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PRELIMINARY RESULTS IN INDUCING MENINGITIS IN BALB/C MICE USING A HUMAN STRAIN OF *Neisseria meningitidis*

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Abstract

Neisseria meningitidis (Nm) is the pathogen carried asymptomatically in the nasopharynx but which under certain conditions can produce meningitis or even multi-organ failure. Experimental induction of meningitis in an animal model is necessary for testing new nanopharmaceutical products. Thus, the aim of the study was to establish the concentration of Nm capable of inducing the disease. We carried out 2 studies where we tested different concentrations of Nm obtained by the nephelometric and spectrophotometric methods. In the first study we included 20 BALB/c mice, and in the second 24. Local and general clinical signs, complete with body temperature values, body weight, hematological, microbiological and histopathological examination were important indicators for the assessment of the establishment of meningitis. The clinical results have pronounced depression in the first 2 days after inoculation, then the general condition will appear. The microbiological and histopathological examination indicated the presence of bacteria at the brain level and specific meningeal lesions only in the case of the second attempt, but the early mortality, requires additional testing of the bacterial concentration that induces meningites in a stable and persistent way so that treatment against the condition can be tested later.

Key words*: intracisternal inoculation, meningitis, mouse, Neisseria meningitidis.*

INTRODUCTION

Bacterial meningitis caused by *Neisseria meningitidis* (*Nm*) is a serious inflammatory disease characterized by acute infection of the brain and cerebrospinal fluid (CSF) and is life-threatening in healthy individuals. The main injuries encountered in infected patients are those of cerebral edema, hernia, cessation of cerebral circulation (Van Deuren, 2000), mental retardation, hearing loss, hydrocephalus, cognitive disorders, sepsis and finally death, which occurs in 20% of cases , in the absence of adequate treatment (Borrow R., 2017). Serogroups A, B, C, W, X and Y are responsible for the occurrence of meningeal disease worldwide (Parikh, 2020). Bacterial strains isolated from patients with specific clinical signs of meningitis are *Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes* (Sáez-Llorens, 1999) and last but not least Nm. It is carried in the upper respiratory tract by adolescents and young adults, representing the main reservoir of the bacteria. Most carried strains never cause invasive disease, but meningococci can be easily transferred through saliva droplets or direct contact from an asymptomatic carrier to an immunocompromised one (Caugant, 2009).
Nm is a Gram-negative, encapsulated, kidney shaped diplococcus that expresses pili, opacity proteins and adhesion molecules. They adhere to the non-ciliated columnar epithelial cells in

the nasopharynx or the epithelium lining the tonsils, adapt and begin to proliferate. Meningococci enter the bloodstream through capillaries and small veins in the underlying submucosal tissue. In a site with low immunity, the survival and rapid proliferation of neisseria in the blood is favored, so that within about 7 days, the disease is clinically expressed (Stephens, 1991), and death can occur in 12-24 hours (de Greeff , 2008) through septic shock or multiple organ failure that occurs in 30% of patients who contract serogroup B and C (Gedde-Dahl, 1983).

Penicillins represent one of the treatment options for meningitis because most isolated neisseria are susceptible to penicillin and ampicillin. However, there are also strains that express an intermediate resistance to these antibiotics, being isolated from patients in Europe, South Africa or the United States. In the absence of an adequate clinical response to the therapy with the mentioned antibiotics, it is recommended to change the therapy with ceftriaxone or cefotaxime (Brandtzaeg, 2012).

Vancomycin or third-generation cephalosporins are adequate for the treatment of moderate to severe meningitis (Bradley, 1997), but cephalosporins have shown little efficacy, with many treatment failures in the management of patients with pneumococcal meningitis (Shultz, 2000; Singhal, 2007). The newer fluoroquinolones seem to have stronger activity in meningitis, although the prevalence of Nm strains resistant to quinolones is increasing. (Schmitz, 2017). Therefore, for the treatment of meningitis caused by Nm and not only, it is necessary to develop antibiotic molecules with accurate drug delivery mechanisms and a broad antibiotic spectrum. In order for this to be fulfilled, researchers in the pharmacological field are testing new variants of drugs on animal models capable of expressing meningococcal disease. The murine model seems to be a suitable one, and for the induction of the disease, numerous methods have been tried through which the expression of the disease similar to humans has been induced. Thus, the pathogen was inoculated intraperitoneally (Tan, 1995), intranasally (Zwijnenburg, 2001), intravenously or intracisterna-magna (Grandgirard, 2007).

The progression of the disease, the reproducibility and the evaluation of the pathogenic characteristics are still limited and largely depends on the inoculated bacterial strain and its concentration so that the disease is reproduced as faithfully as possible. In this sense, we set out to develop a murine model of meningitis using a strain of Nm, serogroup B, on which new drugs can be later tested.

MATERIALS AND METHODS

Ethics statement

All experiments on animals took place within the "Cantacuzino" National for Medico-Military Institute of Research and Development (IC), Baneasa Animal Facility (BAF), Bucharest. The studies were approved by the competent authorities, in accordance with the provisions of Directive EU 63/2010 regarding the care, use and protection of animals used for scientific purposes. The animals included in the study come from the SPF (Specific Pathogen Free) kennel of BAF, they are housed in groups of 5, in individually ventilated cages, receiving water and food *ad libitum*, with cycles of 12 hours light, 12 hours dark. The temperature inside the cages was monitored daily, being maintained in the range of 20-22^{o} C, and the humidity was between 45-65%. The health status of the animals was monitored daily by the veterinary staff by evaluating the expression of clinical signs, measuring body temperature and weight. The conditions for excluding the animals were established *a priori* and referred to weight loss of more than 20% or their inclusion in the coma scale 1 and 2 (1 = coma, 2 = the animal does not return to the quadrupedal position after was positioned on its back, 3 = the animal returns to the quadrupedal position after 30 seconds after turning on its back, 4 = the animal stands up after 5 seconds after turning on its back showing minimal motor activity, 5 = normal behavior).

Preparation of the inoculum of Nm

The experiments were carried out in two stages, which we will call study 1 and study 2, respectively, depending on the concentration of the inoculum used to induce meningitis.

Study 1: *Nm* was grown on chocolate agar medium, 24 h, 37^{o} C, 5% CO_2. The colonies

grown on the plate were inoculated in brain-heart infusion broth (BHI) supplemented with Dextran Iron (5 mg/kg, Intrafer 200 B12, Interchemie werken, Holland), incubated in the same conditions mentioned previously. To read the turbidity of the inoculum, measured in the McFarland index, we used the nephelometric method and for the exact measurement of colony forming units per milliliter agar plating was performed. The concentrated innoculum obtained was 10^7 CFU/mL. The quantity inoculated in the animal was 10 µL, which means that the mice received bacterial concentrations 100 times lower than the bacterial inoculum, respectively 10^5, 10^4 and 10^3 CFU/mouse.

Study 2: Since the highest McFarland index that the nephelometer can express is 14iMF, the second attempt to induce meningitis was by establishing the concentration of the inoculum of *Nm* by spectrophotometry (TECAN spectrophotometer). The fresh bacteria, 24 hours old, was taken off the chocolate agar plates and inoculated in the BHI medium enriched with Dextran Iron. The vortexing process followed, then reading the density of the solution with an exponential increase (by adding additional colonies of *Nm*, until reaching the value of 0.7 optical density at 630 nm, which represents ~10^{10}CFU/mL. The same dose, of 10 µL, was inoculated to the animals, which means that they received 10^7 and 10^5 CFU/mouse.

Animals included in the study were BALB/c mice, female, 8 weeks old, which, depending on the study, were divided according to Table 1.

Table 1. Lotting of animals according to the concentration of *Nm* used

	Animal group	Number of animals/group	Concentration of *Nm* used (CFU/mL)	The method of establishing the *Nm* concentration
1	N0	5	10^5	Nephelometry
	N1	5	10^4	
	N2	5	10^3	
	Control group	5	BHI+ Dextran Iron	
2	S1	12	10^7	Spectrophotometry
	S2	12	10^5	

Inoculation of Nm in animals

Before starting the experiments, the animals were weighed (KERN 440-47N scale, Germany) and rectally thermometered (SCALA SC 28 Flex thermometer, Taiwan). On day 0, the animals were anesthetized with a mixture of Ketamine (50 mg/kg, Vetased, Farmavet, Romania) and Xylazine (3mg/kg, Bioveta, Romania). The fur from the cervical area was trimmed and disinfected with 70% ethanol and 3% betadine. The inoculation procedure took place in a laminar flow hood. Each mouse was placed on a sterile field. The operator held the animal's head in a flexed ventral position with his left hand, so that the median line of the nose was parallel to the work table. The landmark for inoculation was that of the diamond-shaped depression created at the craniocervical junction. The bacteria, regardless of the concentration, was inoculated inside the large tank, with a syringe with a 30G needle. After inoculation, the animals were placed in cages. The clinical monitoring (general clinical signs, temperature and body weight) lasted 7 days, in study 1, the animals being euthanized at the end and in study 2, the culling of 8 mice (4 from each group) at 48 h, 72 h and 7 days after inoculation.

RESULTS AND DISCUSSIONS

One-way Kruskal-Wallis ANOVA (Prism 9, GraphPad LLC, USA) was used for statistical analysis to compare the average values of weight and body temperature between groups of animals included in the study. For all analyses, a p-value < 0.05 was considered significant.

From a clinical point of view, in study 1, the animals presented a deterioration of the general state of health, characterized by weight loss (Figure 1), diarrhea, purulent ocular secretions, lethargy, hypothermia (Figure 2).

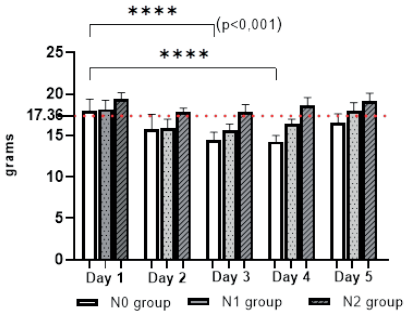

Figure 1. Body weight evolution in Study 1 (dotted red line represents the average value of the animals body weight recorded on day 0)

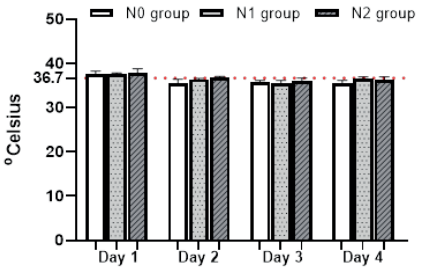

Figure 2. Evolution of body temperature in Study 1 (dotted red line represents the average value of the animals' body temperature recorded on day 0)

Regarding the bacterial concentrations used, the most brutal clinical signs were observed in the animals from the N0 group, followed successively by the other two groups, the symptoms expressed being therefore directly proportional to the aggressiveness of *Nm*. However, no animals succumbed by day 7.

In the case of study 2, the symptomatology was more pronounced, especially in the S1 group, in which mortality occurred after the first inoculation, at 24 h (2 animals). The brain was harvested from these animals to verify the viability of the inoculated bacteria and following the microbiological examination, *Nm* could be isolated. The state of health was visibly altered in the case of S1, unlike S2. Weight loss was significant after the first day of inoculation, $p < 0.001$, increasing until day 4 when $p < 0.0001$. At the end of the experiment, the surviving animals registered weight increases, compared to the previous days, indicating a value of $p < 0.05$, the values approaching those of day 0 (Figure 3).

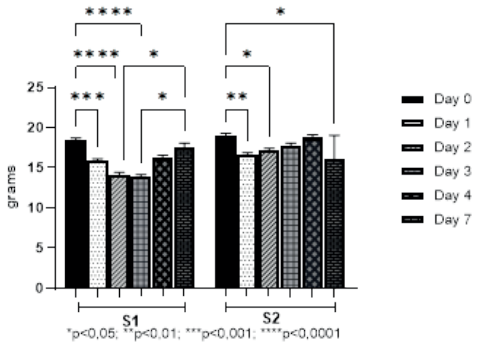

Figure 3. Evolution of body weight in Study 2

Regarding the body temperature value, a statistical significance was observed in the S1 group, on the second and third day after inoculation, when $p < 0.0001$. In the case of the S2 group, the body temperature oscillations showed no significant statistical relevance (Figure 4).

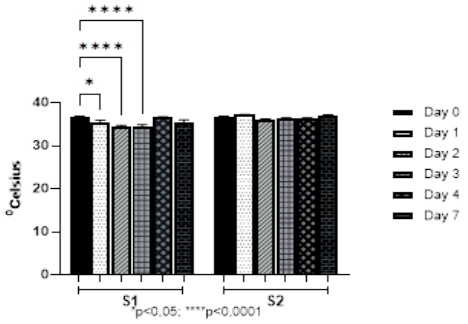

Figure 4. Evolution of body temperature in Study 2

According to the work protocol, the animals were euthanized at 48 h, 72 h and on day 7. 4 types of samples were taken: 10 µL of cerebrospinal fluid (Figure 5) inoculated on chocolate agar, pieces of brain tissue that were spread on a chocolate agar plate (Figure 6), the rest of the brain which was placed in 12 ml of BHI (Figure 7) and some brain samples for histological analysis (Figure 8). At 48h, as well as at 72 h, 2 out of 4 animals from group S1 had viable *Nm*, grown on chocolate agar plates seeded with pieces of brain tissue.

The histological samples showed in the animals of the S1 group, major foci of meningitis, blood vessels with an increased appearance in volume, discrete lesions specific to encephalitis (perivascular sheaths), and strong neutrophilic infiltrate (Figure 9).

Figure 5. Collection of 10 µL cerebrospinal fluid

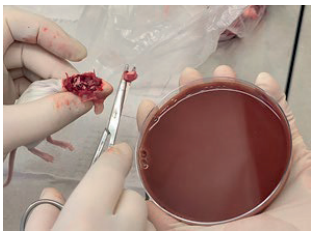

Figure 6. Collection of brain tissue and inoculation on chocolate agar medium

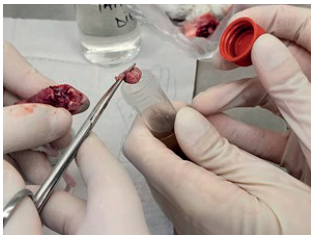

Figure 7. Harvesting brain tissue in BHI medium

Figure 8. Collection of brain tissue for histological analysis

Seven days after the inoculation, the last 6 animals, which no longer showed signs of meningococcal infection, were sacrificed, the same samples being collected, the recovery of the bacteria was no longer possible.

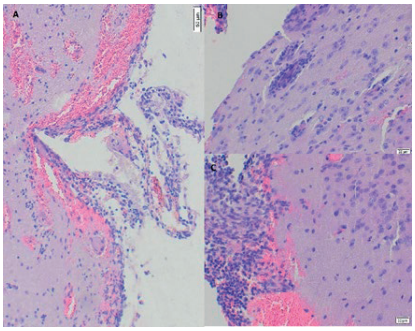

Figure 9. Histological appearance of meningeal sections: A-meningitis focus (ob. 10, Hematoxylin-Eosin staining), B, C- perivascular sheaths, neutrophilic and hemorrhagic infiltrate (ob. 20, Hematoxylin-Eosin staining)

We managed to develop a murine model of meningitis produced by *Nm* in the case of the second study, a fact demonstrated by the number of animals that responded to contact with the bacteria, thus 16.33% of them succumbed to the disease at 24 h. In 33.33% of the animals *Nm* was recovered at 48 h and in 16.6% of the animals after 72 h, the rest of the animals managing to cure the infection, an aspect explained by the fact that *Nm* is a strictly human pathogen. The symptoms expressed along with the microbiological and histopathological findings show that the infection with *Nm* in a concentration of 10^7 UFC/mouse is the closest to human meningitis, but the animals that succumbed to the disease, within the first 24 hours after inoculation, make us believe that the dose tested is not the optimal one for the animals to become stable from the point of view of the establishment of the disease, to then be used for clinical examination or testing of new drugs.

In murine models of meningitis, different ways of inducing the disease were tried, either intranasal or intraperitoneal, but complications such as sepsis or pneumonia (Zwijnenburg, 2001) make the choice of inoculation within the cisterna magna a suitable one, as the bacteria reach directly at the level of the central nervous system. The use of different concentrations of *Nm* for the induction of meningitis aimed to establish the effective level of bacteria that would satisfy the objectives of our study. Like Mook-Kanamori and colleagues (Mook-Kanamori, 2012), we compared the animals' behavior at different concentrations and could observe the aggressiveness of *Nm* at high concentrations or the fact that it is completely harmless when the concentration is low, as demonstrated by the first study tried. From a histopathological point of view, the numerous meningeal infiltrations, hemorrhages or perivascular neutrophilic sheaths, complemented by the stagnant blood that caused dilation of the vessels at the cerebral level, reflect the anatomopathological findings of human patients who died due to bacterial meningitis (Vergouwen, 2010), the same type of lesions being observed in Mtafya's studies (Mtafya, 2019). Brain infarcts or abscesses occur in human patients with meningitis, as well as in experimental rat models (Ostergaard,

2004), but in the case of our studies, this type of specific lesions was not observed and an explanation possible would be the bacterial aggressiveness, the relatively short inoculation-death/euthanasia time, the age of the animals or even the chosen animal model. However, the clinical signs expressed, the isolation of the bacterium from the brain of dead or euthanized animals and the histopathological lesions encourage us towards new attempts to create a murine model of meningitis induced by *Nm*, serogroup B, stable and reproducible. The spectrophotometric method proved to be more efficient than the nephelometric one and easier to implement. An animal model that recapitulates the pathophysiology of a pathogen similar to that in humans would be ideal, but still, hard to find. Today, several inbred and non-inbred mouse strains are available. For the study of meningitis, whether as the disease itself or testing meningococcal vaccines, inbred lines are preferred because, it seems, they better mimic the genetic diversity of humans (Tuttle, 2018). Females are chosen for such studies, they generate robust responses compared to males, the same influence of gender being observed in the case of humans (Klein, 2016). The chosen animal model is also used by other researchers, who obtained good results in the attempt to induce meningitis by inoculation of *Nm* (Pagliuca, 2019), which represents another reason that our study idea is realistic and worthy of being pursue to complete the established objectives.

CONCLUSIONS

The concentrations of *Nm* used in the first study were not effective for inducing meningitis, the animals being able to clear the bacteria. In study 2, the spectrophotometric method was useful to establish the concentration of *Nm* as accurately as possible, which determined the specific symptomatology. The concentration of 10^7CFU/mouse induced the most signs and brain lesions of meningitis and *Nm* could be recovered from the animal brains, at different time intervals. The mortality of the animals even in the first 24h after inoculation shows that 10^7CFU/mouse might be too strong for the disease to progress over longer periods of time, necessary for testing new drugs.

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