IMMUNE RESPONSE TO INFECTION

Protective activity of a murine monoclonal antibody against acute and chronic experimental infection with type IV group B streptococcus

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A murine IgM monoclonal antibody (MAb H11) was developed against the type polysaccharide capsular antigen of group B streptococcus (GBS), serotype IV, after intraperitoneal immunisation of BALB/c mice with heat-killed bacteria. MAb H11 reacted in immunodiffusion with the purified polysaccharide in both its sialylated and desialylated form, giving a line of identity, and opsonised type IV GBS strains in an in-vitro assay. When administered at the time of intraperitoneal lethal challenge with homologous GBS, or 4 h earlier, MAb H11 protected 90% of the mice. Protection was still observed when MAb H11 was given 4 h after the challenge. This MAb was strongly effective in preventing septic arthritis induced by type IV GBS.

Introduction

Streptococcus agalactiae (group B streptococcus; GBS) infection is a life-threatening neonatal disease [1], and there is also evidence of its role in severe adult invasive disease [2, 3]. Clinical features of GBS infection include endocarditis, osteomyelitis, septic arthritis and endophthalmitis. The capsule of GBS is an important virulence factor in systemic disease [4], and antibodies that react with the type capsular antigens are protective in neonatal infections [5]. For this reason, some authors support the use of immunotherapy in GBS infections [6] and intravenous (i.v.) administration of immunoglobulins is effective not only in animal models but also in experimental trials in man [7–9]. Moreover, human and murine monoclonal antibodies (MAb) against type or group B polysaccharide (common to all GBS strains) provide protection in animal models of acute systemic GBS infection [10–15]. A murine model of chronic GBS infection with septic arthritis caused by type IV GBS [16], a serotype not frequently isolated but responsible for human invasive disease, has been described [17, 18]. As with other GBS serotypes, type IV GBS has a type-specific polysaccharide antigen composed of repeated subunits of glucose, galactose, N-acetylglucosamine and sialic acid [19]; this forms a thin and irregularly shaped capsule [20].

To our knowledge, no MAb against type IV polysaccharide has been produced, so this study investigated the protective role of type-specific antibodies in acute and chronic experimental infections with type IV GBS, with MAb produced against the type IV polysaccharide antigen. The immunological and functional characteristics of the selected anti-type IV monoclonal antibody (MAb H11) are described and the efficacy of MAb H11 is compared with rabbit hyperimmune anti-group B and anti-type IV serum.

Materials and methods

Bacterial strains, antigens and antisera

Type IV GBS, reference strain 1/82, was kindly supplied by Dr J. Jelinkova (National Public Health Institute, Prague, Czech Republic). Additional type IV strains (nos 1606 and 1613), isolated from the urine of diabetic patients, were also used. Other GBS strains were O90 (type Ia), NCTC 8187 (type Ib), NCTC 11079 (type II), NCTC 11080 (type III) (kindly supplied by Dr G. Colman, Central Public Health Laboratory, 61 Colindale Avenue, London) and strain 10/84 (type V) (from Dr J. Jelinkova).

All strains were stored at -80°C in Todd-Hewitt Broth (THB; Unipath) containing sheep blood 10% v/v. Frozen cultures were streaked on to sheep blood 5% v/v Columbia agar plates (Unipath) and incubated at 37°C overnight. For experimental infections, the organisms were grown to the stationary phase (18 h)

in THB at 37°C, washed and diluted in serum-free RPMI 1640 medium (Flow Laboratories, McLean, VA, USA). The inoculum size was estimated turbidimetrically at 540 nm and the number of live bacteria was calculated by enumeration of colony-forming units (cfu) on sheep blood 5% v/v Columbia agar plates. Polysaccharides of all group B serotypes and group polysaccharide were produced and purified as described previously [21].

Group- and type-specific GBS antisera were raised in rabbits immunised with formalin-killed whole bacterial cells, as described by Lancefield [22]. Serum titres were determined by ELISA [21]. These were 64 000 and 32 000 for anti-type IV GBS and anti-group B serum, respectively.

Mice

BALB/c and CD1 mice were purchased from Charles River Breeding Laboratories, Calco, Milan, Italy.

Murine monoclonal antibodies

BALB/c female mice (2 months old) were immunised intraperitoneally (i.p.) with 0.2 ml of an overnight culture of a heat-inactivated cell suspension of type IV GBS, strain 1/82 (OD₅₉₀, 0.6: corresponding to 10⁸ cfu/ml). Booster injections were administered on day 7 (0.1 ml of vaccine, i.v.), day 14 (0.1 ml i.p.) and day 130 (0.1 ml i.p. and 0.2 ml i.v.). Four days after the last immunisation, spleens were removed aseptically and the splenic cells were fused, in a ratio of 8:1, with a non-immunoglobulin-secreting murine myeloma X63-Ag8-653, in the presence of polyethylene glycol 1450 (Sigma) 50% v/v. Fusion, hybridoma selection and culture were performed as reported by Malavasi *et al.* [23]. Culture supernates were screened for antibody production by ELISA [16] with purified type IV polysaccharide as coating antigen and anti-mouse polyvalent immunoglobulins alkaline-phosphatase as conjugated antiserum (Sigma). Clones positive for antibody production were subcloned twice by limiting dilution into 96-well tissue culture plates at a concentration of 1.0 and 0.5 cell/well, expanded and kept frozen in RPMI 1640 medium (Flow) supplemented with horse serum 10% v/v and dimethyl sulphoxide 20% v/v. A stable hybridoma (MAb H11) was selected and subsequently grown *in vivo* in pristane-treated BALB/c mice. One-to-two weeks after i.p. injection of 10⁶ hybridoma cells, ascitic fluid was removed, clarified by centrifugation and stored at -40°C.

Determination of specificity, immunoglobulin subclass and MAb concentration

The specificity of the MAbs was checked by ELISA, with group B and GBS types Ia, Ib, II, III and V polysaccharides as coating antigens [16]. MAb

isotyping was performed by ELISA [16] with both goat anti-mouse IgG (γ -chain) and anti-mouse IgM (μ -chain) alkaline phosphatase-conjugated antiserum (Sigma). The concentration of MAb IgM in the ascitic fluids and culture supernates was determined by radial immunodiffusion (Mouse IgM NL RID Plate, The Binding Site Inc., San Diego CA, USA). The double immunodiffusion test was performed in agar as described by Ouchterlony [24].

Opsonic assay

The opsonic activity of the MAb preparation was quantified *in vitro* by the opsonophagocytosis killing assay [21]. Briefly, human polymorphonuclear leukocytes (PMNL) were mixed with bacteria from an exponentially growing culture in a ratio of 3:1, in the presence or absence of GBS-absorbed guinea pig serum 10% v/v as complement, and the relevant MAb (or rabbit type IV-specific antiserum) as antibody. The mixture was incubated at 37°C and the number of cfu was counted on Columbia blood agar at the beginning and 1 h after incubation. The opsonic activity was reported as the percentage decrease in the initial number of cfu. All experiments were performed in triplicate.

Neuraminidase treatment

Type IV polysaccharide was treated for 3 h at 37°C with an immobilised preparation of neuraminidase (from *Clostridium perfringens*; Sigma) to cleave sialic acid, as described previously [21]. After enzymic hydrolysis of the polysaccharide, the free sialic acid was assessed by high performance anion-exchange chromatography coupled with amperometric detection [21].

Determination of GBS lethal dosage

The virulence of type IV GBS was assessed by injecting bacteria i.p. or i.v. in two different animal models used in the protection experiments. The LD₅₀ was calculated by a standard method for estimating 50% endpoint [25] and represented the mean of three separate experiments, i.p. and i.v.

i.p. route. The virulence of type IV GBS strain 1/82 by the i.p. route was assessed by injecting 10²–10⁸ cfu/mouse into groups of 10 CD-1 male mice, 4 weeks old. Mortality was recorded for 5 days. The LD₅₀ was 3.2 (0.1) $\times$ 10⁶ cfu/mouse.

i.v. route. The virulence of type IV GBS strain 1/82 by the i.v. route was assessed as described previously [16]. Groups of 20 CD-1 female mice, 8–10 weeks old, were given 10⁵–10⁹ cfu/mouse by i.v. injection and mortality was recorded for 60 days. The LD₅₀ was 1.94 (0.15) $\times$ 10⁷ cfu/mouse [16].

In-vivo protection studies

Two murine models were used to examine the protective efficacy of Mab H11.

GBS acute infection. The protective activity of Mab H11 against lethal infection by type IV GBS was assessed in groups of eight CD-1 male mice (4 weeks old). Ascitic fluid (0.25 ml), at different dilutions, was injected i.p. 4 h before or 4 h after lethal i.p. challenge (5 LD₅₀ in 0.25 ml). Simultaneous injection of both Mab and bacteria was performed in parallel. Mortality was recorded at 24-h intervals for at least 15 days.

GBS chronic infection. The efficacy of Mab H11 against type IV GBS chronic arthritis infection was assessed with a murine model described previously by Tissi *et al.* [16]. Groups of 20 CD-1 female mice, 8–10 weeks old, received Mab i.v. (0.5 ml of ascitic fluid *via* the tail vein) 4 h before injection of 10⁷ cfu (0.5 ml/mouse) of type IV GBS. In parallel, other groups of mice received the polyclonal rabbit antisera directed against type IV GBS and group B polysaccharides, with the same treatment schedule. Both the Mab and the polyclonal antibodies were used at a dilution corresponding to an ELISA titre of 6400. The animals were observed daily for illness and death for 2 months. On days 1, 5 and 10 after challenge, cultures from blood, kidneys and joints of antibody-treated or untreated mice were performed to verify group B streptococcal infection. Blood samples were obtained by retro-orbital sinus bleeding before the animals were killed. Kidneys were removed aseptically and placed in a tissue homogeniser with 3 ml of sterile RPMI medium. Appropriate dilutions of kidneys or blood were plated in triplicate on Islam agar (Unipath) plates containing inactivated horse serum 5% v/v and cfu were enumerated after incubation for 48 h under anaerobic conditions. Results were expressed as the number of cfu/ml of blood or whole organ. Joints were removed, ground in a mortar and resuspended in 1 ml of sterile RPMI medium. All samples were plated on Islam agar plates and the results were expressed as the number of cfu/ml of homogenate and as the number of positive or negative cultures from the animals examined. Control animals received an unrelated Mab (AF1, IgM antibodies against a polysaccharide epitope of *Candida albicans* mannoprotein) that did not react with GBS.

Statistical analysis

Differences in data of survival rates and the incidence of arthritis in the protection studies were evaluated by Fisher's exact test. Differences in the number of cfu were determined according to Student's *t* test. Each experiment was repeated three times.

Results

Production and characterisation of MAbs to GBS

Fourteen days after immune spleen-cell fusion with myeloma cells, nine hybridoma cultures secreting IgM antibodies against the type-specific polysaccharide of type IV GBS were obtained. One of the positive cultures was selected for cloning and propagation into pristane-treated mice. A stable and high antibody-producing clone was used as the source of the Mab (hereafter referred to as Mab H11). In ELISA, Mab H11 reacted with type IV GBS polysaccharide but not with the other type-specific or group B polysaccharides. In immunodiffusion, Mab H11 gave a single precipitin line of identity with the native type IV polysaccharide and its desialylated form (Fig. 1A); Mab H11 and rabbit hyperimmune anti-type IV serum gave a line of identity with the type IV antigen (Fig. 1B).

Opsonophagocytosis assay

The functional activity of Mab H11 was tested against viable GBS in an in-vitro neutrophil-mediated bactericidal assay (Table 1). In the presence of human neutrophils and complement, Mab H11 (86 µg/ml)

Fig. 1. A, Immunodiffusion in agar of type IV native polysaccharide (well 1) and the desialylated polysaccharide obtained after neuraminidase treatment (well 2) with Mab H11 (well 3). B, Immunodiffusion in agar of type IV native polysaccharide (well 3) with rabbit hyperimmune anti-type IV GBS (well 1) and Mab H11 (well 2).

Table 1. In-vitro opsonophagocytic killing of type IV GBS reference strain by Mab H11 in the presence of human PMNL and complement

PMNL	Complement	Mab H11 (µg/ml)	Number of cfu (10 ⁶ /ml) at *		Killing (%)
			0 min	60 min	
–	+	+(86)	2.4	3.8	0
+	+	–	2.5	2.5	0
+	–	+(86)	2.9	3.9	0
+	+	+(86)	2.6	0.5	80
+	+	+(8.6)	2.6	2.1	20

– absent; + present.

*Data are from a representative experiment of three performed.

promoted 80% of killing of the GBS type IV strain, a degree of killing similar to that obtained with a rabbit IgG anti-type IV serum (diluted 1 in 100) under the same experimental conditions. No killing of type Ia, II or III GBS strains was observed (data not shown).

Mouse protection studies

Two experimental murine models of GBS infection were used to examine the protective efficacy of MAb H11 in comparison to that of the polyclonal rabbit antisera directed against the type IV and group B polysaccharides. These models were represented by mice with acute, lethal GBS infection and by mice with a chronic GBS arthritis.

Protective efficacy of MAb H11 against acute lethal GBS infection. The protective efficacy of MAb H11 was determined in 4-week-old CD-1 mice infected i.p. with 5 LD₅₀. MAb H11 ascitic fluid administered i.p. with the infecting bacteria, at doses of antibodies ranging from 0.3 to 1.8 mg/kg (ELISA titre, 32 000–6400), offered significant protection against a lethal challenge with type IV GBS. Survival at the lowest dose of MAb H11 was 75%, and 90% at the highest. MAb preparation was also tested for prophylactic and therapeutic activity. When administered at the highest

dose (1.8 mg/kg) 4 h before challenge with 5LD₅₀, MAb H11 was highly protective with a 90% survival rate; when the antibody was administered 4 h after lethal challenge, 28% of the animals were still protected ($p < 0.05$) (Fig. 2).

The protection afforded by MAb H11 was examined also against i.p. infection with type IV GBS clinical isolates. Of the animals given the antibody 4 h before infection, 80–90% survived compared with no survival of the control animals. In contrast, no protection could be provided by anti-type IV GBS MAb H11 against lethal challenge of serotypes Ia, Ib, II, III or V (data not shown).

Protective efficacy of MAb H11 against GBS chronic arthritis infection. The ability of MAb H11 to confer protection against chronic GBS infection was investigated in comparison with anti-type IV and anti-group B rabbit sera. CD-1 mice, 8–10 weeks old, were given 10^7 type IV GBS by i.v. injection; this produced 40% mortality and arthritis in approximately 70% (Table 2). The administration of MAb H11 at a dose of 1.8 mg/kg (ELISA titre, 6400) offered excellent protection against both mortality and arthritis (Table 3). The hyperimmune type IV rabbit serum also reduced significantly the number of animals with

Fig. 2. Protective i.p. administration of 0.25 ml of anti-type IV polysaccharide specific MAb H11 (1.8 mg/kg) 4 h before (●), simultaneously (○) or 4 h after (□) i.p. inoculation of 5 LD₅₀ (1.63×10^7 cfu/mouse) of GBS type IV strain 1/82. Control mice were treated with an unrelated MAb (△) and with GBS alone (▲). Data are from three independent experiments. SE < 10% have been omitted.

arthritis. In contrast, the anti-group B serum only slightly reduced the incidence of articular lesions (Table 2). Evaluation of cfu on day 1 after infection in the joints of animals revealed 9.2×10^1 cfu/ml of joint homogenate in mice pretreated with MAb against

1.9×10^6 cfu/ml of joint homogenate in controls (Table 3). In MAb H11-treated mice, bacteria were completely cleared, as demonstrated by the absence of positive mice on day 5 (Table 3). The number of bacteria found after pretreatment with type IV antiserum or anti-group B serum on day 1 was lower than in controls ($p < 0.001$), but higher than in MAb-treated animals.

As chronic systemic GBS infection in adult mice is characterised by the presence of high numbers of live bacteria in kidneys [16], cfu enumeration in blood and kidneys at different times after protective treatment with MAb, type- or group-specific antisera was also performed. Whereas all the treated animals cleared the organisms from the blood within 5 days, control mice had the first negative blood culture only on day 10 (Table 4). Kidneys of all mice were colonised by GBS at each examination but in the controls and in the anti-group B serum-treated mice, cfu strongly increased during the first 10 days of infection. In contrast, in MAb H11- and type IV antiserum-treated mice, the numbers of cfu decreased significantly in the same period.

Table 2. Effect of MAb H11, group B and type IV GBS antiserum administration on mouse survival and on incidence of arthritis induced by i.v. injection of 10^7 cfu of type IV GBS, strain 1/82; the results were obtained at the end of the observation period (60 days)*

Treatment†	Surviving (%)	Mice with articular lesions (%)	Mean day onset
Control	12 (60)	14 (70)	2
MAb H11	20 (100)‡	0 (0)‡	—
Type IV antiserum	20 (100)‡	3 (15)‡	4
Group B antiserum	16 (80)	8 (40)§	2

*Values are the mean of three separate experiments. In each experiment 20 mice were used. (SE, always $< 10\%$, has been omitted.)

†Mice were pretreated by i.v. injection of 0.25 ml of identical quantities of antibody preparations (ELISA titre 6400) 4 h before GBS challenge.

‡ $p < 0.001$ (treated mice *versus* control).

§ $p < 0.05$ (treated mice *versus* control).

Table 3. Growth of type IV GBS in the joints of CD1 mice pretreated with MAb H11, group B- or type IV-specific antiserum on days 1, 5 and 10 post-infection

Treatment*	Number (SD) of cfu recovered and number (n) of positive mice out of 10†					
	Day 1 (n)		Day 5 (n)		Day 10 (n)	
Control	$1.9 (0.3) \times 10^6$	10	$1.1 (0.2) \times 10^9$ §	10	$6.7 (0.9) \times 10^{10}$ §	10
MAb H11	$9.2 (1.2) \times 10^1$ §	10	0	0	0	0
Type IV antiserum	$9.8 (1.2) \times 10^4$ ‡	8	$4.4 (1.0) \times 10^3$ ‡§	6	$4.2 (0.3) \times 10^5$ ‡§	2
Group B antiserum	$2.6 (0.2) \times 10^5$ ‡	10	$1.7 (0.2) \times 10^6$ ‡§	7	$6.1 (1.1) \times 10^7$ ‡§	4

*All pretreatments were performed 4 h before GBS infection with 10^7 cfu/mouse.

†Ten mice/group were killed at each time point. The numbers of cfu/ml of joint homogenate are reported. Values are an average of three independent experiments.

‡Values significantly different from control ($p < 0.001$).

§Values significantly different from day 1 ($p < 0.001$).

Table 4. Growth of type IV GBS in blood and kidneys of CD-1 mice pretreated with MAb H11, group B- or type IV-GBS specific antiserum*

Treatment	Number (SD) of cfu recovered †					
	Day 1		Day 5		Day 10	
	Blood	Kidneys	Blood	Kidneys	Blood	Kidneys
Control	$5.3 (0.5) \times 10^3$	$5.8 (0.5) \times 10^6$	$3.3 (0.2) \times 10^3$	$3.3 (0.5) \times 10^8$ §	0	$1.8 (0.5) \times 10^{11}$ §
MAb H11	$1.8 (0.2) \times 10^3$ ‡	$4.2 (0.8) \times 10^5$ ‡	0	$6.3 (0.8) \times 10^4$ ‡§	0	$3.6 (0.2) \times 10^3$ ‡§
Type IV antiserum	$2.5 (0.5) \times 10^3$ ‡	$6.3 (1.1) \times 10^5$ ‡	0	$1.8 (0.2) \times 10^5$ ‡§	0	$2.1 (0.2) \times 10^4$ ‡§
Group B antiserum	$3.1 (0.5) \times 10^3$ ‡	$1.4 (0.2) \times 10^6$ ‡	0	$4.2 (0.2) \times 10^7$ ‡§	0	$1.8 (0.2) \times 10^9$ ‡§

*Mice were pretreated i.v. with 0.25 ml of identical quantities of antibody preparation (ELISA titre 6,400) 4 h before GBS challenge (10^7 cfu/mouse).

†Eight mice/group were killed at each time point. The number of cfu/ml of blood or for both kidneys are reported. Values are an average of three independent experiments.

‡Values significantly different from controls ($p < 0.001$).

§Values significantly different from day 1 ($p < 0.001$).

Discussion

The present study demonstrated that a murine monoclonal IgM antibody (MAb H11) to type IV antigen is protective in mouse models of acute and chronic type IV group B streptococcal infections. MAb H11 was specific for both sialylated and desialylated type IV polysaccharide. This finding confirms that sialic acid does not influence the expression of the native immunodeterminant type IV polysaccharide [19]. In fact, the two forms of type IV antigen gave a reaction of identity in immunodiffusion both with the MAb and the polyclonal rabbit anti-type IV GBS serum. It was not possible to obtain anti-polysaccharide antibody isotypes other than IgM. On the other hand, the majority of the described MAbs against GBS type polysaccharides (Ia, Ib, II and III) belong to the IgM class and only a few to the IgG class [10, 14].

The MAb was protective in acute infections only when given simultaneously or before challenge. The role of type-specific antibodies in the prevention of acute infection has been suggested by Lancefield *et al.* [26], who demonstrated that various mouse protective antibodies can be produced in the sera of experimentally immunised rabbits. Furthermore, low levels of maternal type-specific antibodies are shown to increase the risk of neonatal infections on man [5]. Infants transfused with blood containing opsonic antibody against the infecting serotype of GBS are more likely to survive than those transfused with blood without antibody to the infecting organism [27]. These results indicate a key role for type-specific antibodies in the first stages of infection, probably by facilitating phagocytosis and killing by PMNL. Furthermore, to be effective, the protective treatment should begin very early, because antibodies cannot overcome active bacterial multiplication. MAb H11 was able to prevent the establishment of chronic septic arthritis. This is particularly important as septic arthritis is a serious clinical manifestation associated with GBS infection not only in infants, but also in adults [1, 3, 28]. Furthermore, the early administration of MAb H11, or type-specific serum, significantly reduced infection in kidneys and completely cleared GBS from blood from day 5. The isotype difference between the antibodies of the rabbit anti-type IV serum (IgG class) and the MAb H11 preparation (IgM) might account for the higher protective activity of the MAb. Similar results on the protective activity of antibodies have been observed by Raff *et al.* [29], who reported that more effective protection against the virulent strain type III GBS was obtained with human IgM MAb than with a genetically engineered IgG with the same antigen combining regions.

In conclusion, the present results demonstrate that the effective neutralisation of the capsular type-specific polysaccharide is important in protection against both acute or chronic GBS infection. The presence of

antibodies in the early stages of GBS infection reduces the lethality of the disease, as well as kidney and joint colonisation.

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