Experimental rabbit model of meningitis produced by *Haemophilus influenzae* serotype c

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Summary. The virulence of *Haemophilus influenzae* type c when inoculated intracisternally (i.c.) into rabbits was evaluated. Rabbits are relatively resistant to infection with *H. influenzae* type b, such that inocula of the order of 10^{6-9} cfu are required to produce meningitis in this model. In contrast, fatal meningitis was produced in this study when 10^3 cfu of a type-c strain were injected i.c. into rabbits. Numbers of bacteria in cerebrospinal fluid (CSF) of control (untreated) animals generally increased to 10^7 cfu/ml. Increases in white blood cells, protein and lactate in the CSF were similar to those which had been observed during meningitis due to *Streptococcus pneumoniae* in rabbits. The infection was amenable to therapy with ampicillin 50 mg/kg given intravenously 12 h after infection. Numbers of bacteria in CSF were reduced to 2.2×10^3 cfu/ml (SEM 0.2×10^3) at 8 h after treatment with a single dose of ampicillin. Two doses of ampicillin, given 12 and 20 h after infection, significantly increased the mean survival time. In contrast to previous experimental studies with rabbits, the penetration of ampicillin into the CSF was high—46 (SEM 10) % of the blood level. Since considerable replication of *H. influenzae* type c occurred within the CSF in this model, the nature of the meningeal damage produced was likely to be similar to that which takes place in man. Hence, *H. influenzae* type c meningitis in rabbits may provide a useful model in which therapeutic and other experimental studies of *H. influenzae* meningitis can be performed.

Introduction

Although rabbits are susceptible to meningeal infection with *Streptococcus pneumoniae* when the organism is administered intracisternally (i.c.)¹ they are relatively resistant to experimental meningeal infection with *Haemophilus influenzae*.² Consequently, high inocula (10^{6-9} cfu) of *H. influenzae* type b have been used to produce a rabbit meningitis model for therapeutic studies with antimicrobial agents³⁻⁹ and adjunct anti-inflammatory agents,¹⁰ and for evaluation of the importance of tumour necrosis factor in the pathogenesis of meningitis.¹¹ After the introduction of large numbers of organisms into the cerebrospinal fluid (CSF), apparently in order to overcome the naturally occurring antibodies¹² which are thought to render the animal resistant to infection,² little or no increase in numbers of bacteria occurs. Therefore, such infections may not closely mimic natural infections with *H. influenzae* in which considerable increases in numbers of bacteria in the CSF occur.

H. influenzae serotype c causes meningitis only occasionally in man.^{13,14} Although the clinical presentation appears similar to cases caused by type-b

strains, *H. influenzae* type-c strains are considered to be much less virulent than type-b strains.¹⁵ The chemical composition and antigenicity of the type-c capsule differs from that of the type-b polysaccharide.¹⁶ Furthermore, type-c strains differ from type-b strains in outer membrane protein¹⁷ and lipooligosaccharide¹⁸ composition, which may also result in a different antigenic profile between type-c and type-b strains. Therefore *H. influenzae* type c may elude the naturally occurring antibodies in rabbits that react with type-b strains. The purpose of the present study was to examine the virulence of a type-c strain in a rabbit model of meningitis, and to determine whether the infection produced could be modified by antimicrobial therapy.

Materials and methods

H. influenzae type-c

The organism used in all experiments was *H. influenzae* P27, a clinical isolate confirmed to be of capsule type c by slide agglutination with type-specific antisera (Difco); it was stored at -70°C in Brain Heart Infusion Broth (BHI; Difco) supplemented with IsoVitalex (Becton-Dickinson) 1% v/v and fetal calf

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serum 10%. Inocula for the production of animal infections were prepared by diluting overnight cultures into freshly supplemented BHI broth. Organisms were allowed to grow to mid-exponential phase ($c. 10^8$ cfu/ml, estimated by turbidimetry) before being harvested by centrifugation at 2000g for 10 min and resuspended in pyrogen-free saline (PFS) to $c. 5 \times 10^3$ cfu/ml. Numbers of viable bacteria were determined by further dilutions of these suspensions in BHI broth which were then plated on to "chocolate" agar plates.

Model of meningitis

The experimental protocols were approved by the ethical committee of Vetinäramt für Kanton Basel Stadt. Chinchilla rabbits, 2–3 kg in weight, (specific pathogen free, obtained from Thomae, Biberach and der Riss, Germany) were fitted with prostheses as described previously.¹ Before infection, rabbits were given ethyl carbamate (urethane) 1.75 g/kg by subcutaneous (s.c.) injection, followed by pentobarbital 10 mg/kg intravenously (i.v.) to induce long-term anaesthesia. The animals were then positioned in stereotactic frames and 3.5-in spinal needles (25 g) sited in the cisterna magna were used to withdraw 0.3 ml of CSF and to introduce 0.2 ml of bacterial suspension. Deeply anaesthetised animals remained in the frames up to 24 h after infection to facilitate repeated CSF sampling and were then removed to cages. Subsequent CSF sampling was performed after repeat anaesthesia with pentobarbital. The rate of removal of CSF did not exceed the rate of CSF formation ($c. 0.4$ ml/h.¹⁹) Blood samples were taken into syringes containing heparin (0.15 U/ml of blood, final concentration) by ear or saphenous vein puncture.

Determination of indices of meningitis

Numbers of white (WBC) and red (RBC) blood cells in CSF and blood were determined with a Sysmex cell counter (model CC-170M, TDA Corp., Kobe, Japan). Counts of viable bacteria were estimated by dilution of CSF in BHI broth and plating on to "chocolate" agar plates. Concentrations of protein, lactate and glucose in CSF were determined as described previously.²⁰ Histological examination of paraffin-embedded sections of brains which had been fixed by immersion in formalin 4% was performed after staining with haematoxylin and eosin (H and E).

Antimicrobial therapy of *H. influenzae* type c meningitis

At 12 and 20 h after infection, eight animals were given ampicillin 50 mg/kg as an i.v. bolus; controls ($n=5$) received an equivalent volume of PFS. Ampicillin present in CSF and blood samples obtained for the determination of bacterial numbers was inactivated by the addition of penicillin-amido- β -lactam-hydrolase (EC 3.5.2.6, from *Bacillus cereus*; Genzyme

Biochemicals Ltd, Maidstone, Kent) 1 U/ml to the BHI broth used for diluting samples. Concentrations of ampicillin in samples taken for pharmacokinetic studies were determined by bioassay; indicator organisms were either *Micrococcus luteus* ATCC 9341 (CSF) or *B. subtilis* ATCC 6633 (plasma). The MIC of ampicillin for *H. influenzae* P27 was determined by a macro broth dilution technique with an inoculum of 10^6 cfu/ml. The MBC (99.99% kill) was determined by subculture of 10- μ l volumes on to "chocolate" agar plates.

Statistical methods

Where indicated, values given are geometric means (cfu and WBC counts); other mean values are arithmetic means. Variation is depicted by the SEM represented by numbers in parentheses. The paired Student's *t* test was used to compare data obtained before and after ampicillin treatment. The Wilcoxon rank sum test was used to compare mean survival times.

Results

Characteristics of experimental *H. influenzae* type c meningitis

Preliminary experiments demonstrated that i.c. inocula of 3.2×10^8 , 6.3×10^7 , or 3.2×10^6 cfu resulted in no increase in bacterial counts in CSF and that inoculation with 5×10^5 cfu resulted in only a limited extent of growth to $c. 10^6$ cfu/ml. Figs. 1–4 present the data obtained from a representative experiment in which the inoculum was $c. 10^3$ cfu. Inoculation of rabbits with 1.6×10^3 cfu was followed by bacterial replication such that numbers increased to around 10^7 cfu/ml. Numbers of bacteria in the CSF doubled approximately every 250 min between 1 h and 12 h after infection, with a very rapid increase during the late stages of untreated infections leading to death of the animals (fig. 1). All untreated animals died; the mean survival time was 34 (SEM 3) h after infection

Fig. 1. Growth of *H. influenzae* type c following i.c. injection of 1.6×10^3 cfu of *H. influenzae* strain P27 into rabbits that were given either saline ($n=5$, $\square$) or ampicillin (50 mg/kg i.v.) 12 and 20 h after infection ($n=8$, $\blacksquare$). Symbols represent mean values and vertical bars represent SEM.

Fig. 2. Pleocytosis after i.c. injection of 1.6×10^3 cfu of *H. influenzae* type c into rabbits that were given either saline ($n=5$, $\circ$) or ampicillin (50 mg/kg i.v.) 12 and 20 h after infection ($n=8$, $\bullet$). Symbols represent mean values and vertical bars represent SEM.

Fig. 3. CSF protein content changes during the course of experimental *H. influenzae* type c meningitis in rabbits given 1.6×10^3 cfu of strain P27 and either saline ($n=5$, $\square$) or ampicillin (50 mg/kg i.v.) 12 and 20 h after infection ($n=8$, $\blacksquare$). Symbols represent mean values and vertical bars represent SEM.

Fig. 4. CSF lactate and glucose content changes during the course of experimental *H. influenzae* type c meningitis in rabbits given 1.6×10^3 cfu of strain P27. The lactate ($\circ$, $\bullet$) and glucose ($\triangle$, $\blacktriangle$) content of CSF from animals given saline ($\circ$, $\triangle$; $n=5$) or ampicillin (50 mg/kg i.v.) ($\bullet$, $\blacktriangle$; $n=8$) treatment 12 and 20 h after infection were determined enzymatically. Symbols represent mean values and vertical bars represent SEM.

(range 27–42 h). Bacteraemia ($> 1 \times 10^2$ cfu/ml) was not detected until 10–12 h after infection. *H. influenzae* was always cultured from blood taken *post mortem*, but numbers varied from 10^6 to 10^9 cfu/ml. For reasons that are unknown, the bacterial density in CSF of animals that were sampled at 12 h after

infection was higher than in those that had been repeatedly sampled.

Meningitis was accompanied by a marked increase in WBC in the CSF, of which more than 80% of the total WBC were granulocytes (fig. 2). An increase in WBC content preceded any rise in protein concentration (fig. 3). Concentrations of lactate rose slowly until 12 h after infection, after which there was a rapid rise (fig. 4). CSF glucose levels rose rapidly at first before stabilising at a concentration which was more than 150% of initial levels (fig. 4).

Histological examination of brains demonstrated acute purulent meningitis and meningo-encephalitis characterised by the presence of exudate containing numerous polymorphonuclear cells (rabbit heterophilic cells) and by the formation of thrombi, especially at the base of the brain.

Ampicillin therapy of H. influenzae type c meningitis

The MIC and MBC of ampicillin for *H. influenzae* P27 was 0.25 mg/L. Ampicillin administration (50 mg/kg i.v.) to eight animals 12 and 20 h after infection resulted in a decrease in bacterial numbers in the CSF (fig. 1), as well as a reduction in numbers of bacteria in the blood to below the limit of detectability (< 20 cfu/ml). Before the second dose of ampicillin, bacterial counts in CSF declined, on average, by $2.1(\text{SEM } 0.2) \times 10^3$ cfu/ml in six animals and counts declined below the limit of detectability ($< 1.2 \times 10^2$ cfu/ml) in the other two rabbits. Ampicillin therapy resulted in significant ($p < 0.001$) decreases in measured bacterial counts in CSF, compared to numbers of organisms present immediately before administration of the drug. Therapy was associated with an increase in mean survival time to 52 (SEM 3) h (range 32–72 h) significantly ($p < 0.05$) longer than in untreated animals. Numbers of organisms in CSF and blood were below detectable limits in three of eight treated animals *post mortem*. *H. influenzae* type c was cultured from the CSF (range 10^3 – 10^6 cfu/ml) and from the blood (range 10^2 – 10^4 cfu/ml) of the other five treated rabbits.

Ampicillin therapy was associated with WBC counts in CSF similar to those observed in untreated animals (fig. 2). However, at 36 h after infection, or *post mortem*, WBC populations in ampicillin-treated animals consisted of granulocytes 60% and lymphocytes and monocytes 40%. Although the protein concentration in the CSF increased after therapy, this change was similar to that seen in untreated animals (fig. 3). Ampicillin therapy resulted in a decrease in CSF lactate concentrations at 20 h after infection compared with controls, but this difference was not statistically significant. There was no difference in CSF glucose concentrations between treated and untreated animals (fig. 4).

Studies of the pharmacokinetics of ampicillin, estimated in five animals, demonstrated that the

concentration of ampicillin in plasma was 31 (SEM 10) mg/L 0.5 h after i.v. injection. Ampicillin was eliminated from the blood with $t_{1/2\alpha}=0.11$ (SEM 0.03) h and $t_{1/2\beta}=1.6$ (SEM 0.3) h. The area under the curve (AUC) was estimated to be 112 (SEM 19) mg $\times$ h/L. The peak CSF concentration of ampicillin was 3.8 (SEM 1.1) mg/L (approximately $15 \times$ MBC) 1 h after injection, with a CSF elimination $t_{1/2}$ of 1.5 (SEM 0.1) h. The AUC was 8.4 (SEM 3) mg $\times$ h/L. The peak CSF/plasma ratio of 46 (SEM 10) % was found 1 h after injection. Accordingly, the ampicillin levels in CSF were above the MBC for c. 6.4 h after injection.

Discussion

In studies of *H. influenzae* type b meningitis in the rabbit model of Darcey and Sande,¹ very high inocula (10^{6-9} cfu) have been required to establish infection. Following the introduction of large numbers of organisms, the pattern of bacterial growth (or persistence) within the CSF was variable. Some workers have reported no increase, or even a decrease, in bacterial numbers;^{3-5,7} others have detected a moderate, apparently immediate increase.⁹ A marked increase in bacterial numbers has also been reported after a period of 14–16 h during which little or no bacterial growth occurred.^{6,8} In contrast, the present study has demonstrated that increases in bacterial numbers continued for at least 24 h after introduction of 10^3 cfu of *H. influenzae* serotype c into rabbit CSF. The doubling time for bacteria in the CSF was slow (250 min) relative to the growth rate observed *in vitro* in supplemented BHI broth (40 min). This net growth rate *in vivo* is considerably slower than the net growth rate *in vivo* of *H. influenzae* type b in rat blood (82 min).²¹ However, the reasons for the relatively slow growth rate and for the virulence of this strain in this model are not clear.

The increases in WBC count and concentration of lactate in the CSF which followed *H. influenzae* type c infection were similar to those which have been observed when *H. influenzae* type b has been used in this model.⁹ The concentration of protein in the CSF was higher than that reported after i.c. injection of amounts of lipo-oligosaccharide²² or outer membrane vesicles²³ equivalent to c. 10^7 cfu *H. influenzae* type b, but similar to that observed when rabbits were infected with *S. pneumoniae*.²⁴ These differences may be explained by the continuous stimulation of an inflammatory response during prolonged infection in contrast with the transient inflammatory response which results from injection of various cell components^{24,25} or pro-inflammatory cytokines.²⁶ The increase in CSF glucose concentration observed was similar to that found when infant rats were infected with *H. influenzae* type b.²⁷ While the reasons for this difference between man and experimental animals is unclear, it may be

accounted for partly by the use of anaesthetics in the present study.

The meningitis model described was affected by therapy with moderate doses of ampicillin (50 mg/kg) which resulted in an increased mean survival time of infected rabbits. The decline in bacterial density— 2.1 (SEM 0.2) $\times 10^3$ cfu/ml by 8 h after ampicillin administration—was similar to that observed in studies of *H. influenzae* type b infections (7.9×10^3 cfu/ml³; 3.9×10^4 cfu/ml⁴; 7.9×10^4 cfu/ml⁷; all rates determined by comparing pre-treatment bacterial counts with those observed at 8 h after ampicillin treatment). Generally, bacteriological cure of meningitis in man can be obtained with appropriate antibiotic therapy, and ampicillin is still widely used to treat *H. influenzae* meningitis in areas where susceptibility is still likely to be observed in the clinic.²⁸ In the case of this model, successful therapy of an infection with a strain sensitive to ampicillin indicated that the animal was not so severely compromised due to anaesthetic, infection route, an excessively virulent strain, or an inoculum dose so high that therapy with ampicillin was unsuccessful; this suggests that the use of this model for evaluation of novel antimicrobial therapy would indeed be valid.

During the initial stages of *H. influenzae* type c meningitis, the population of WBC present in the CSF was composed of 80% granulocytes. Interestingly, although the total number of WBC in the CSF changed little, the composition of WBC in the CSF of ampicillin-treated animals changed to only 60% granulocytes, the remainder being lymphocytes. This may indicate a change in the nature of the inflammatory response between that occurring at the onset of infection and that occurring during the course of treatment. This shift in the qualitative composition of WBC in the CSF has also been observed during sterile meningitis induced by the i.c. injection of cytokines.²⁶

Although changes in CSF protein were similar in untreated and treated animals, CSF lactate concentrations tended to decrease after administration of ampicillin. This decrease in lactate has also been observed during ampicillin therapy of *S. pneumoniae*²⁹ and ceftriaxone therapy of *H. influenzae* type b meningitis.¹⁰

Ampicillin pharmacokinetics were characterised by a CSF ampicillin concentration which exceeded that deemed necessary to achieve bacteriological cure of meningitis ($>10 \times$ MBC)³⁰ with the CSF $t_{1/2}$ being similar to the $t_{1/2\beta}$ of serum—1.5 (SEM 0.1) and 1.6 (SEM 0.3) h, respectively. However, the penetration of ampicillin into CSF—46 (SEM 10) % of blood levels—was much higher than that reported in studies which have employed either bolus injection (3% penetration;³ 15% penetration⁵) or continuous infusion (19% penetration;⁴ 13% penetration⁷) for treatment of experimental *H. influenzae* type b meningitis. Although the penetration of ampicillin into the CSF of patients with inflamed meninges is considered to be quite low at 8–13%,³¹ a recent study demonstrated

that the CSF concentration of ampicillin was 39% of the blood level when meningitic children were given 400 mg/kg/day,³² results similar to those found here. The prolonged and progressive infection due to *H. influenzae* type c in the present model may induce meningeal damage such that the penetration of ampicillin is similar to that which occurs in a clinical case. This is likely to be in contrast to the situation which develops when meningeal inflammation results from a sudden infection with a large inoculum of organisms. However, in the present study, we cannot exclude the possibility that the urethane anaesthetic contributed to the high penetration of ampicillin into the CSF.

In conclusion, a reliable model of meningitis in rabbits was established by the i.c. inoculation of *H. influenzae* type c. In contrast to infections in rabbits produced by type-b strains, in the present model, inoculation of only 10³ cfu of *H. influenzae* type c i.c.

led to a fatal infection accompanied by marked increases in bacterial numbers in the CSF. With the exception of the concentration of glucose, cytochemical changes in the CSF were similar to those seen in patients. The penetration of ampicillin was higher than that observed previously when meningitis has been triggered by injection of large inocula of *H. influenzae* type-b strains. These observations suggest that progressive infection, associated with increasing CSF bacterial density after injection of low numbers of *H. influenzae* type c, provides a rabbit model of *H. influenzae* meningitis which more closely mimics the clinical situation. Hence, the use of *H. influenzae* type c to establish meningitis in rabbits may be a better experimental model than that which results from infection with *H. influenzae* type b.

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