RESEARCH

Using reibergrams to evaluate the intrathecal synthesis of C3c and C4 in children affected with Neisseria meningitidis *meningoencephalitis*

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ABSTRACT

Meningococcal meningoencephalitis was a serious pediatric health problem in Cuba before the successful introduction of a Cuban vaccine against *Neisseria meningitidis*. The present paper assesses the role of the complement system in this disease in unvaccinated sick children, using a novel methodology developed by the authors. Seven children were used, of an average of 5.8 years of age with *N. meningitidis* meningoencephalitis diagnosed by traditional microbiological methods. Serum and cerebrospinal fluid samples were obtained at the same time point, and used to quantify major immunoglobulins, IgG subclasses, C3c, C4 and albumin by radial immunodiffusion with commercially available plates. No C3c was found in one of the two deceased patients. Measurable intrathecal synthesis of C3c was observed in the remaining patients, however, intrathecal synthesis of C4 was found in all patients, as demonstrated with the corresponding reibergrams. The measurement of the intrathecal synthesis of these components of the complement system is useful for discriminating immunodeficiencies and to better understand the behavior of the disease.

Keywords: meningoencephalitis, *Neisseria meningitidis*, C3c, C4, intrathecal synthesis, reibergrams

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RESUMEN

Utilización de reibergramas para evaluar la síntesis intratecal de C3c y C4 en niños con meningoencefalitis por *Neisseria meningitidis*.La meningoencefalitis por *Neisseria meningitidis* afectó sensiblemente a la población infantil cubana antes de la exitosa campaña con la vacuna producida en Cuba contra esta bacteria. El objetivo del presente trabajo es evaluar, por medio de métodos novedosos desarrollados por los autores, el papel del sistema de complemento en el desarrollo de la enfermedad en niños con la enfermedad que no fueron vacunados. Siete niños con edad promedio de 5.8 años con meningoencefalitis por *N. meningitidis,* diagnosticados por los métodos microbiológicos tradicionales. Las muestras de suero y líquido cefalorraquídeo se obtuvieron simultáneamente y se cuantificaron las inmunoglobulinas mayores, las subclases de IgG, C3c, C4 y la albúmina por inmunodifusión radial en placas comerciales. Uno de los dos pacientes que fallecieron no poseía C3c en tales líquidos biológico s. Se observó síntesis intratecal de C3c en los otros pacientes y síntesis intratecal de C4 en todos los pacientes estudiados, lo cual se demostró a partir de los correspondientes reibergramas diseñados en Cuba para estos componentes del sistema de complemento. La determinación de la síntesis intratecal de estos componentes del sistema de complemento es útil para discriminar inmunodeficiencias y entender el comportamiento de la enfermedad.

Palabras clave: meningoencefalitis, *Neisseria meningitidis*, C3c, C4, síntesis intratecal, reibergramas

*I*ntroduction

Meningococcal meningoencephalitis was a serious pediatric health problem in Cuba before the successful introduction of a Cuban vaccine against *Neisseria meningitidis* [1, 2]. Although this disease no longer represents a significant problem for our country, it still causes thousands of deaths worldwide, reaching particularly high fatality rates in the so-called 'meningitis belt' of Africa [3]. Today, Cuba manufactures antimeningococcal vaccines for these African countries.

The complement system, with the help of immunoglobulins, is the most effective defense mechanism against this bacterium. These defensive molecules bind to the target surface for the antibody-mediated destruction of target cells or microorganisms; a process known as opsonization that in turn makes the target attractive to phagocytes. Also, the binding of antibodies to the surface of the microorganism may activate the complement cascade, especially when the antibodies belong to the IgM or IgG classes [4].

The polyclonal production of different immunoglobulins occurs in cerebrospinal fluid during the acute phase of meningococcal encephalitis [5, 6]. The subsequent activation of the complement system generates several products, mainly C3b and C4b, which are deposited on cell surfaces and are recognized by macrophages expressing receptors for these proteins [7, 8]. Additionally, the cells or microorganisms already opsonized with IgG, such as bacteria, are recognized by the Fc receptors of phagocytes and subsequently lysed [8, 9]. Therefore, it is important to evaluate the capacity of the central nervous system in the synthesis and activation of the complement system during infections with bacteria such as *N. meningitidis*, especially in early age groups where it is possible that the presence of primary immunodeficiencies may lead to the death of the patient [9-12].

The methods currently available to define the involvement in this disease of the intrathecal synthesis of complement components actually introduce important errors that may potentially lead to contradictory results, hindering the treatment of specific cases and the possible vaccine strategies [9-13]. These methods

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are based on the calculation of indexes such as the quotient of the concentrations of complement components in cerebrospinal fluid (CSF) or serum, and the albumin quotient. Since the use of these indexes can lead to false positives, they are not reliable for crucial diagnoses during the usually limited time course of the treatment of infectious neurological diseases.

The use of reibergrams is the most recent methodology for the discrimination of intrathecal protein synthesis. They were first defined for the main immunoglobulin classes: IgA, IgM and IgG [14], and have quickly become, with their formula and chart, an essential element for the study of CSF as evidenced by their inclusion in automatic equipment -such as nephelometers- supplied by the top medical equipment manufacturers.

The first reibergrams, however, did not include several other proteins which are fundamental in understanding the mechanisms of the immune response in the central nervous system. Our group has therefore designed reibergrams for IgE [15], IgG subclasses [16] and for the intrathecal synthesis of C3c and C4 [17, 18]. These methods take into account the conditions of the blood-CSF barrier, according to the most recent concepts, eliminating the error sources for the indexes employed by the old formulas.

The aim of this study is to assess the function of the complement system in the development of meningoencephalitis in a group of unvaccinated sick children, using the new methods developed in Cuba.

Materials and methods

Patients

Seven children averaging 5.8 years of age (range: 3 months to 8 years) with meningoencephalitis by *N. meningitidis* were studied. They were diagnosed by the conventional microbiological methods, consisting of CSF culture, blood culture and Gram staining. The patients were admitted either into the Pediatric Hospital of San Miguel del Padrón or into the Leonor Pérez Cabrera Pediatric Hospital, both in the city of Havana. The identity of the infectious agent was confirmed in all cases by sending the isolated strains to a reference laboratory at the Provincial Hygiene and Epidemiology Laboratory of Havana. They were also stored at the Central CSF Laboratory in the CSF strain collection of the before the inoculation of the Cuban vaccine against this bacterium, as each sample analyzed in this laboratory is recorded and preserved for later analyses. The records contain all tests and assays performed on each isolate.

In all cases, the patients arrived at the emergency units of these hospitals with fever, headaches, vomiting and photophobia. In patients less than 1 year of age there was also bulging of the fontanels and irritability; and there was neck stiffness in 60% of the cases. The children were transferred to the intensive care units of their respective hospitals and had blood samples taken during the acute phase of the disease with to identify the causal biological agent, if any. The patients lived in suburban area in Havana municipalities of San Miguel del Padrón, El Cotorro and Boyeros.

This study was approved by the Research Bioethics Committee of the Dr. Miguel Enríquez Medical School belonging to the Medical Sciences University of Havana. The informed consent was requested and obtained from the parents and/or tutors before performing diagnostic lumbar punctures.

Methods

Serum and cerebrospinal fluid samples

The CSF samples were obtained by lumbar puncture, at the same time point used in collecting blood samples to obtain the serum. In both cases (CSF and serum) the cells were eliminated by centrifugation, discarding all hemolyzed samples and storing the remaining samples at -70 °C for up to 30 days. In all cases the samples were separated into aliquots for storage to avoid the effects on their protein content of the frost/defrost cycles.

Analysis of serum and cerebrospinal fluid

The levels of IgA, IgM, IgG, C3c, C4 and albumin in the serum and cerebrospinal fluid (CSF) were quantified by radial immunodiffusion in NOR PARTIGEN® and LC PARTIGEN® plates (Behringwerke, now Siemens, Marburg, Germany) respectively. The IgG subclasses were quantified in LL RID® (The Binding Site, Birmingham, United Kingdom) radial immunodiffusion plates in the case of serum samples, or in NANORID® (The Binding Site, Birmingham, United Kingdom) plates in the case of CSF samples.

C3c and C4 reibergrams

When carrying out the assays, indexes were used for the determination of the intrathecal synthesis of complement components and immunoglobulins. There are, however, several inconveniences in their use; these include the fact that they cannot be employed when the CSF-blood barrier is dysfunctional, they produce variable results depending on the volume of the CSF sample extracted, and the absence of reports on their pediatric use.

The quotients of the concentrations in CSF and serum (CSF/serum) for each patient were calculated for the levels of C3c, C4 and albumin.

Functional status of the blood-cerebrospinal fluid barrier

The examination of the functional status of the blood-cerebrospinal fluid barrier was performed by calculating the albumin coefficient Q: Q Albumin = CSF Albumin/Serum Albumin. The upper limit of this parameter varies with age, and is calculated by the formula: $QAlb = (4 + \text{age (in years)}/15) \times 10^{-3}$.

The albumin, C3c and C4 quotients are placed in a CSF/serum chart, also known as a reibergram [17, 18]. The darkest curve on the reibergram is a boundary discriminating the portion of protein synthesized in blood from that synthesized in the CSF. If the values calculated fall above this hyperbolic curve then it can be affirmed that intrathecal synthesis has taken place. The broken percentile lines with 20, 40, 60 and 80% represent the fraction of this protein that has been synthesized in the CSF in relation to the total amount of the molecule in this biological fluid.

The darkest curve represents 0% synthesis in the CSF, indicating that all of the specific protein in CSF was synthesized in the blood. Three vertical lines, present in every reibergram, indicate the normal limit for 3 different ages, for a faster interpretation of the

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results: Q Albumin 5.5×10^{-3} (up to 15 years old), Q Albumin up to 6.5×10^{-3} (up to 40 years old) and Q Albumin up to 8×10^{-3} (up to 60 years old) [17, 18].

Results

No immunodeficiencies due to deficits of immunoglobulins A, M or G were observed. Likewise, there were no altered IgG subclass patterns. The concentrations of these proteins were higher than normal, as is typically observed in patients undergoing this clinical process.

Based on the Q Albumin values, there were 5 patients with CSF-blood barrier dysfunctions when the diagnostic lumbar punctures were performed, since their Q Albumin was higher than 5×10^{-3} (Figures 1 and 2).

The results of the C3c and C4 proteins of the complement system revealed that one of the patients did not have quantifiable levels of C3c in the serum or CSF. These samples were from one of the two deceased patients, although the death of the other patient could not be related to any immunodeficiency. There were measurable amounts of intrathecal synthesis of C3c in the remaining patients (Figure 1). Based on the reibergram for C4, there was intrathecal C4 synthesis in all patients (Figure 2).

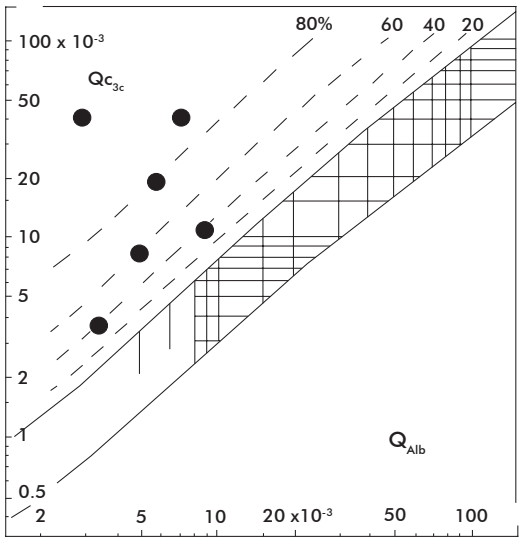

Figure 1. Reibergram for the determination of the intrathecal synthesis of C3c. Note the presence of only 6 points corresponding to 6 patients, due to the immunodeficiency for C3 in the other patient. There is intrathecal synthesis of C3c in all patients, since all points fall above the thickest hyperbolic curve. Five of the patients have Q Albumin values above 5 x 10^{-3}, which is the normal value for children; evidencing the presence of a CSF-blood barrier dysfunction. These dysfunctions are typical of the acute phase of meningoencephalitis.

Discussion

Immunoglobulins are important in the resolution of infectious diseases affecting the central nervous system [19, 20], since they are effective mediators of the lysis of encapsulated microorganisms, such as *N. meningitidis*.

Although the prevalence and incidence of primary immunodeficiencies is not high, some patients are found to have selective immunodeficiencies for IgA [21] or specific IgG subclasses, such as IgG_2, which are associated to meningoencephalitis caused by microorganisms with a polysaccharide capsule [22]. The levels of major immunoglobulins and IgG subclasses, however, remained high in both biological fluids in our patients, thus discarding these immunodeficiencies as a causal factor.

We were able to confirm, in this group of patients, that the frequency of CSF-blood barrier dysfunctions is high in this disease, since only two patients had values below the normal upper limit.

The dysfunction of the CSF-blood barrier observed in this disease facilitates the transit of immunocompetent cells and cells involved in the inflammatory response between both compartments. However, the host organism cannot tolerate this disequilibrium for a long time and therefore tries to restore the vital functionality of this barrier [23]. The two deceased patients had very high immunoglobulin levels and a marked CSF-blood barrier dysfunction.

Reibergrams are clinical charts that facilitate the graphic visualization of the status of the CSF-blood barrier and the intrathecal synthesis of the protein under study. They were originally described for the main immunoglobulins, *i.e.* IgA, IgM and IgG [14], and later additional reibergrams were designed in Cuba for IgG subclasses [16], IgE [15], C3c [17] and C4 [18]. These reibergrams are the latest method used in CSF studies, outperforming earlier formulas and methods such as the use of indexes. Reibergrams are based on the theory of molecular diffusion/flow of CSF [24, 25].

The indexes assumed that protein transport across the membrane follows a linear distribution. However, it has been demonstrated that this distribution is actually hyperbolic, depending on the molecular weight of the protein crossing the CSF-blood barrier. The use of indexes, therefore, may lead to erroneous results.

The application of reibergrams for C3c and C4, which is the purpose of this study, enables the detec-

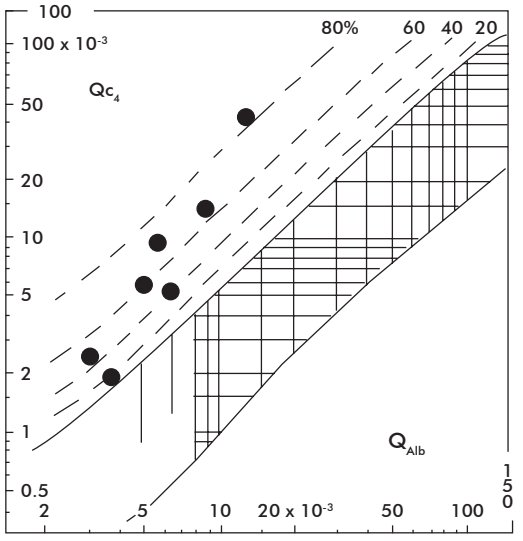

Figure 2. Reibergram for the determination of the intrathecal synthesis of C4. All points of the chart are located above the strongest hyperbolic curve, indicating the intrathecal synthesis of C4 and also demonstrating the activation of the complement by the classical or lectin pathways, which share this component.

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tion of the synthesis of these components of the complement system in the central nervous system. This makes it possible to learn if these patients are able to synthesize and activate these molecular structures in the CSF. The role of these proteins in the appearance and development of the disease is essential for the evaluation of the immune status of patients, and in general for understanding the pathophysiologic mechanism underlying this disorder.

We were able to verify the existence of measurable intrathecal synthesis of C4 in all patients, which is a marker for the start of the complement activation process via the classical pathway through the interaction of IgM, IgG or the MBL-MASP2 complex with the initial fragments of the pathway. This process, however, does not take place with the same intensity in all patients due to the intrathecal fraction synthesized that may depend on a large number of biological variables [26].

Starting with the activation of C4, a number of byproducts are generated that interact with other components of the complement cascade [8]. However, there was an immunocompromised patient with a C3 deficiency, whose levels of C3 in either CSF or blood remained below the detection limit.

C3c is a stable degradation product of factor C3 of the complement system. It does not degrade in serum or CSF, which makes it a consistent indicator. Two important biological inferences can be made by following the formation of C3c: one, since this fragment is a stable byproduct of C3 degradation, it can be used to indirectly estimate C3 concentration; second, it indicates that all the C3c produced in the central nervous system is the product of the biological activation of the system by the classical, alternative, or lectin pathways.

The presence of intrathecal synthesis of C3c is a revealing sign of the activation of this system, indicating the possible presence of an immunological event associated to a type II or cytotoxic hypersensitivity. This is essential for understanding the pathophysiological mechanisms started by infectious or autoimmune neurological disorders [12, 27], and helps confirm the diagnoses for neurological disorders with these characteristics.

Since C3 is a component that is present in all three complement activation pathways [28], problems in the synthesis of this molecule can lead to a serious immunodeficiency. One such deficiency resulted in the death of one of the patients during the first months of age, due to the child's inevitable exposure to multiple infectious agents in normal life and the absence of the complement system. This system is central for bacterial lysis, being one of the main defense mechanism of the host against infectious agents. Therefore, complement deficiencies of this magnitude, as in most primary immunodeficiencies, are incompatible with life [29]. The deceased patient had already suffered a bacterial meningoencephalitis produced by *E. coli*, and the meningoencephalitis analyzed here led to his death.

There have been attempts to use the complement inhibitors of meningococci as antimeningococcal vaccines due to the importance of complement function [30].

Those patients that develop defense mechanisms, verified at the intrathecal level by this work, had a successful recuperation.

The reibergrams for C3c and C4 can be combined to improve the clinical immunological picture regarding the complement system, enabling the evaluation of the intrathecal synthesis of these components, the examination of the functionality of the CSF-blood barrier and the search for intrathecal synthesis patterns characteristic of a specific disease that may lead to the dissection of links with other disorders. The latter can provide new possibilities for the evaluation of autoimmune or infectious neurological diseases.

The use of reibergrams, however, also has its limitations. Reibergrams assume that proteins do not undergo structural modifications or changes in molecular weight as they cross the CSF-blood barrier; however, this assumption does not always hold true. Also, when using reibergrams the same analytical method must be used for serum and CSF samples, which must be taken at the same time point, and it demands highly sensitive analytical assays for the often low concentrations at which these analytes are usually found in CSF. In addition, sample size in terms of the number of patients is also low, since the mass vaccination campaigns in Cuba against this disease have greatly reduced its incidence. This has led to the use and study of the retained samples available and stored at our research center.

Nonetheless, the use of reibergrams has undeniable advantages, such as the fact that the results do not change with the volume of CSF extracted, together with the possibility of their use in lumbar, ventricular or cisternal CSF. They are therefore much more reliable than earlier methodologies to examine the presence of intrathecal synthesis.

Another advantage of reibergrams is the fact that the CSF/serum quotients calculated do not depend on the analytical method used for quantification, as long as they are performed on the same analytical run and with the same method. In addition, they can be used at any range of Q Albumin, unlike previous methodologies which did not provide conclusive results if Q Albumin had higher values than expected for the age of the patient. In contrast, reibergrams are applicable regardless of the status of the CSF-blood barrier, in the presence or absence of dysfunctions. Reibergrams are therefore a methodology that overcomes the limitations of earlier analytical tools.

Conclusions

The use of reibergrams to determine the intrathecal synthesis of these components of the complement system is useful to reveal the presence of immunodeficiencies and offers a better understanding of meningococcal meningoencephalitis as well as other autoimmune and infectious diseases. The reibergram methodology is the most up-to-date, novel and useful tool for the determination of C3c and C4 when studying these complement components in the central nervous system.

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