Reibergram of Intrathecal Synthesis of C4 in Patients with Eosinophilic Meningitis Caused by *Angiostrongylus cantonensis*

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Abstract. *Angiostrongylus cantonensis* produces eosinophilic meningitis in humans and is endemic in Thailand, Taiwan, China, and the Caribbean region. During infection with this parasite, it is important to know if the complement system may be activated by the classical or lectin pathway. Cerebrospinal fluid and serum samples from 20 patients with meningeal angiostrongyliasis were used to quantify C4 levels and albumin. Results were plotted on a C4 CSF/serum quotient diagram or Reibergram. Twelve patients showed intrathecal synthesis of C4. Antibody-dependent complement cytotoxicity should be considered as a possible mechanism that destroys third-stage larvae of this helminth in cerebrospinal fluid of affected patients.

INTRODUCTION

Angiostrongylus cantonensis, a nematode parasite, is the most common cause of eosinophilic meningitis in Thailand, Taiwan, and China and in the Caribbean region. The worm was discovered in the pulmonary arteries and hearts of domestic rats in Guangzhou (Canton), China, by Chen in 1935.¹ The first report of this zoonosis was published in Cuba and in the Western Hemisphere more than 25 years ago.^{2–5} If an infection occurs in non-permissive hosts, including humans and mice, development of parasites will terminate at the young adult worm stage in the brain and cause eosinophilic meningitis or meningoencephalitis.^{6–9}

Complement is a central component of the innate immune system comprising at least 35 proteins that collaborate in an intricate manner in the elimination of microorganisms and removal of apoptotic cells, and also serves as a natural adjuvant, enhancing and directing the adaptive immune response.¹⁰ The final effect of activation of this system was destruction of microorganisms, but it might also have led to the host tissue damage. Initiation of the complement system may occur by three pathways: the classical pathway, the alternative pathway, and the lectin pathway.¹⁰

One study reported that intrathecal synthesis of C₃ or C₄ may contribute to the differential diagnosis of central nervous system (CNS) disorders.¹⁰ Another study reported C₄ intrathecal synthesis in patients with eosinophilic meningitis caused by *A. cantonensis*.¹¹

A similar design to determine intrathecal synthesis of C₄ was not available until recently. A cerebrospinal fluid (CSF)/serum quotient graph for these purposes has been reported,¹² and now it is possible to quantify the intrathecal fraction of C₄ produced in CNS.

The objective of the present study was to determine whether the complement system may be activated by the classical or lectin pathway in patients with eosinophilic meningitis and whether this system might be involved in natural removal of third-stage larvae when the parasite reaches the CNS.

MATERIALS AND METHODS

Patients and sample collection. Samples of CSF and serum were obtained from 20 patients with a diagnosis of eosinophilic meningitis caused by *A. cantonensis*. The mild clinical symptoms and rapid recovery of the patients indicated that there were no cases of encephalitis in these patients. The patients were admitted to different hospitals in Havana during 2003–2009 and had typical symptoms of meningitis. Ten patients were children (average age = 4.5 years) and 10 patients were adults. Samples were obtained at the time of admission at the onset of the symptoms and were kept in small aliquots at –80°C until analysis. Ten control samples were obtained from CSF and serum collections in our laboratory from patients with suspected meningitis. However, these patients were subsequently found to have febrile convulsions without biological agents or verified autoimmune causes. The controls were not age matched because C4 levels do not vary with age of the patients.

C₄ levels in serum were quantified by using a radial immunodiffusion technique by NOR Partigen plates (Dade-Behring, Marburg, Germany), and C₄ levels in CSF were quantified by using LC Partigen plates (Dade-Behring). To identify whether there was C₄ intrathecal synthesis and determine its relationship to the blood-CSF barrier, albumin in serum and CSF was quantified by using the same procedures.

Data analysis. Results were plotted on a CSF/serum quotient diagram (Reibergram) designed for C₄, which was introduced for quantification of intrathecal synthesis of this component of the complement system and constructed on the same principle as quotient diagrams for CSF immunoglobulins.¹³ This procedure is based on molecular diffusion/CSF flow theory, and its fundamental principle is that a decrease in the flow rate is always accompanied by an increase in molecular diffusion from blood to CSF.^{14–16} The Reibergram (Figure 1) was constructed by using the ratios Q albumin (CSF albumin/serum albumin) plotted on the ordinate axis and the Q C₄ (CSF C₄/serum C₄) plotted on the abscissa axis.^{17,18}

The upper hyperbolic curve (thick line) represents the discrimination line between brain-derived and blood-derived proteins. Values above this upper line represent intrathecal C₄ synthesis. Reference ranges for the normal CSF/serum ratios are depicted by the thicker upper hyperbolic line (Q lim) and the thinner lower hyperbolic line (Q low). They include a reference population without intrathecal C₄ synthesis.¹² Between

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FIGURE 1. Quotient diagrams for C_4 (C_4 Reibergram),¹² with data from patients infected with *Angiostrongylus cantonensis*. Reference ranges of blood-derived C_4 in cerebrospinal fluid are between the upper discrimination line (Q_{lim}) and lower discrimination line. Upper hyperbolic line, Q_{lim} , represents discrimination line between brain-derived and blood-derived C_4 fractions, in function of increasing Q_{Alb} . Values above Q_{lim} indicate intrathecal fractions, which can be read from diagrams (dashed lines for intrathecal synthesis). Line Q_{lim} represents 0% synthesis.

these lines is zone of normal biological variation for Q_{C_4} .¹⁹⁻²¹ The dashed lines indicate the extent of intrathecal synthesis as intrathecal fractions (C_4 IF) as 20%, 40%, 60%, and 80% of the measured total C_4 concentration in CSF. The limit of the reference range for $Q_{albumin}$ between normal and increased CSF protein concentrations caused by the blood-CSF barrier dysfunction is indicated by the age-dependent vertical lines at $Q_{albumin} = 5.5 \times 10^{-3}$ (≤ 15 years), at $Q_{albumin} = 6.5 \times 10^{-3}$ (≤ 40 years), and at $Q_{albumin} = 8 \times 10^{-3}$ (≤ 60 years).

RESULTS

Review of clinical files of the 20 patients showed that the main symptoms were fever, vomiting, and some meningeal signs in 50% of patients (8 children and 2 adults). The average number of cells found in the CSF of patients with a diagnosis of infection with *A. cantonensis* was 425×10^{-6} cells/L, and the mean percentage of CSF eosinophils was 41.5%. Our patients had meninges inflammation with the classic triad of diagnostic signs of nuchal rigidity, sudden high fever, and altered mental status. There were no patients with seizures. Patients generally had mild signs and symptoms and rapid recovery. Ingestion of third-larvae of *A. cantonensis* may have occurred accidentally because persons in Cuba generally do not consume raw snails. Cases were found in children who lived in semirural areas that have terrestrial snails. These children often played with snails (*Bradybaena similis honkonensis*, *Subulina octona*, and *Veronicella cubensis*) that have been reported as intermediate hosts of the parasite.²² Adults

TABLE 1
Eosinophils and C_4 in 20 patients with eosinophilic meningitis caused by *Angiostrongylus cantonensis*, Cuba*

Patient	Age, years	Blood eosinophils, (%)	CSF eosinophils, (%)	Blood C_4 , g/L	CSF C_4 , mg/L
1	0.9	10	30	2.89	1.14
2	1	8	36	0.43	1.16
3	4	9	41	0.38	3.91
4	3	7	32	0.52	0.56
5	8	12	45	0.59	1.04
6	0.8	6	32	0.42	4.3
7	4	9	41	0.53	0.17
8	0.08	10	38	0.64	2.03
9	10	8	51	0.87	2.41
10	6	10	31	0.41	1.45
11	28	6	58	5.6	12.0
12	33	10	62	0.19	1.91
13	39	21.4	51	0.25	1.57
14	15	4	48	0.23	1.0
15	36	6	41	0.23	1.0
16	18	5	51	0.39	1.0
17	16	5	16	2.0	2.0
18	20	6	43	4.85	0.91
19	15	5	36	1.0	6.25
20	22	9	38	5.0	35.7
Mean	14.0	8.3	41.5	0.98	4.02

* CSF = cerebrospinal fluid.

in these areas often consumed raw vegetables that were not washed.

Cerebral angiostrongyliasis is generally diagnosed from the clinical history of the patient and CSF eosinophilia and supported by possible exposure to the infective larvae. The gold standard in the diagnosis is detection of the agent that causes the disease, although this diagnosis is often difficult.

Levels of C_4 in serum and CSF, age of the patients, and CSF and serum percentages of eosinophils for each patient are shown in Table 1. Serum and CSF C_4 levels did not show a statistically significant correlation with blood and CSF eosinophil percentages and patient age (Table 2). Nevertheless, there was a statistically significant correlation between serum C_4 and CSF C_4 .

The C_4 quotient diagram (C_4 Reibergram) showed that intrathecal synthesis of C_4 was observed in 12 patients (Figure 1). Six patients had a high C_4 intrathecal fraction (i.e., $> 80\%$), three patients had fraction $> 60\%$, and three patients had a fraction $> 30\%$. Eight patients did not have detectable C_4 in CSF. Eight patients had a blood-CSF barrier dysfunction with

TABLE 2
Correlation between C_4 levels and eosinophils and age in patients with eosinophilic meningitis caused by *Angiostrongylus cantonensis*, Cuba*

Variable		CSF C_4 , mg/L	Serum C_4 , g/L
Age, years	R	0.21	0.19
	95% CI	-0.25 to 0.59	-0.26 to 0.58
	P	0.3714	0.406
CSF eosinophils, %	R	0.01	0.0229
	95% CI	-0.42 to 0.45	-0.4240 to 0.4607
	P	0.9341	0.9238
Blood eosinophils, %	R	-0.0295	-0.17
	95% CI	-0.4659 to 0.4185	-0.57 to 0.28
	P	0.9018	0.449
Serum C_4 , g/L	R	0.61	
	95% CI	0.23 to 0.83	
	P	0.004	

* CI = confidence interval; CSF = cerebrospinal fluid.

FIGURE 2. Mechanisms involved in the immune response and C_4 intrathecal synthesis against *Angiostrongylus cantonensis* larvae invasion throughout the damaged blood-brain barrier (BBB). Larvae take advantage of existing factors such as metalloproteinases (MMPs) and adhesion molecules to enter the cerebrospinal fluid space. Antigen-presenting cells (APCs) are important in the innate response. C_4 can play an essential role by the interaction with mannose-binding lectin (MBL) or C_2 from the classical pathway. Complement component 2 bound to C_{4b} is cleaved by classical ($C1s$) or lectin (MASP2) proteases to produce $C_{4b}C_{2a}$. The final products of complement activation forming the membrane attack complex with the interaction of Th1 and Th2 mechanisms produce inflammation, necrosis, and apoptotic cells. Neurotoxins produced by eosinophils during the inflammation process including antibody production should be the most responsible for the central nervous system damage. This figure appears in color at www.ajtmh.org.

a Q albumin value greater than the normal range that was dependent on age.

The percentile hyperbolic lines above the stronger discrimination line in the quotient diagram indicated that six patients had a high intrathecal fraction of C_4 ($> 80\%$). This finding was observed even though there was no relationship between intrathecal fraction and the time when third-stage larvae of *A. cantonensis* invade the CNS or other clinical factors. When C_4 is synthesized in CSF, it shows that the classical and lectin pathways are used to eliminate *A. cantonensis* larvae (Figure 2). However, some of the CSF C_4 is derived from the blood (Table 2).

DISCUSSION

The patients in this study were from different municipalities in Havana. They live in urban, rural, and semi-rural areas. All affected children lived in houses with dirt backyards where it is common to find snails and rats, which are, respectively, the intermediate and definitive hosts of the parasite.²³ Affected adults lived in poor sanitary conditions in which rats were commonly found. There was no evidence that the patients ate uncooked mollusks and snails. Eating raw mollusks and snails is uncommon in Cuban. In contrast, it is common in Southeast Asia and South America.

Presumptive diagnosis of human angiostrongyliasis is based on clinical symptoms, medical history, laboratory findings in blood and CSF, brain imaging results, and serologic tests. A history of eating intermediate or paratenic hosts or uncooked vegetables is suggestive for diagnosis of *A. cantonensis* infection. Our patients were infected during 2003–2009, and their

diagnosis was based mainly on clinical findings because there is no other parasite that can cause eosinophilia in the CSF in Cuba.^{24–26}

There were no cases of gnathostomiasis in our patients because of differences in the history of exposure and clinical symptoms and because the Caribbean region and Cuba are free of this disease. *Gnathostoma spinigerum* infection is endemic in Southeast Asia.²⁷ Infection with *Gnathostoma* species is typically categorized into cutaneous, visceral, and CNS forms.²⁷ *Gnathostoma* infection can cause symptoms that recur for 10–12 years, whereas *Angiostrongylus* infection can cause symptoms that recur for several months.²⁸ Our patients did not have dermatologic manifestations and skin lesions typical of *Gnathostoma* infection.²⁹ In addition, tissue damage in our patients was not found. Unlike gnathostomiasis, angiostrongyliasis is usually a self-limited clinical entity. In comparison with angiostrongyliasis, gnathostomiasis can persist for many years with cutaneous, visceral, or neurologic manifestations.²⁹ In our patients, recovery occurred within two weeks. Neurologic manifestations of gnathostomiasis usually include radiculomyelitis, encephalitis, paralysis, and hemorrhage; those characteristics were not observed in our patients.^{28,29} A history of exposure to gnathostomiasis is different from that of angiostrongyliasis, and third-stage larvae migrate to host muscle tissue and encyst. This migration does not occur in angiostrongyliasis.

The advantage of using the Reibergram for demonstrating C_4 intrathecal synthesis is that it identifies the fraction of C_4 produced in the CNS, indicating the local response to the *A. cantonensis* third-stage larvae. This method takes into account C_4 that enters the CSF from serum and shows the additional amount found in the CSF caused by local production. The Reibergram suggests that although C_4 is a blood-derived protein, during the inflammation process, there was an increased amount produced in the brain. This study showed that 60% of patients with meningitis had intrathecal C_4 . Also noteworthy is that none of the evaluated patients was placed below the lower hyperbolic curve, which is an area with no biological explanation. In medical practice, because a technical error in protein quantification is suspected when a case falls in this area, the Reibergram is a quality control procedure.

Using the previously used C_4 -index, which is different from the Reibergram, may produce erroneous results.¹² In addition, indexes cannot be applied when there is a dysfunction of the blood-CSF barrier, which greatly limits their application in inflammatory diseases that usually involve barrier dysfunction. One report has demonstrated the superior diagnostic value of the Reibergram.¹² Under all conditions of the blood-CSF barrier, the CSF/serum quotient graph can identify the occurrence of intrathecal C_4 synthesis. Also, it has been demonstrated that because the Reibergram uses protein quotients normalized to quotient (CSF/serum), these results are not affected by the extracted CSF volume.^{20,21,30–32}

From a practical point of view, information for a patient who was able to synthesize C_4 intrathecally has enormous importance because it indicates that this immunologic response have involved classical and/or lectin complement pathways. This information is important for clinical diagnosis because it can aid in the detection of immunodeficiency caused by the complement system. Conversely, the high mortality rate reported in adults in Cuba with this disease²⁵ indicates that it is important to identify what occurs in the adult response against larvae and during complement activation and synthesis.

Use of the C4 Reibergram in clinical practice may help obtain answers about the participation of complement in patients with blood-CSF dysfunction. In this study, samples from eight patients showed a dysfunction of the blood-CSF barrier, and two of the samples showed C4 intrathecal synthesis. Because use of the C4 Reibergram is relatively new, knowledge about C4 intrathecal synthesis under these conditions is also new. For the same reasons, the meaning of its high C4 fraction synthesis has up to now has not had biological significance.

It is important to identify additional cases of this disease to correlate and identify clinical differences between patients in whom a high C4 fraction develops and those who have a smaller fraction. For the same reasons, patients with undetectable C4 in CSF should be further investigated.

Synthesis of intrathecal C4 should be studied in combination with markers in other complement pathways because a general view may help understand the observed destruction of larvae in human CSF. Third-stage larvae are usually destroyed in human CSF, in which complement may have an important role. The complement system could be one of the most important elements in the primary defense mechanism of the brain against infections, especially in the early stage, when the specific antibodies are not yet synthesized.³³ The alternative pathway of complement activation and the classical pathway could be activated, even before the appearance of antibodies, by other factors such as trypsin-like enzymes and myelin-binding and mannan-binding lectins.¹⁰

The basic mechanisms involved in the immune response and C₄ intrathecal synthesis against *A. cantonensis* larvae invasion throughout the damaged blood-brain barrier follows the following pattern. Larvae take advantage of existing factors such as metalloproteinases and adhesion molecules to enter the CSF space. Antigen-presenting cells are important in the innate response to activate defense mechanisms against larvae. The classical and lectin pathways of complement activation can lead to the inflammatory process during meningitis. C₄ can play an essential role by interaction with mannose-binding lectin or C₂ from the classical pathway. Complement component 2 bound to C_{4b} is cleaved by classical or lectin proteases to produce C_{4b}C_{2a}. The final products of complement activation form the membrane attack complex by interaction of Th1 and Th2 mechanisms to produce inflammation, necrosis, and apoptotic cells. Neurotoxins produced by eosinophils during the inflammation process, including antibody production, should be the primary causes of CNS damage.

A type II hypersensitivity process is activated during larvae migration into the CSF. This process may also activate the complement system as part of the cytotoxic process against *A. cantonensis* larvae by phagocytosis.^{34,35} Although intrathecal synthesis of IgG may be not found in CSF during the acute phase of the disease,¹⁷ specific IgG synthesized in the blood may be found in the CSF. C₄ activation may occur in the lectin pathway and be identified as innate immunity.³⁶ Antibody-dependent complement cytotoxicity may also occur. This type of cytotoxicity, rather than phagocytosis,³⁷ may eliminate *A. cantonensis* larvae.

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