening of the alveolar septa and leucocyte infiltration. The alveolar configuration was quite irregular with an alternation of emphysematous alveoli and alveoli compressed and obliterated (Fig. 3). The liver presented perilobular congestion with fatty and hydropic degeneration, as well as diffuse activation of the reticulo-endothelial system to confluent hepatocellular necrosis (Fig. 4). The spleen was generally fibrous and congested with conspicuous areas of haemorrhage and emperipolesis of white pulp (not shown). In the brains of the control mice, collapsed neurones with pyknotic nuclei and strongly stained eosinophilic cytoplasm were detected. Such neurones were diffusely identified in the cortical layers of the brain and sometimes in the Purkinje cells of the cerebellum to feature an ischaemic encephalopathy (not shown).

The organs of MAb-treated mice that died within 24 h of challenge presented pathological features similar to those described for the challenged control mice. However, mice that had been treated with the MAb mixture and died within 24 h showed a lower degree of hydropic degeneration of liver, mainly perilobular, but no signs of necrosis (Fig. 5). In contrast, the organs of the MAb-treated mice that survived the infection and were killed at 24 h after challenge displayed less severe alterations. The lungs presented focal but inconspicuous thickening of the pulmonary alveolar walls (Fig. 6). The livers displayed minor signs of inflammation, such as mild hepatic reticulo-endothelial cell activation with microvacuolar degeneration (not

Fig. 3. Section of the lung of a control mouse that died within 24 h of challenge with *E. coli* O6:K⁻. The alveolar damage is evidenced by marked thickening of the alveolar septa and obliteration of the lumina (H&E $\times 100$).

Fig. 4. Section of the liver of a control mouse that died within 24 h of challenge with *E. coli* O6:K⁻. The organ shows confluent necrosis. (H&E $\times 100$).

Fig. 5. Section of the liver of a mouse which was pre-treated with the MAb mixture and died within 24 h of challenge with *E. coli* O6:K⁻. There are no signs of confluent hepatocellular necrosis, but hydropic degeneration is present (H&E $\times 100$).

Fig. 6. Section of the lung from a mouse pre-treated with MAb ARM1-4 and killed 24 h after challenge with *E. coli* O6:K⁻. The alveoli appear nearly normal (H&E $\times 100$).

shown). In surviving mice, pre-treated with the MABs either singly or in combination, the organs showed a complete restitutio ad integrum after 10 days (not shown).

Discussion

The results of the present study confirmed that MABs MT1F and ARM1-4, elicited in mice after immunisation with antibiotic-treated *E. coli* O6:K⁻, offered full protection against lethal challenge with *E. coli* O6:K⁻ and a certain degree of cross-protection against lethal challenge with different *E. coli* serotypes [19, 20]. When the MABs were administered together, the cross-protective capacity was significantly improved. MABs MT1F and ARM1-4 recognised protein epitopes on the surface of untreated *E. coli* O6:K⁻ [19, 20]. Previous reports showed that antibodies to outer-membrane proteins (OMPs) were protective in experimentally induced infection in mice [16–21]. Other authors found anti-OMP antibodies that cross-reacted with protein epitopes of different gram-negative serotypes [7, 24, 25]. Furthermore, Hofstra et al. showed cross-reactivity among OMPs of members of the Enterobacteriaceae in crossed immuno-electrophoretic studies [26], and recent studies [14] showed that an antiserum raised to *E. coli* J5 contained high titres of IgG antibodies that bound to at least three major OMPs on many clinically relevant gram-negative bacteria. Therefore, it is possible that the simultaneous recognition of different protein epitopes on the bacterial surface could

account for the enhanced cross-protection observed in mice pre-inoculated with the MAB mixture.

MABs MT1F and ARM1-4, given i.p. alone or in combination, controlled the bacterial spread to organs after i.p. challenge. This finding was confirmed by the histological examination of the mice from different experimental groups, which showed that the organs (brain, liver, spleen, lung and kidney) from the surviving MAB-treated mice showed only moderate damage. In contrast, organs from control mice showed diffuse alterations similar to those encountered in the early stages of septic shock [27]. Therefore, it seems that pre-administration of MABs prevented the cascade of events leading to septic shock which is strongly related to the systemic release of pro-inflammatory cytokines such as TNF- α [27]. Indeed, in the sera of mice pre-treated with MABs, TNF- α levels were lower than those in untreated mice at 90 min after bacterial challenge. Furthermore, serum levels of TNF- α correlated with the survival rates of the animals, as high values of the cytokine were associated with high mortality rate. It is widely accepted that endotoxin is the major stimulator of the release of TNF- α by monocytes and macrophages and that endotoxin is continuously released into the bloodstream during a systemic gram-negative bacterial infection [28]. Consequently, it could be hypothesised that pre-administration of MABs controlled the systemic release of endotoxin. However, the MABs used in this study were devoid of a direct anti-endotoxin activity. In fact, they recognised bacterial proteins [19, 20] and displayed a

high protective capacity despite the elevation of systemic endotoxin levels after bacterial challenge. The latter effect was probably due to the capacity of the MAbs to activate complement [19, 20], thus causing enhanced bacterial death and subsequent raising of systemic endotoxin levels. Therefore, it seems that high levels of circulating endotoxin are not necessarily correlated with a high mortality rate nor with an overproduction of TNF- α , especially in the presence of antibodies which may significantly contribute to clearance of the bacteria from the body.

In conclusion, the findings of the present study confirm that MAbs MT1F and ARM1-4 directed against protein epitopes of *E. coli* provided protection against systemic infection by homologous and heterologous *E. coli*. The heterologous protection was improved by the use of a mixture of the MAbs. The mechanism of protection implied an enhanced bacterial clearance promoted by the MAbs, which were able to prevent both the systemic release of pro-inflammatory TNF- α and the histological lesions normally encountered during a systemic life-threatening gram-negative bacterial infection. These events occurred in the presence of high levels of circulating endotoxin, which highlights the importance of the bacterial cell as a whole in the pathogenesis of gram-negative bacterial infections.

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