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Relationship between penicillin and cephalosporin resistance of *Streptococcus pneumoniae* strains and its inflammatory activity in the experimental model of meningitis

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**Abstract** Using a rabbit model of meningitis, we sought to compare the inflammatory activity induced by three pneumococcal strains with different susceptibilities to penicillin and cephalosporins, belonging to the serotypes 3, 6B and 23F at different inoculum sizes. These serotypes are prevalent in Western Europe and are believed to produce a moderate-to-severe cerebrospinal fluid (CSF) inflammatory response. Only minor differences were observed in the inflammatory activity evoked by the three strains in the subarachnoid space, and most were probably related to differences in bacterial counts. Infection by serotype 23F caused secondary bacteremia in all challenged animals. Our findings reinforce the concept that resistant pneumococci are not more virulent, a fact that should be taken into account when evaluating the efficacy of different anti-pneumococcal therapies. However, the frequent induction of secondary bacteremia by the resistant serotype 23F requires further study.

**Keywords** *Streptococcus pneumoniae* · Meningitis · Penicillin resistance · Inflammation

**Introduction**

The emergence of penicillin and cephalosporin resistance among pneumococci today constitutes a therapeutic problem worldwide and presents a major obstacle to the treatment of pneumococcal diseases, especially meningitis [1, 7, 9, 12, 14, 15]. It has been shown that serotype influences the severity of experimental pneumococcal meningitis, and three different levels of inflammatory response have been defined. Based on inflammatory cerebrospinal fluid (CSF) parameters, serotypes could be classified as producing a mild, moderate or severe response [4, 13]. Penicillin resistance has also been suggested as another possible mechanism influencing severity. Although clinical evidence does not support this hypothesis, it is not clear whether differences in susceptibility to antimicrobial agents of pneumococcal strains might lead to differences in the inflammatory response they provoke.

Penicillin and cephalosporin resistance is more frequent among some serotypes, such as 23F, 6B, 9 and 14 [5, 6, 8, 10]. Serotypes 23F and 6B have been shown to induce a severe CSF inflammatory response in the experimental rabbit model [4]. Serotype 3, usually susceptible to penicillin and a frequent cause of pneumococcal bacteremia, has been shown to promote a moderate CSF inflammatory response [13].

In the present study, using a rabbit model of meningitis, we sought to compare the inflammatory activity induced by three pneumococcal strains with different susceptibilities to penicillin and cephalosporins, belonging to the serotypes 3, 6B and 23F, at different inoculum sizes. These serotypes are prevalent in Western Europe and are believed to produce a moderate-to-severe CSF inflammatory response.

**Material and methods**

**Bacterial strains**

Three pneumococcal strains with different susceptibilities to penicillin and third-generation cephalosporins were studied. Strain A was a serotype 3, fully sensitive to these antimicrobial agents, with an MIC of 0.01 μg/ml to penicillin and cefotaxime. Strain B was a serotype 6 and had an MIC of 0.25 μg/ml to penicillin and 2 μg/ml to cefotaxime. Finally, strain C belonged to serotype 23 and had an...
MIC of 4 μg/ml to penicillin and 2 μg/ml to cefotaxime. Bacterial suspensions were prepared from fresh overnight cultures on 5% blood agar plates made from frozen stock cultures. The inoculum was prepared by suspending the colonies in Mueller-Hinton broth, adjusted to 0.5 McFarland standard (10⁵ cfu/ml). To prepare the different size inocula, bacterial suspensions were prepared making a tenfold dilution in saline to obtain concentrations of 10⁴, 10⁵, 10⁶ and 10⁷ cfu/ml. For each experiment, the inoculum size was determined after making a tenfold dilution in saline, and plating 0.1 ml on blood agar plates. The procedure was then checked by subsequent counting of the colonies after 24-48 h of incubation at 35°C.

Meningitis model

The rabbit model of meningitis was performed in accordance with an established protocol [3]. For all experiments, rabbits were challenged in groups of 4 animals per group. A total of 16 animals (4 per inoculum size) were inoculated with each strain (total 48 rabbits). On the first day of the experiment, they were anesthetized intramuscularly with ketamine (35 mg/kg) and xylazine (5 mg/kg), a dental acrylic helmet was affixed to the calvaria, and the animals were returned to their cages. After 24 h, the rabbits were anesthetized again by the same method and placed in a stereotaxic frame. A 25-gauge spinal needle was introduced in the cisterna magna and 200 μl of CSF was withdrawn as a sterile control (hour 0 of experiment). For each group, meningitis was induced by instillation into the subarachnoid space of a bacterial suspension adjusted to 10⁴, 10⁵, 10⁶ or 10⁷ cfu/ml of one of the three different strains. After 18 h, the rabbits were anesthetized subcutaneously with urethane (1.75 g/kg) and phenobarbital (5 mg/kg). Blood cultures were taken at this time to detect secondary bacteremia; 0.1 ml of blood collected from the central ear vein was suspended in 5 ml of trypticase soy broth (TSB) and incubated overnight at 37°C. Serial CSF samples were taken at 18, 20, 24 and 42 h post inoculation. Brain edema, expressed as g water/100 g dry weight, was determined after killing the rabbits with an overdose of phenobarbital at 42 h.

Sample processing

CSF bacterial titers, leukocyte counts, and protein and lactate concentration were determined. For colony counts, direct cultures and serial tenfold dilution were performed (limit of detection of 10⁷ cfu/ml). For leukocyte counts, 10 μl of each sample was diluted 1:1 with Turk solution and read with a Neubauer camera. After centrifugation, CSF was stored at -80°C before determining the rest of the inflammatory parameters. CSF protein concentration was determined by Bradford’s method (Bio-Rad protein assay), and CSF lactate concentration with Lactate PAP (bioMérieux, France).

In vitro studies

The possibility of different growth kinetics for the three pneumococcal strains was studied by performing in vitro growth curves in Mueller-Hinton broth with 5% lysed horse blood, and in a rabbit pool of non-inflamed CSF, to compare these with those observed in the in vivo model. Glass tubes containing 5 ml of medium or CSF were inoculated with approximately 5×10⁶ cfu/ml microorganisms and incubated at 35°C in a shaking water bath. Samples were taken at 0, 2, 4, 8, 14, 24, 30 and 48 h of incubation. The number of cfu/ml was determined after making a tenfold dilution in saline, and 0.1 ml was spread onto 5% blood agar plates. All procedures were performed in duplicate. Colony counts were performed after 24-48 h of incubation at 35°C.

Statistical analysis

One-way ANOVA was performed for in vivo and in vitro growth curves data, and one-way Kruskal-Wallis analysis of variance on ranks was applied for all the inflammatory parameters; in the case of significance (P < 0.05), Student-Newman Keuls multiple comparison method was used. Fisher’s exact test was performed to compare categorical variables.

Results

CSF bacterial concentrations

At 18 h post inoculation, all groups of rabbits presented positive CSF cultures. Bacterial titers for strains A and C were not affected by the inoculum size, and log cfu/ml at different time point were similar. Strain B exhibited a different behavior; the highest inoculum, 10⁶, induced the lowest bacterial counts at 18 h, 2.84 ± 2.33 log cfu/ml vs 4.38 ± 0.01 with 10³, 4.41 ± 0.80 with 10⁴ and 4.3 ± 0.90 with 10⁵.

Considering the different strains, bacterial titers for the three strains with 10⁶ inoculum size are shown in Fig. 1. There were statistically significant differences (P < 0.05) in bacterial titers at 18 and 20 h with strain B vs C and A. At 18 h strain B showed 2.84 ± 2.33 log cfu/ml, strain A 5.67 ± 0.84 log cfu/ml and strain C 6.33 ± 0.54 log cfu/ml. At 20 h strain B showed 2.92 ± 2.07 log cfu/ml, strain A 5.95 ± 1.42 log cfu/ml, and strain C 6.62 ± 0.55 log cfu/ml.

This possible kinetic difference found in the in vivo experiments was also studied performing in vitro growth curves, however, no differences between strains were observed either in the logarithmic stage of growth or in the lysis rate. Highest log cfu/ml was 8.8 at 14 hours for strain A, 9, 3 log cfu/ml at 8 hours for strain B and 9, 3 log cfu/ml at 8 hours for strain C.

![Fig. 1](image-url)  
Log (CFU/mL) of S. pneumoniae at different time points (18, 20, 24 and 42 h) after inoculation of 10⁶ cfu/ml of three different strains of Streptococcus pneumoniae with different susceptibilities to penicillin and third-generation cephalosporins. Strain A: serotype 3, MIC of 0.01 μg/ml to penicillin and cefotaxime. Strain B: serotype 6, MIC of 0.25 μg/ml to penicillin and 2 μg/ml to cefotaxime. Strain C: serotype 23, MIC of 4 μg/ml to penicillin and 2 μg/ml to cefotaxime. Four determinations per time point were performed. *P < 0.05 strain B vs C and A at 18 and 20 h.