IMMUNOLOGICAL RESPONSE TO INFECTION

Murine monoclonal antibody elicited with antibiotic-exposed *Escherichia coli* exerts protective capacity in experimental bacterial infections

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Summary. *Escherichia coli* pre-exposed to a sub-minimal inhibitory concentration (sub-MIC) of several antibiotics elicits an enhanced humoral response which is protective against challenges with untreated homologous and heterologous bacteria. To characterise the specificity of this response we produced murine monoclonal antibodies (MAbs) to aztreonam-treated *E. coli* O6:K-. This resulted in the identification of MAb MT 1F, of isotype IgG₁, that recognised a 12-kDa protein component of the untreated bacterial cells. After passive transfer, the MAb displayed protective activity in mice infected with lethal doses of live *E. coli* O6:K- and *E. coli* O111:B4. In ELISA experiments the MAb cross-reacted with structures located on whole cells of *E. coli* O6:K-, *E. coli* O111:B4, *E. coli* J5 and *Salmonella minnesota* Re595 and it also exerted a bactericidal activity against live *E. coli* O6:K-. The modifications induced by antibiotic treatment may unmask bacterial epitopes that may elicit the production of MAbs endowed with protective capacity.

Introduction

Gram-negative bacterial infections are associated with high morbidity and mortality rates despite appropriate antibiotic treatment.¹ Therefore, interest has arisen as to the possibility of immunotherapy of such infections.^{2,3}

A previous study showed that mice immunised with *Escherichia coli* O6:K- pre-exposed to sub-minimal inhibitory concentrations (sub-MICs) of several antibiotics presented enhanced protection towards homologous and heterologous bacterial lethal challenges, compared to mice immunised with untreated bacteria.⁴ This protection was transferred to normal mice through serum of animals immunised with antibiotic-treated bacteria, thus suggesting a role for antibodies in the mechanism of protection.⁵ Indeed, in addition to quantitative differences between the immunisation groups, qualitative differences in the antibody reactivity pattern were also observed in the group of mice immunised with antibiotic-treated bacteria.

To gain further insight into the specificity of the protective antibodies, monoclonal antibodies (MAbs) were prepared for use as molecular probes and an examination made of the serological and immuno-

chemical characteristics of MAb MT 1F, which protected normal animals against lethal challenges with homologous and heterologous bacteria.

Materials and methods

Bacteria

E. coli O6:K- (ATCC 25922, Difco Laboratories) was used for immunisation of mice. *E. coli* O111:B4, *E. coli* J5 and *Salmonella minnesota* Re595 were a kind gift of Professor J. Verhoef (Eijkman-Winkler Laboratory for Medical Microbiology, University Hospital of Utrecht, The Netherlands).

Immunisation procedure

E. coli O6:K-, grown overnight in Mueller Hinton Broth (Difco) in the presence of 0.5 × MIC of aztreonam (Bristol-Myers Squibb, Rome, Italy) (MIC = 0.078 mg/L) was washed three times in sterile saline and resuspended in saline containing formalin 0.2% to an optical density (OD) equivalent to 10⁹ cfu/ml. The suspension was incubated for 1 h at 37°C and the cells were washed once again in saline. After re-adjustment of the suspension to an optical density equivalent to 10⁹ cfu/ml, amounts were injected into female

Fig. 1. Immunoblot of *E. coli* O6:K- after reaction with: lane 1, polyclonal sera of mice immunised with untreated *E. coli* O6:K-; 2, polyclonal sera of mice immunised with antibiotic-treated *E. coli* O6:K-; 3, MAb MT 1F; 4, MAb MT 1F after digestion of *E. coli* O6:K- with proteinase K.

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, 10^8 cfu/mouse.⁵

Production and selection of hybridomas

Hybridomas were produced by a slight modification of the method of Fazekas De St Groth and Scheidegger.⁶ 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 antibody directed against untreated *E. coli* O6:K-, *E. coli* J5, *E. coli* O111:B4 and *S. minnesota* Re595 as described.⁷ The antibody-producing clones were subcloned by limiting dilution and 3×10^6 cells were injected intraperitoneally (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). 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 the double immune diffusion technique. MAb from ascitic fluid was purified by protein G column chromatography (Immunopure plus IgG purification Kit; Pierce).⁸

ELISA

The binding of MAb to bacterial cells was measured by ELISAs performed as described previously.⁷ Viable, untreated *E. coli* O6:K-, *E. coli* O111:B4, *E. coli* J5 and *S. minnesota* Re595 (10^7 cfu/ml in coating buffer solution) were used to coat the microtitration plates (Nunc; Nunc, Denmark) and the reactivity was measured spectrophotometrically at 492 nm with a spectrophotometer (Easy Beam, STL-Lab Instrument, Innsbruck, Austria) and peroxidase labelled goat anti-mouse conjugates (BioRad Laboratories, Milan, Italy). ODs 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).⁹

Immunoblotting

Proteins of untreated *E. coli* O6:K- were resolved by polyacrylamide gel electrophoresis under reducing conditions in slab gels.¹⁰ The proteins were transferred to nitrocellulose paper¹¹ and strips were incubated for 18 h at room temperature with ascitic fluid to link antibodies. The reactivities were detected by enhanced chemiluminescence (ECL, Amersham International PLC, Arlington Heights, IL, USA), as described previously.¹²

In further experiments, protein (1 mg/ml) of untreated *E. coli* O6:K- was digested with proteinase K ($2.5 \mu\text{g/ml}$) for 1 h and then transferred to nitrocellulose by electrophoresis.

Bactericidal assay

The bactericidal activity of ascitic fluids was estimated as described.¹³ 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 ($5 \mu\text{g/ml}$) in the presence of $200 \mu\text{l}$ of guinea-pig serum as complement source. After incubation for 45 min at 37°C in a shaking water bath, $100 \mu\text{l}$ of the mixture was plated on blood agar and incubated overnight at 37°C . The MAb dilution giving 50% bacterial killing was considered to be the bactericidal titre. Mouse SP1 myeloma-inducing ascitic fluid and guinea-pig serum alone were used as controls.

In-vivo protection experiments

Ninety female 6–8-week-old BALB/c mice (Nossan, Correzzana, Italy) of average weight 25 g, were used. The mice were housed in groups of 10 and provided with food (Standard pellets, Dottori Piccioni Laboratories, Gessate, Italy) and water *ad libitum*. In passive protection experiments, 2, 4 and $8 \times \text{LD}_{50}$ doses of live *E. coli* O6:K- and *E. coli* O111:B4 (the LD_{50} for these strains was 3×10^7 cfu/mouse and 1×10^8 cfu/mouse respectively) were injected into mice 90 min. after i.p. injection of MAb ($5 \mu\text{g/mouse}$). Ten female BALB/c mice given sterile saline instead of MAb served as the control group. Mice were checked daily for 7 days and deaths were recorded.

Fig. 2. Reactivity of sera from control mice and MAb MT 1F investigated in ELISA with *E. coli* O6:K-, *E. coli* O111:B4, *E. coli* J5 and *S. minnesota* Re595. Control sera ■; MT 1F □.

Statistical analysis

Data obtained after bacterial challenge were analysed with the χ^2 test. Data obtained in ELISA were expressed as the mean DF and the SD of three independent experiments done in duplicate; *p* values < 0.005 were considered significant.

Results

MAb characterisation

More than 100 hybrid clones obtained by fusing splenocytes of mice immunised with antibiotic-treated *E. coli* with SP1 plasmacytoma cells were studied. The MAbs were first screened by ELISA and then immunoblotted for their reactivity to untreated *E. coli* O6:K-. Mice immunised with antibiotic-treated *E. coli* O6:K- presented a different pattern of antibody response from that of mice immunised with untreated *E. coli* O6:K-. Therefore, among the MAbs reacting with structures of *E. coli* O6:K-, one was selected (MT 1F) that bound to a bacterial antigen recognised only by the sera of mice immunised with antibiotic-treated bacteria.

MT 1F, isotype IgG₁ as determined by the double immunodiffusion technique, reacted specifically with a 12-kDa antigen of the untreated *E. coli* O6:K-. To determine whether the antigen was a bacterial protein or a lipopolysaccharide (LPS), an immunoblot analysis was performed on an antigenic preparation of proteinase K-digested *E. coli* O6:K-. After the enzymic digestion of bacterial proteins, no antigen-antibody reaction could be detected, thus indicating that the 12-kDa antigen reacting specifically with MT 1F was a protein (fig. 1).

To determine whether MT 1F cross-reacted with other gram-negative strains, the binding of the MAb

to whole bacterial cells was investigated by ELISA. The MAb presented a significantly higher reactivity (DF > 20) against untreated *E. coli* O6:K- than that of sera from mice immunised with antibiotic-treated *E. coli* (DF = 13.9, *p* < 0.001) (fig. 2). Furthermore, the MAb bound also to *E. coli* O111:B4, *E. coli* J5 and *S. minnesota* Re595 (DF 16.4, 17.8 and 14.5 respectively).

Protection studies

The protective capacity of MT 1F towards lethal challenges with live homologous or heterologous bacterial strains was investigated. In a pilot experiment, several dilutions of MAb (from 1 to 10 µg/ml) were injected into mice 90 min before lethal challenge (2 × LD₅₀) with *E. coli* O6:K-. The best protection pattern was observed with 5 µg of the MAb/ml. Therefore, in the following experiments, non-immunised mice were given 5 µg/ml of MAb MT 1F by i.p. injection and challenged 90 min later with 2 × LD₅₀, 4 × LD₅₀ and 8 × LD₅₀ of either *E. coli* O6:K- or *E. coli* O111:B4. A group of mice given sterile saline instead of MAb and subsequently given the same lethal doses of bacteria by i.p. injection acted as the control.

The onset of mortality in the control group was observed after 4 h, and within 24 h all control mice (30 animals) were dead. In contrast, in mice passively immunised with MT 1F and challenged with 4 × LD₅₀ and 8 × LD₅₀ of *E. coli* O6:K-, the onset of mortality was delayed for six of 10 mice given 4 × LD₅₀ and for two of 10 mice given 8 × LD₅₀ until 24 h, but all these mice died within 48 h. However, of the 10 mice challenged with 2 × LD₅₀ *E. coli* O6:K-, two died in the first 24 h, whereas the other eight survived and remained healthy 7 days later.

Fig. 3. The mortality of BALB/c mice after challenge with different lethal doses (2, 4, 8 $\times$ LD50) of live *E. coli* O6:K- and *E. coli* O111:B4. Control mice ■; mice protected with MT 1F and challenged with *E. coli* O6:K- ▨; mice protected with MT 1F and challenged with *E. coli* O111:B4 □.

In the experiments in which the mice were challenged with 2 $\times$ LD50 of *E. coli* O111:B4, 50% of the mice ($n = 10$) died within 24 h, whereas the remainder survived. Two of 10 mice survived a challenge of 4 $\times$ LD50 whereas with a challenge of 8 $\times$ LD50 all mice ($n = 10$) died within 24 h (fig. 3).

Bactericidal assay

The complement activating capacity of MT 1F was investigated. In the presence of 50% guinea-pig serum only, no bacterial killing was observed. Similarly, the addition of SP1 mouse myeloma-induced ascitic fluid to the reaction mixtures induced no bacterial death. However, MT 1F exerted bactericidal activity against live *E. coli* O6:K- with a bactericidal titre > 100 .

Discussion

E. coli is the commonest cause of gram-negative bacteraemia and, despite appropriate treatment with new and potent antibiotics, the incidence of gram-negative bacteraemia is increasing. The possibility of an immunological approach to treatment of these infections has long been envisaged.^{14,15}

Encouraging results in terms of reduction of mortality and morbidity in man have been obtained through the administration of plasma rich in antilipopolysaccharide (LPS) antibodies.^{16,17} Antibodies directed to LPS, lipid A and core regions of LPS as well as antibodies to epitopes located on outer-membrane proteins have been shown to exert a protective effect in animals.³

In a previous study we demonstrated that passive transfer of hyperimmune serum from mice immunised with antibiotic-treated bacteria was capable of pro-

tecting normal animals against viable homologous and heterologous lethal bacterial challenges to a greater degree than did serum from mice immunised with untreated bacteria.⁵ These results suggested that antibiotic treatment induced changes in the immunogenicity of the bacterial cell-surface components, leading to an enhanced production of antibodies to epitopes that were better exposed after antibiotic treatment.¹⁸⁻²⁰

In this study, the previous work was extended and MAbs were produced from the spleens of mice immunised with 0.5 $\times$ MIC aztreonam-treated *E. coli* O6:K-. Attention was focused on MAb MT 1F of IgG₁ isotype, which reacted with a cell-surface determinant of 12 kDa on untreated *E. coli* O6:K- cells. This reactivity was revealed in Western immunoblot analysis only with the sera pooled from mice immunised with antibiotic-treated bacteria.⁵ The treatment of *E. coli* with proteinase K resulted in the loss of MAb binding, thus indicating a reaction of the MAb to protein components, but not to other bacterial structures such as LPS.

MT 1F showed protection against lethal challenge with two different O-serotypes of *E. coli* and the protective efficacy depended on the number of bacteria used for challenge. To our knowledge, this is the first report of a MAb of IgG₁ isotype, raised against a bacterial antigenic determinant expressed after antibiotic treatment, having a protective capacity. Because of the reactivity of MT 1F expressed in ELISA against whole bacterial cells, which was abolished in immunoblotting experiments after enzymic digestion of bacterial proteins, it seems probable that MT 1F recognised protein structures on the bacterial surface. This hypothesis is supported by the observation that the accessibility of the major outer-membrane proteins

was enhanced when members of the Enterobacteriaceae were grown in the presence of β -lactam antibiotics.²⁰ It is possible that epitopes on proteins on the outer surface of *E. coli* were better exposed after antibiotic treatment, thereby permitting the immune system to produce a different antibody response.

Our data seem to show that the epitope recognised by MT 1F is present on different serotypes of some members of the Enterobacteriaceae. Hofstra *et al.*,²¹ in crossed immuno-electrophoretic studies, showed cross-reactivity among OMPs of members of the Enterobacteriaceae. Further studies showed that antibodies to OMPs were protective in experimentally induced infections in mice.^{22, 23} In ELISA experiments, reactivity of the MAb against whole *E. coli* J5, *E. coli* O111:B4 and *S. minnesota* Re595 was observed. Therefore, it is possible that if MT 1F recognises common bacterial epitopes, it might bind to cell-wall determinants of heterologous strains and offer a degree of protection against lethal challenges of bacteria. The

mechanism of protection observed in this study was probably due to an improvement of the opsonic capacity of the passively immunised mice, because it was observed that MAb MT 1F had bactericidal capacity *in vitro* against live *E. coli*.

In conclusion, the present findings confirm that treatment of *E. coli* with antibiotics is capable of inducing qualitative changes in the antigenic presentation of the bacterial surface components. These structures may induce a protective immune response towards homologous and heterologous *E. coli* strains. Although at an early stage, the experimental approach adopted in this study appears to be capable of directing at the molecular level the complexity of the immune response to antibiotic-treated bacteria, thus opening up the possibility of identifying immunogenic molecules of biological and clinical interest.

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