BACTERIAL CHARACTERISATION AND PATHOGENICITY

Virulence properties of type VII *Streptococcus agalactiae* (group B streptococci) and immunochemical analysis of capsular type polysaccharide

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Strains of a new polysaccharide type of group B streptococci (GBS), type VII, have been isolated from human carriers and invasive infections. Some of these strains bear the protein antigen c or R, as do other GBS serotypes. The capsular type polysaccharide is sialylated and this residue is involved in the immunodeterminant structure. All type VII strains examined were virulent in CD-1 mice; the LD₅₀ after intraperitoneal (i.p.) challenge was $4.57 (SD\ 0.12) \times 10^7$ cfu for the reference strain and $5.49 (SD\ 1.5) \times 10^7$ cfu for clinical isolates. A particular feature of this serotype was the ability to induce septic arthritis not only when injected intravenously (i.v.), but also when injected i.p. Rabbit antiserum against the capsular type VII polysaccharide exhibited opsonic activity in a phagocytosis assay and protective activity against infection.

Introduction

Group B streptococci (GBS; *Streptococcus agalactiae*) remain, despite advances in diagnosis and treatment, an important cause of perinatal morbidity and mortality, as well as the aetiological agent of severe invasive diseases in non-pregnant adults [1–4].

The major virulence factor of GBS is the antiphagocytic polysaccharide capsule, which also allows the classification of GBS into well-defined serotypes [1]. Some of these serotypes, such as type Ia, Ib, II and III, were described by Lancefield in 1934 [5]. During the last three decades, GBS strains not reacting with these typing sera have been isolated from carriers and from invasive infections [6–8]. Therefore, some new serotypes were defined on the basis of the structural differences of the capsular polysaccharide [9–13].

In the 1970s and early 1980s, types Ia, Ib and III were the most common serotypes associated with neonatal

GBS diseases [1, 2]. More recently, important changes in serotype distribution have been observed in the USA and in Japan where serotypes V and VIII (JM9), respectively, have emerged as significant causes of diseases both in children and in adults [8, 14–17].

In this framework, the recognition and characterisation of new GBS serotypes is an important issue. The present study describes the virulence characteristics of a new GBS serotype, type VII, and immunochemical properties of the type VII capsular polysaccharide.

Materials and methods

Bacterial strains

S. agalactiae type VII reference strain 7271 was obtained from the Czech National Type Culture Collection (Prague). Additional type VII GBS clinical isolates were kindly provided by E. Gunther (Institute of Experimental Microbiology, Jena, Germany): 123348, 124055 (type VII/c), 132157 (type VII/R) and 132158 (type VII/R). Other reference strains included in this study were GBS 090R (group B, non-capsulate variant of strain 090), 090 (type Ia), NCTC 11078 (type Ia/c), NCTC 8187 (type Ib), NCTC

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11079 (type II), NCTC 11080 (type III), NCTC 9828 (type III/R), 1/82 (type IV), 10/84 (type V) and 118754 (type VI). The last three strains were obtained from the Czech National Type Culture Collection (Prague).

Type-specific antiserum preparation and specificity

Type VII GBS antiserum was prepared by intravenous (i.v.) immunisation of New Zealand White rabbits with formalin-killed whole bacterial cells as described by Lancefield [5]. The antiserum was absorbed with organisms of the non-capsulate type Ia variant GBS strain 090R to remove anti-group B polysaccharide antibodies [5]. Serotype specificity of the serum was checked against all GBS reference typing strains by Ouchterlony double diffusion in agar [18]. Briefly, 20 μ l of type VII serum were loaded in the centre well and 20 μ l of type-specific antigen (obtained by extraction of the bacteria in 0.2 N HCl for 2 h at 50°C) in the peripheral wells. After overnight incubation at room temperature (RT), the immunodiffusion test was examined for bands of precipitate.

Serum specificity was also checked by ELISA inhibition experiments. Type VII GBS antiserum was absorbed separately with all GBS reference typing strains, then assayed by ELISA for reactivity with purified type VII polysaccharide as described previously [11].

The serum titre was determined by ELISA. Microtitration plates (Immunol A, Dynatech) were coated overnight at 4°C with 100 μ l of GBS type VII bacterial suspension ($OD_{520} = 0.33$) in 200 mM carbonate-bicarbonate buffer, pH 9.6. After three washes with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) containing Tween-20 (Sigma) 0.01%, plates were blocked with skimmed milk (Difco) 5% in PBS for 1 h at 37°C. After washing, 100 μ l of antiserum diluted in PBS plus gelatin 0.01% (PBS-1) were added and plates were incubated for 2 h at 37°C. After washing, 100 μ l of a goat anti-rabbit IgG alkaline phosphatase-conjugated antiserum (Sigma) diluted in PBS-1 were added followed by incubation for 1 h at 37°C. After washing, *p*-nitrophenylphosphate (Sigma; 1 mg/ml in 1 M diethanolamine-HCl, pH 9.8) was added and incubated for 40 min at RT. The OD_{405} was read with a microtitre reader (Victor-1420 Multilabel Counter; Wallace, Milan, Italy). The titre achieved for type VII antiserum was 64 000.

Other serological methods

The identity and immunospecificity of type VII polysaccharide were tested by double immunodiffusion assay [18]. Electrophoretic migration of the type VII polysaccharide was performed in agarose 1% w/v and was run at 15 mA for 40 min at RT in 0.04 M barbital

buffer, pH 8.6 [19]. At the end of the run, anti-type VII serum was added in a trough cut parallel to the path of electrophoretic migration. After overnight incubation at RT, gels were examined for bands of precipitate.

Electron microscopy

To demonstrate the presence of type VII-specific polysaccharide in the capsule, bacteria were incubated with homologous type-specific antiserum diluted 1 in 100, washed with phosphate-buffered saline (PBS; pH 7.2) and incubated with gold-labelled protein A. Fixation, dehydration in acetone and embedding in Epon were performed as described previously [20].

Purification of type VII GBS capsular polysaccharide

Bacteria were grown in 50 L of ultrafiltered (DC10 LA Amicon filtration system with 10 000-M_r cut-off hollow fibre cartridges) Todd-Hewitt Broth (Oxoid), containing glucose 2% v/v with continuous titration to pH 7.2. The polysaccharide was purified from culture supernates by gel filtration and anion-exchange chromatography as described previously [11].

High-performance size-exclusion chromatography (HPSEC)

The purified polysaccharide was chromatographed by HPSEC to confirm the absence of contaminating substances. The chromatography was performed on a Zorbax SE-450 column (9.4 $\times$ 250 mm; Dionex, Sunnyvale, CA, USA) equilibrated in 200 mM Na₂HPO₄ (pH 7.0), at a flow rate of 1 ml/min. The column was serially connected with an SE-Diol guard column (4 $\times$ 12.5 mm). Column fractions were monitored with a variable wavelength detector (Dionex) at 214 nm to reveal the type polysaccharide and at 260 and 280 nm to detect the presence of contaminating nucleic acids and proteins, respectively. The type VII polysaccharide peak was confirmed by immunodiffusion with type VII-specific antiserum.

Analytical procedures

The cell-bound type polysaccharide was assessed by determining the sialic acid content of the bacteria as described elsewhere [21].

The monosaccharide composition of the purified type VII polysaccharide was determined by high-performance anion-exchange chromatography (HPAEC) coupled with a pulsed amperometric detector (PAD; Dionex) [11]. Briefly, neutral and amino sugars were identified after hydrolysis of the polysaccharide with 2 N trifluoroacetic acid at 100°C for 5 h; sialic acid was detected after hydrolysis of the sample with 100 mM HCl at 80°C for 1 h.

The purified polysaccharide was analysed for protein content by the BioRad protein assay (BioRad Laboratories, Munich, Germany) and spectrophotometrically at 260 nm for nucleic acids.

Desialylation of polysaccharide by neuraminidase

Type VII polysaccharide (130 µg) was incubated with an immobilised preparation of neuraminidase (from *Clostridium perfringens*; 80 mU; Sigma) with agitation at 37°C. After treatment for 3 h, the enzyme was removed by centrifugation and the amount of free sialic acid (i.e., that cleaved from the polysaccharide by the enzyme) was measured directly in the test solution by HPAEC-PAD and compared to total sialic acid of intact polysaccharide.

In-vitro opsonophagocytosis assay

The assay was performed as described in detail elsewhere [11]. Briefly, human peripheral blood leucocytes (PMNLs) were mixed with bacteria (ratio 3:1) in the presence or absence of GBS-absorbed guinea-pig serum (10%) as complement source, and different dilutions of type VII-specific serum. Duplicate samples were removed for quantitative culture at the beginning and after incubation for 1 h at 37°C. Opsonophagocytic killing was reported as the percentage decrease in the initial number of cfu. All experiments were performed in triplicate.

Virulence determination

A murine model of GBS infection was used to evaluate the virulence of different type VII strains [22]. Bacteria were grown overnight at 37°C in Todd-Hewitt broth, washed and diluted in serum-free RPMI 1640 medium (Gibco, Life Technologies, Milan, Italy). The inoculum size was estimated turbidimetrically at 540 nm and the number of live bacteria was calculated by counting the cfu on sheep Columbia agar containing blood 5% v/v.

CD-1 female mice (Charles River Breeding Laboratories, Calco, Milan, Italy), aged 8–10 weeks were inoculated intraperitoneally (i.p.) or i.v. with 10^7 – 10^9 cfu of GBS (0.5 ml/mouse) and mortality was recorded at 24-h intervals for 60 days. The LD50 was calculated by the method of Reed and Muench [23] and represents the mean of three experiments.

Clinical evaluation of arthritis. Mice challenged i.v. or i.p. with the different type VII strains were examined at least twice during day 1 after inoculation and then daily for 2 months to evaluate the presence of joint inflammation. Arthritis was defined as visible joint swelling or erythema, or both, of at least one joint. The time of onset, incidence, number of joints involved, duration of arthritis and ankylosis were observed.

Histological evaluation of arthritis. Groups of mice

inoculated i.v. with 10^7 cfu or i.p. with 10^8 cfu of the different type VII GBS strains were examined every 2 days for histological features of arthritis. Joints were removed aseptically, fixed in formalin 10% v/v for 24 h and then decalcified in trichloroacetic acid 5% v/v for 7 days, dehydrated, embedded in paraffin, sectioned at 5–7 µm and stained with haematoxylin and eosin.

GBS growth in blood and joints. Blood and joint infections were determined by viable counts after challenge as described previously [24]. Samples from a group of mice inoculated i.v. with 10^7 cfu or i.p. with 10^8 cfu of the different type VII GBS strains were collected on days 1, 5 and 10 after challenge. Blood samples were collected from the retro-orbital sinus before the mice were killed. Joints were removed, ground in a mortar and resuspended in 1 ml of sterile RPMI medium. All samples were streaked on Islam agar (Oxoid) plates containing inactivated horse serum 5% v/v and cfu were counted after incubation for 48 h under anaerobic conditions. The results were expressed as cfu/ml of blood or joint homogenate.

Mouse protection test

The protective activity of type VII-specific serum against infection with type VII GBS was assessed in groups of 10 mice. Immune serum (0.5 ml), diluted 1 in 10, was administered i.v. or i.p. 4 h before an i.v. or i.p. injection of 10^7 or 10^8 cfu/mouse of type VII reference strain 7271. Animals were observed daily for 2 months to evaluate the presence of arthritis and mortality. Control mice were given absorbed serum only.

Statistical analysis

Differences in survival rates and the incidence of arthritis in the protection test were evaluated by Fisher's exact test. Each experiment was repeated three times.

Results

GBS type VII antiserum specificity

Rabbit antiserum to whole type VII GBS cells was assayed for specificity by double diffusion against the acid cell extract of all GBS reference typing strains. Type VII antiserum reacted with the extract of the homologous strain and cross-reacted with the serotype V cell extract. No line of identity was obtained between the acid cell extract of type VII and type V (data not shown). Absorption of type VII antiserum with GBS type V cells removed only the cross-reaction.

Serum specificity was also confirmed in ELISA inhibition experiments. Antiserum raised against type VII GBS organisms was absorbed separately with

organisms of each capsular type, then assayed by ELISA for reactivity with purified type VII polysaccharide. Absorption of the serum with type VII organisms removed the type VII specific antibodies, while absorption with organisms of heterologous capsular types had no effect (data not shown).

Immune electron microscopy

When incubated with homologous antiserum, GBS type VII strains, whether characterised or not by the presence of protein c or R, were shown to have a large capsule surrounding the bacteria (Fig. 1A). The type-specific polysaccharide was localised within the capsule by pre-embedding labelling (Fig. 1B). Incubation of type VII GBS with non-immune serum followed by protein A-gold treatment showed no labelling and no capsular layer (data not shown).

Cell-bound sialic acid

The capsular type polysaccharide of all GBS serotypes described to date contains residues of sialic acid (N-acetylneuraminic acid) on side chains and its amount can be used as an indirect measure of the degree of capsulation. Thus, the presence of cell-bound sialic acid content was determined in the reference strain and in different type VII clinical isolates. Its concentration was 0.55 (SD 0.05) $\mu\text{g}/\text{mg}$ of dry cell weight for the reference strain and 0.45–0.57 $\mu\text{g}/\text{mg}$ of dry cell weight for four other type VII GBS strains.

Immunochemical properties of type VII polysaccharide

Purified type VII polysaccharide chromatographed on Zorbax SE-450 with a UV absorbance at 214 nm showed a single major absorbance peak (data not shown). Chromatograms performed at an absorbance of 260 and 280 nm revealed minor peaks indicating low residual levels of proteins and nucleic acid (<2.0%). The carbohydrate composition of the purified native polysaccharide was analysed by HPAEC-PAD. The

polysaccharide contained glucose (26%), galactose (26%), N-acetylglucosamine (19%) and N-acetylneuraminic acid (24%) which together accounted for 95% of the material weight and which were present in the molar ratio 1.4:1.4:0.8:0.8. The purified native polysaccharide had a molecular size distribution coefficient (K_D) of 0.4 and a M_r of 250 000, as calculated by gel filtration.

The native type polysaccharide reacted in Ouchterlony diffusion with type VII-specific rabbit antiserum, forming a single precipitin line (Fig. 2). No reaction was observed between the native type antigen and the immune sera against all other GBS serotypes.

Removal of sialic acid residues from type VII polysaccharide by treatment with neuraminidase did not alter substantially the reaction of the polysaccharide with type VII antiserum (Fig. 2). In fact, only a very small spur could be observed. The efficacy of neuraminidase treatment was assessed by HPAEC-PAD to determine the amount of free sialic acid that

Fig. 2. Immunodiffusion in agar of type VII native polysaccharide (well 1), neuraminidase-treated polysaccharide (5), type antigen extracted from the reference strain after treatment of the bacteria with 0.2 N HCl for 2 h at 50°C (2 and 3) or for 10 min at 100°C (4) with type VII antiserum (centre).

Fig. 1. Localisation of capsule and type specific-polysaccharide on ultra-thin sections of type VII GBS reference strains ($\times 40\,000$). (A) Capsule after stabilisation with type-specific antiserum; (B) demonstration of type-specific polysaccharide by pre-embedding labelling with type-specific antiserum followed by protein A-gold.

was measured directly in the test solution. The enzymic treatment cleaved 40% of the total sialic acid content of the polysaccharide.

Type VII polysaccharide extracted from the bacterial cells by 0.2 N HCl, according to the procedure used for GBS typing, was obtained in two forms, depending on the temperature used. The antigen extracted at 50°C for 2 h formed a clear line of identity with the native purified polysaccharide, while that extracted by Lancefield's method [5], at 100°C for 10 min, gave a precipitin line of partial identity both with the neuraminidase-treated antigen and with the 50°C acid extract (Fig. 2).

Immuno-electrophoresis of the native, neuraminidase-treated and hot HCl-extracted type VII polysaccharide revealed differences in the mobility of these polysaccharides. The native type VII antigen showed a negative charge migrating towards the anode, whereas the HCl-extracted antigen migrated in the opposite direction (Fig. 3). The homologous neuraminidase-treated antigen presented a spur towards the cathode (Fig. 3).

Opsonophagocytic killing assay

In the presence of complement and human PMNs, no change in the number of cfu of type VII GBS was

Fig. 3. Immuno-electrophoresis of type VII native polysaccharide (centre well), neuraminidase-treated polysaccharide (upper well), type antigen extracted from the reference strain after treatment of the bacteria with 0.2 N HCl for 10 min at 100°C (lower well); troughs contain GBS type VII antiserum.

observed after incubation for 1 h (Table 1). In contrast, the addition of specific-type polysaccharide antibodies to the in-vitro model resulted in a 90% decrease in bacterial numbers (Table 1).

Virulence of type VII GBS

The ability of GBS type VII to cause systemic infection and septic arthritis was studied in mice. The virulence of the type VII GBS reference strain is shown in Table 2. A higher mortality rate was observed in mice inoculated i.v. than in those inoculated i.p. The LD₅₀ was $1.93 (SD 0.23) \times 10^7$ cfu/mouse for mice inoculated i.v. and $4.57 (SD 0.12) \times 10^7$ cfu/mouse for those inoculated i.p. ($p < 0.001$ i.v. versus i.p.). The LD₅₀ assessed for three clinical isolates gave mean values of $2.18 (SD 1.8) \times 10^7$ and $5.49 (SD 1.5) \times 10^7$ cfu/mouse when inoculated i.v. or i.p., respectively (data not shown).

One day after i.v. or i.p. injection of type VII GBS, mice manifested articular swelling. The kinetics of appearance and the incidence of articular lesions are shown in Table 3. The injection of 10^7 or 10^8 cfu/mouse by the i.v. route determined the onset of arthritis, while only the highest concentration (10^8 cfu/mouse) induced articular lesions by the i.p. route. The major histopathological changes in the affected joints of mice inoculated i.v. with 10^7 cfu were the presence of an acute exudative synovitis, starting 2 days after the infection, and a PMNL-monocyte infiltrate of the subsynovial and periarticular

Table 2. Effect of i.v. or i.p. injection of different doses of type VII GBS reference strain on survival of CD-1 mice

Inoculum size (cfu/mouse)	Route of inoculation	MST	D/T
10^9	i.v.	1	20/20
10^8	i.v.	7	20/20
10^7	i.v.	> 60	6/20
10^9	i.p.	1	20/20
10^8	i.p.	13	14/20
10^7	i.p.	> 60	2/20

MST, median survival time expressed in days;
D/T, dead mice at day 60 and total mice tested.

Table 1. In-vitro opsonophagocytic killing of GBS serotype VII reference strain by type VII-specific antiserum in the presence of human PMNL and complement

PMNL	Complement	Type VII antiserum (dilution)	Mean cfu (SD) $\times 10^6$ /ml at*		Percentage killing
			0 min	60 min	
—	+	+ (1 in 100)	2.60 (0.01)	8.60 (0.02)	0
+	+	—	2.68 (0.02)	5.48 (0.04)	0
+	—	+ (1 in 100)	3.28 (0.03)	4.64 (0.02)	0
+	+	+ (1 in 100)	2.68 (0.01)	0.28 (0.01)	90
+	+	+ (1 in 1000)	2.36 (0.01)	0.31 (0.01)	87
+	+	+ (1 in 10 000)	2.36 (0.03)	0.80 (0.02)	66

—, absent; +, present.

*Data are from a representative experiment of three performed. Values are the means and SD of triplicate determinations.

Table 3. Time course of joint arthritis in CD-1 mice after i.v. or i.p. injection of different doses of type VII GBS reference strain

Inoculum size (cfu/mouse) and route	Number (%) of mice with articular lesions at given days after infection*			
	1	5	10	30
10 ⁸ i.v.	12 (30)	40 (100)	—	—
10 ⁷ i.v.	12 (30)	36 (90)	36 (90)	36 (90)
10 ⁸ i.p.	8 (20)	30 (75)	30 (75)	—
10 ⁷ i.p.	0	0	0	0

*Mice were inoculated i.v. or i.p. on day 0; 40 mice were used in each experiment. Values are an average of three independent experiments (SE, always <10%, have been omitted).

connective tissue. After 1 week, the articular cavity was filled with purulent exudate and joint destruction progressed until fibrous ankylosis was observed on day 60. In the joints of mice inoculated i.p. with 10⁸ cfu, the histopathological features of the lesions were similar to those observed in the joints of mice inoculated with 10⁷ cfu.

In vivo, infection, after i.v. or i.p. injection of 10⁷ or 10⁸ cfu/mouse, was monitored by determining the level of bacteraemia and growth in joints at days 1, 5 and 10. All animals treated i.v. and i.p. cleared the micro-organisms from the blood within 10 days, while GBS were constantly isolated from the affected joints during the whole course of infection (Table 4).

The type of infection and the incidence of arthritis induced by type VII GBS clinical isolates were similar to those induced by the reference strain (Table 5).

When mice were inoculated i.v. with 10⁸ cfu/mouse, the administration of type VII specific antiserum resulted in a significant ($p < 0.001$) increase in the median survival time; total protection from death and arthritis was observed when the animals were inoculated i.p. with 10⁸ cfu or i.v. with 10⁷ cfu/mouse. As expected, when absorbed with type VII GBS, the specific immune antiserum was not protective (data not shown).

Discussion

The reference strain of this new GBS serotype was originally isolated in 1979 by Perch and co-workers [7]

Table 5. Effect of i.v. or i.p. injection of different doses of type VII GBS clinical strains on survival and on induction of arthritis in CD-1 mice

Strain no.	Inoculum size (cfu/mouse)	Route of injection	MST	D/T	Articular lesions (%)*
123348	10 ⁸	i.v.	6	20/20	—
	10 ⁷	i.v.	> 60	5/20	85
	10 ⁸	i.p.	13	13/20	70
	10 ⁷	i.p.	> 60	3/20	0
124055	10 ⁸	i.v.	7	20/20	—
	10 ⁷	i.v.	> 60	7/20	90
	10 ⁸	i.p.	14	14/20	70
	10 ⁷	i.p.	> 60	3/20	0
132157	10 ⁸	i.v.	6	20/20	—
	10 ⁷	i.v.	> 60	6/20	90
	10 ⁸	i.p.	12	15/20	75
	10 ⁷	i.p.	> 60	4/20	0

MST, median survival time expressed in days. All mice inoculated i.v. or i.p. with 10⁹ cfu/mouse died at day 1 (data not shown). D/T, dead mice at day 60 and total mice tested.

*Incidence (%) of articular lesions at day 10 after infection. Twenty mice were used in each experiment. Values are an average of three independent experiments (SE, always <10% have been omitted).

and designated number 7271. Strains of this serotype have been isolated not only from urogenital or rectal specimens, but also from cerebrospinal fluid and blood samples of severe invasive GBS diseases in many European countries, the USA and East Asia [25, 26 and personal observations]. Like the other GBS serotypes, type VII is also surrounded by a capsule that, as demonstrated by electron microscopy immunogold technique, is formed by the type-specific polysaccharide. The capsule contains sialic acid and the content of cell-bound sialic acid of this new serotype is similar to that of GBS strains of serotypes III, IV and VI grown under similar culture conditions [21, 27].

GBS capsular polysaccharides of serotypes Ia, Ib, II, III, IV and V are polymers of repeating units containing glucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid [9, 10, 28]. These polysaccharides are immunologically distinct, as the ratio and the linkages between the sugars are peculiar for each serotype. HPAEC-PAD analysis of the monosaccharide component of type VII polysaccharide indicated the presence of sugars identical to those found in all the other GBS serotypes. The monosaccharides were present at a molar ratio corresponding with the smallest likely whole-integer ratio of 2 glucose, 2 galactose, 1 N-acetylglucosamine and 1 N-acetylneuraminic acid. This

Table 4. Growth kinetics of type VII GBS in the blood and joints of CD-1 mice*

Inoculum size (cfu/mouse) and route	Site of infection	Number of cfu (SD) recovered on post-infection days		
		1	5	10
10 ⁷ i.v.	Blood	7.5 (1.2) × 10 ²	1.7 (0.4) × 10 ³	0
	Joints	6.3 (1.7) × 10 ⁵	7.7 (0.6) × 10 ⁸	1.5 (0.4) × 10 ⁹
10 ⁸ i.p.	Blood	6.5 (1.2) × 10 ³	3.6 (0.3) × 10 ²	0
	Joints	9.1 (0.9) × 10 ³	6.5 (0.6) × 10 ⁶	7.1 (0.3) × 10 ⁹

*Mice were inoculated i.v. with 10⁷ or i.p. with 10⁸ cfu/mouse on day 0. Eight mice/group were killed at each time point. The number of cfu/ml of blood or joint homogenate are reported. Values are an average of three independent experiments.

molar ratio of sugars was identical to that of type IV polysaccharide [9].

Serological analysis of type VII native purified polysaccharide revealed that it is antigenically distinct from the other GBS type antigens. In immunodiffusion experiments, the native antigen showed no cross-reaction with sera specific for the other GBS type polysaccharide. ELISA inhibition experiments confirmed the specificity of type VII antiserum for type VII antigen, as its reactivity was abolished only after absorption with the homologous antigen. Thus, the cross-reactivity observed in immunodiffusion between type VII antiserum and the acid extract of type V cells is not dependent on the type V polysaccharide, but probably on other superficial antigens of this serotype. Therefore, to make the serum monospecific for serotype VII, it is necessary to absorb the type VII antiserum with type V bacteria; other GBS type-specific sera (type Ia, Ib) also need to be absorbed with cells of heterologous serotypes to become monospecific [29].

The relationship between the crude HCl-extracted polysaccharide used for serotyping and the native antigen has been demonstrated by immunodiffusion. The acid treatment, performed on bacteria at 50°C, extracted a polysaccharide that formed a precipitin line of identity with the native type VII antigen when both reacted with type VII antiserum. The antigen extracted, according to Lancefield's method with hot HCl, gave a line of only partial identity and was desialylated, as demonstrated by the opposite electrophoretic mobility with respect to the native antigen. Desialylated polysaccharides of GBS types Ia, II, III and VI also showed a partial serological identity or no identity with the native form of the respective polysaccharides [11, 30–33]. The specificity of antibodies for the native form of GBS type polysaccharides is dependent on the presence of sialic acid residues. These residues are immunodominant, as they exert conformational control over determinants responsible for this specificity [30–33]. Thus, the sialic acid also exerts a conformational control for the native type VII antigen. This finding, based on the immunological relationship between type VII specific antiserum and its homologous antigen, is in agreement with nuclear magnetic resonance (NMR) spectroscopy structural studies which showed that sialic acid was the terminal residue of the side chains of the type VII polysaccharide [12].

Neuraminidase-treatment of type VII antigen has been proved to be incomplete, releasing only 40% of the total sialic acid. The inability of neuraminidase to remove all the sialic acid residues has also been observed for type III polysaccharide and other sialic acid-containing polysaccharides [30, 34].

The results of the present study indicate that the type VII polysaccharide is also an important virulence

determinant for serotype VII GBS. In fact, phagocytosis of type VII by neutrophils occurred only in the presence of type-specific antiserum and protection from infection was conferred by administration of specific anti-capsular type antibodies, as described for all other GBS serotypes [11, 35–38]. Type VII strains were virulent in this mouse model of invasive infection and were shown to induce joint infections, which are a known complication of GBS diseases both in infants and in adults [1]. A very high percentage of mice (90%) developed articular lesions after i.v. injection of GBS type VII. Joint lesions were also observed after injection of GBS by the i.p. route. This last finding is peculiar to type VII strains, as GBS serotypes II, III, IV, V and VI induced arthritis only when administered by the i.v. route [27]. GBS type polysaccharides play a critical role in the onset of septic arthritis, as mutants lacking type capsule were not arthritogenic [27]. The type VII GBS strains used in this study were capsulate, but their ability to induce septic arthritis even when injected i.p. might also be related to other virulence factors that have not yet been elucidated.

In conclusion, except for its high arthritogenicity, this new serotype shows similar behaviour *in vitro* and *in vivo* to that of the other GBS serotypes. At present, this serotype is rarely isolated from infants and adults, but the real incidence might be underestimated as not all laboratories routinely use type VII antiserum in GBS serotyping. However, another GBS serotype (type V), that was previously considered rare, has recently become a frequent cause of GBS infections in all age groups [39]. Thus, the possibility of future emergence of type VII organisms should not be excluded.

The finding of new non-typable strains suggests that the epidemiology of GBS infections continues to change; therefore, new serotypes need continuous monitoring in order not to exclude important polysaccharides from the formulation of new vaccines that are being developed against invasive GBS infections [40–41].

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