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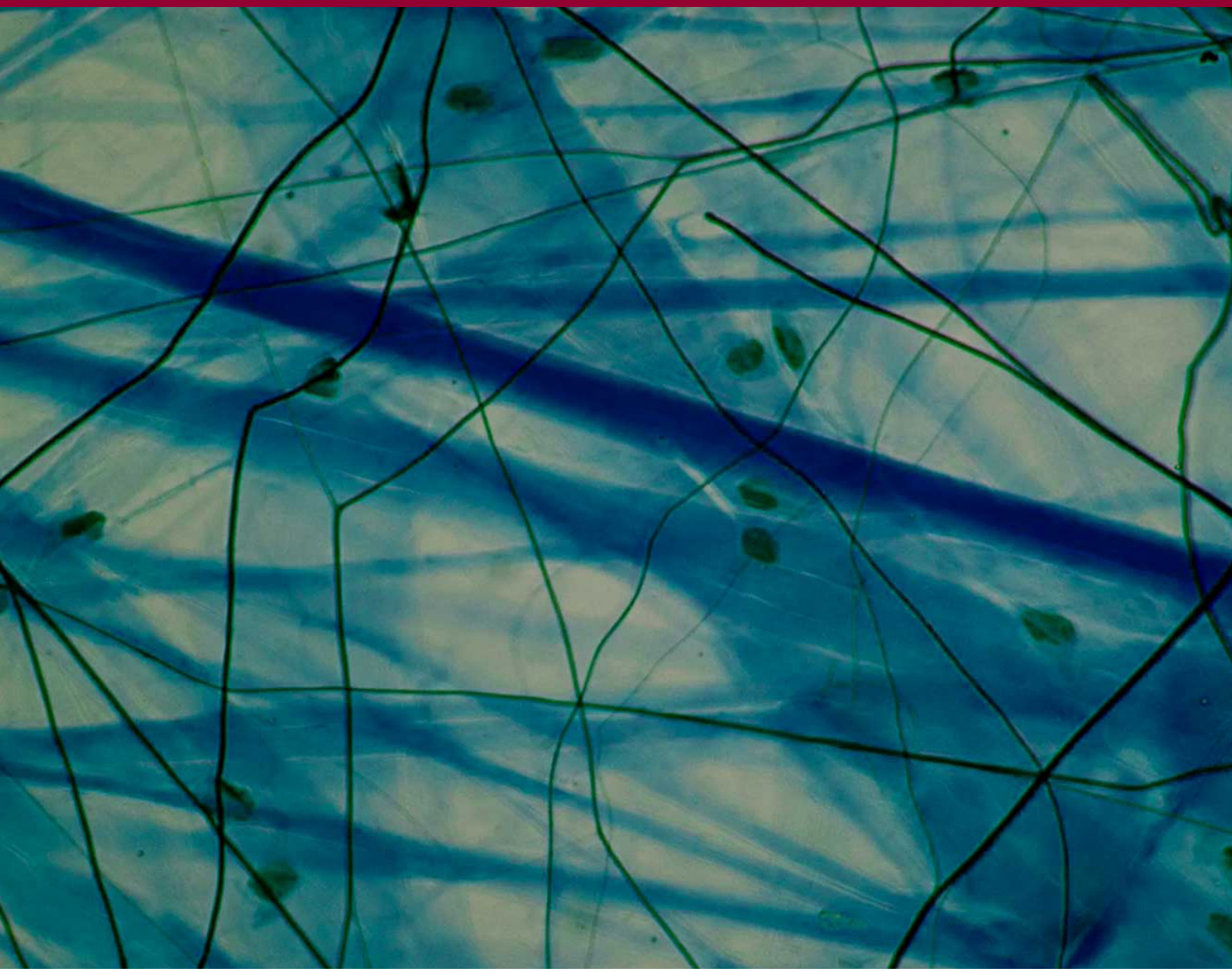

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REIBERGRAMS. USEFUL TOOLS FOR NEUROIMMUNOLOGICAL STUDIES

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ABSTRACT
Reibergrams are clinical charts widely employed in Neuroimmunology laboratories. When the first reibergrams were developed, they were used for the major immunoglobulins. Since then, other reibergrams have been reported for the theory of molecular diffusion/ cerebrospinal fluid flow and the molecular weight of different proteins involved in the central nervous system immune response, like IgG subclasses, IgE, C3c, C4, soluble C5-9 complex and MBL, and for some clinical applications in neurological disorders like infectious, autoimmune and neurodegenerative diseases.
Key words: reibergram, major immunoglobulins, IgG subclasses, IgE, C3c, C4, C5-9 complex, MBL.

RESUMEN
Los reibergramas son cartas clínicas ampliamente utilizadas en los laboratorios de Neuroinmunología. Los primeros reibergramas se describen para las clases más importantes de inmunoglobulinas. Desde ese momento se han reportado estudios de otros reibergramas basados en la teoría de la difusión molecular/flujo del líquido cefalorraquídeo y el peso molecular de las diferentes proteínas involucradas en la respuesta inmune en el sistema nervioso central como las subclases de IgG, IgE, C3c,C4, el complejo soluble C5-9 y MBL, y algunas aplicaciones clínicas en enfermedades neurológicas infecciosas, autoinmunes y neurodegenerativas.
Palabras clave: reibergrama, immunoglobulinas mayores, subclases de IgG, IgE, C3c, C4, complejo C5-9, MBL

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Introduction

Cerebrospinal Fluid. Composition and functions.

The cerebrospinal fluid (CSF) is a biological clear fluid that contains few or no cells. The protein concentration is about 200 times lower than in serum but its salt concentration is similar to the blood.

The main physiological functions are to protect the brain against sudden movement or physical shock and to drain brain-and blood derived CSF molecules into the venous blood.

In clinical medicine it contributes to the diagnosis of neurological disorders, being an element to obtain information about the brain and a useful tool to help in treatment monitoring.

Immune response in the central nervous system.

The immune response in the central nervous system is different from the general immune response. For instance, in the brain the isotype switch from IgM class to IgG class reaction is not produced. IgM, IgG and IgA class response patterns depend on the microorganism, the course of the disease and the localization of the pathological mechanism. Intrathecal IgM is not an indicator of acute disease.

Another example is the intrathecal immune response against Angiostrongylus cantonensis. This is a parasite that accidentally affects humans after eating non-cooked terrestrial snails containing larvae of the parasite or contaminated unclean vegetables. The intrathecal immune response develops according the following pattern: In the acute phase there is an intense IgE and C3c intrathecal synthesis but not of the major immunoglobulins IgA, IgM and IgG. Only eight days later there is intrathecal synthesis of IgA and IgG and of IgM in less proportion.

Additionally, to know about the intrathecal synthesis of immunoglobulins it is also quite important to point out about the complement system. The complement system is a complex molecular system consisting of approximately sixty factors and components present in plasma and CSF or as surface receptors of many cells of the innate and adaptive immune system. There are three complement pathways: the classical, the alternative and the lectin pathway. The first two ones belong to the innate immune system whereas the last one is under construction at the present time, because of the appearance of new components and the discovery of their function in the last 20 years.

This is the reason for the interest in to increasing the knowledge about the immune response in the central nervous system. In this scenario, the CSF analysis plays an important role in the laboratory-supported diagnosis of neurological diseases. The relevance depends on knowledge-based interpretation of the results.

Reibergrams

Reibergrams are clinical charts widely employed all over the world in Neuroimmunology laboratories mainly since these tools were incorporated to different nephelometers and other instruments in order to perform quantitative analysis in serum and cerebrospinal fluid (CSF) for neuroimmunological studies.

Reibergrams allow to determine whether there is intrathecal synthesis of immunoglobulins and to assess the functioning of the blood-brain barrier in patients suffering from diverse neurological diseases among other applications.

1. Need to calculate the fraction of the immune response synthesized in the Central Nervous System.

It is essential to know in every neuroinflammatory process if there is intrathecal synthesis of immunoglobulins and of other components of the local immune response.
The local synthesis of these components in the CSF is quite important to arrive to a diagnosis or to follow up a neurological disorder like infective, autoimmune and neurodegenerative disease.
The most important fact to make a real diagnosis is to know about what is going on at the central nervous system and this is obtained by the local fraction of the immune response. Consequently, there is need to create useful tools from the empirical information of a local central nervous system disorder.

2. The Reduction of a biological function to a mathematical formula. The first approaches.

It is very complicated to try to reduce a natural phenomenon with the extreme complexity of the biological world into a mathematical formula. However, there is real need to determine the immune response at the local level.
This required information cannot be simply obtained from the blood levels of the immune response components. For instance, major immunoglobulins and other proteins that take part in the neurological process like the complement components can be synthesized in blood and also in CSF. The discrimination between blood protein and protein locally synthesized in the brain is essential to explain the central nervous system production.
The first formulae that appeared in the early 1970's are very simple and reductionist and, because of these difficulties, they have serious disadvantages. The most employed formula is, nowadays, the so-called Index [1]. The Index is not recommended when there is a blood-brain barrier dysfunction or in pediatric patients and the results vary with the extracted volume of CSF, among others problems.
All the formulae that exist up to now need a protein marker to evaluate the blood fraction that diffuses throughout the blood-brain barrier to increase the CSF concentration. Albumin is the selected protein marker due to its molecular characteristics, the impossibility to be catabolized in the CSF and its exclusive hepatic source. That means that all the CSF albumin content comes from the blood.
The albumin ratio or quotient (Q Albumin), which is the result of dividing the CSF albumin concentration by the serum albumin concentration, is widely employed as a measure of blood CSF barrier function.
The Q ratio is employed for different proteins in general and it is calculated in the same way.
Lower concentrations of serum proteins appear in the ventricular CSF, secreted by the coroid plexus. Additionally, serum proteins enter into the CSF throughout the subarachnoid space. For this reason, the lumbar protein concentration is three times larger than the ventricular protein concentrations. The content of the CSF flow drains the proteins along with the cells and other components and passes through the arachnoid villi and granulations to the venous blood.
These proteins are transferred from the blood to the CSF in a controlled diffusion process through their concentration gradients, which are established depending on their molecular size [2]. The gradient is more pronounced for larger molecules (~ 200: 1 for albumin at 3000: 1 for IgM). This protein retention is called the blood-CSF barrier.
The difference in protein gradients depending on their molecular size is defined as selectivity of the barrier function. The equilibrium concentration of a plasmatic protein depends on the absolute level of the serum concentration, the gradient and the CSF flow

rate. The ratio of CSF/serum concentration or CSF/plasma concentration describes the concentration gradient with increased values when there is an increase in the protein concentration in the CSF

The rate is independent of the absolute individual concentration in the serum, but reflects all the influences related to the blood-CSF barrier (molecular mass, CSF flow and the effective diffusion path), and the CSF extraction conditions (volume of extraction or area of the CSF extraction, either the cerebral ventricle or the lumbar region).

The rate for the CSF albumin/serum albumin concentration (Q Alb) has been recognized as a suitable parameter to characterize the individual variations of the blood-CSF barrier function and has been introduced as a suitable reference for a more sensitive evaluation of other plasmatic proteins in the CSF, due to the reduction of the individual variables.

Many formulations to discriminate between the brain-derived proteins and blood-derived IgG fraction have been reported [3] There have been formulae of linear discrimination by Link and Tibbling in 1977 [1] or the one formulated by Tourtellotte and Ma in 1978 [4] - bifunctional lines [5] or nonlinear functions [6, 7] Empirically, the hyperbolic function has proven to be the best suited to the clinical data.

With the application of Fick's laws of diffusion for the transport of blood proteins to the CSF, a theoretical support for the hyperbolic function is achieved, as the exact pathophysiological description of the fractional changes between the two ratios CSF/serum (QIgG: QAlb) with increased protein content in the CSF.

3. Reibergrams. Principles from experimental data to the new formula and graphs.

In 1980, Hansotto Reiber [5] announced his first formula that was better than the previous ones because it attempted to discriminate in a graph the different areas where intrathecal synthesis of IgG could occur and the possible dysfunction of the blood-CSF barrier. Although this formula was also linear, this attempt to distinguish between what was synthesized locally from the simultaneous evaluation of the blood-CSF barrier constituted a step forward.

However, no biological process responds linearly to a mathematical equation. The characteristics of the biological complexity that includes chemical, physical and biological changes make it impossible for any natural phenomenon to be reduced to a linear equation.

That is why Hansotto Reiber, initially with Klaus Felgenhauer in 1987, [6] decided from the empirical observation of the concentrations of the major immunoglobulins and albumin in thousands of apparently healthy persons, without proven neuroinflammatory processes, to evaluate their profile, plotting the values of Q IgG or IgA or IgM as a function of Q alb.

The result obtained indicated that the points described a curve instead of and not linear relationship; therefore, the experimentally relationship was adjusted to a curve that fitted properly.

From Fick's laws of diffusion (**Figure 1**) it was considered that the proteins of greater molecular weight diffused less and that the amount of immunoglobulins that passed from the blood to the CSF could theoretically be calculated according to their molecular weight.

Different equations were essayed to fit the data. A hyperbolic function was the one that best described the relationship, and an iterative procedure was used to obtain the combination of hyperbolic function parameters that minimized the standard error of the mean. Thus, the constants a/b, b2 and c ratios were calculated and finally adjusted to a hyperbolic curve for each of the major classes of immunoglobulins. This equation and its corresponding graph were able to explain the experimental fit. The average values of

these points gave an average curve that moved between normal values according to the empirically observed natural distribution.
Then, having this adjusted distribution, it was decided that in order to consider that there was an intrathecal synthesis of any of these immunoglobulins, they had to exceed 3 standard deviations from the higher value that marked the limit of what naturally happened by diffusion from what was synthesized locally.

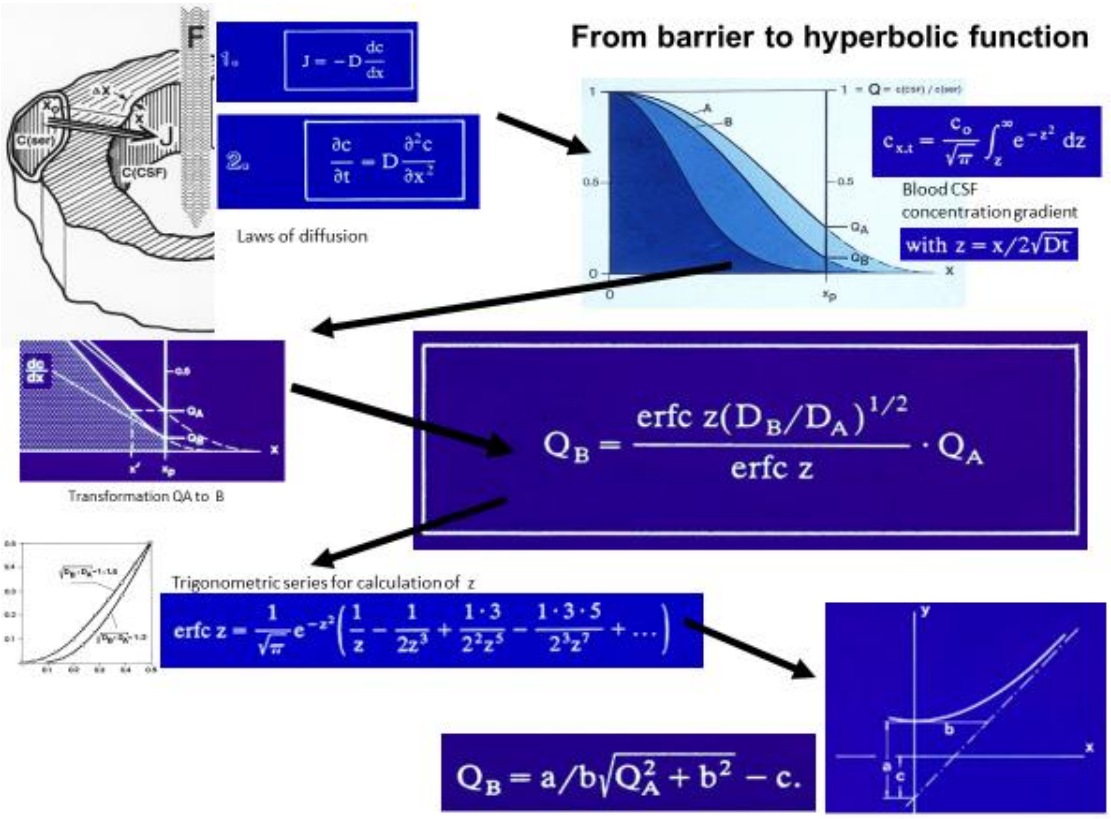

Figure 1. Diagram of the process followed for the creation of the reibergram (Reiber, H, 1980)

4. Reibergram for the major immunoglobulin classes.

The quotient diagram published in 1987 by Felgenhauer and Reiber [6] and later improved by Reiber in 1994, was developed [8]. This meant a step forward for the quantification of the major immunoglobulins and due to these studies, their use was extended throughout the world, and were later baptized with the name of reibergrams honoring their creator.
The diffusion of proteins of molecular weight similar to or greater than that of albumin achieve their passage through the blood-CSF barrier affected only by the molecular weight of the protein according to Fick's laws and based on the theory of the molecular diffusion-CSF flow [9]. Then, other reibergrams could be made knowing this molecular characteristic of the protein that passes from the blood to the CSF.
The reibergram (**Figure 2**) has five clearly defined areas with a different interpretation according to where the point of Q albumin and Q Ig values of a given patient meet.
Zone 1. It is the area between the boundaries of the uppermost line called Q lim and the lower line. This area is limited by the value of the normal Q albumin for the patient's age. In the figure it is limited by Q albumin = 7 which is the normal value for age between 15

and 40 years. The interpretation is that there is no intrathecal synthesis of the immunoglobulin and there is no dysfunction of the blood-CSF barrier.
Zone 2. It is the area between the Qlim line and the lower line, and limited to the Q albumin values above the normal value for the patient's age. The interpretation is that there is no intrathecal synthesis of the immunoglobulin and there is a dysfunction of the blood-CSF barrier
Zone 3. It is located to the right of the normal value of Q albumin and above the Q limit. This means that there is intrathecal synthesis of the immunoglobulin and blood-CSF barrier dysfunction.
Zone 4: It is located in the area limited by the normal value of Q albumin for the age and above the boundary curve. This means that if a point falls in that area the patient has intrathecal synthesis of the immunoglobulin and has no blood-CSF barrier dysfunction.
Zone 5: Zone that has no biological significance. In these cases, an error in the quantification procedures of albumin and immunoglobulin in the serum or in the CSF or in both may have occurred, or the sample was collected at different times, i.e. if more than 6 hours have passed between the venous puncture and the lumbar puncture in order to obtain CSF for adults. In children this difference cannot be greater than two hours between one procedure and the other.

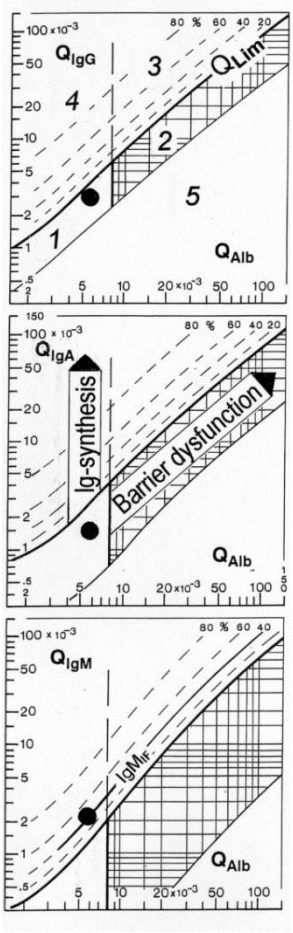

Figure 2. Reibergrams for the major classes of immunoglobulins.
CSF/serum quotient diagrams for IgG, IgA, and IgM. The upper hyperbolic curves (thick lines) represent the discrimination lines between brain-derived and blood-derived immunoglobulin fractions. Values above these upper discrimination lines represent intrathecal IgG, IgA, or IgM synthesis. The dashed lines indicate the extent of intrathecal synthesis as intrathecal fractions (IgGIF, IgAIF, or IgMIF) with 20, 40, 60, and 80% of the measured total immunoglobulin concentration in CSF, with reference to the discrimination line as 0% intrathecal synthesis. The limit of the reference range for QAlb between normal and increased CSF protein concentrations due to blood-CSF barrier dysfunction is indicated by the age-dependent vertical lines at QAlb 5 5 z 1023 (up to 15 years), at QAlb 5 6.5 z 1023 (up to 40 years), and at QAlb 5 8 z 1023 (up to 60 years). The diagrams depict the following ranges: 1, normal; 2, blood-CSF barrier dysfunction (i.e., reduced CSF turnover); 4, intrathecal immunoglobulin synthesis with no change in CSF turnover; 3, intrathecal immunoglobulin synthesis with reduced CSF turnover. Values below the lower hyperbolic line in range 5 indicate a methodological fault. The data of 15 control patients (F) are representative of the age-related normal range with normal blood-CSF barrier function and no intrathecal immunoglobulin synthesis. With permission from Elsevier.

Then from the first reibergrams, a family has emerged for the determination of intrathecal synthesis of the different components of the immune response that have been applied to the clinic of neurological diseases.

5. Reibergrams for IgG subclasses

The theory of molecular flow/CSF flow on which the reibergram is based is not restricted to immunoglobulins.
For the determination of intrathecal synthesis of IgG subclasses, the IgG reibergram was adopted for the IgG1, IgG2 and IgG4 subclasses. However, IgG3 has great similarity with the molecular weight of IgA, so the IgA constants are used for the new reibergram [10].

TABLE 1. Molecular characteristics of the IgG and IgA subclasses [10]

	IgG3	IgA	IgG1	IgG2	IgG5
Molecular mass (KDa)	170	160	146	146	146
Hydrodynamic radius (Å)	58	58	52	52	52
Mean concentration in serum (g/L)	0,51	3	6,98	3,8	0,56

The proposed reibergram uses the constants of IgA as follows: IgG3 (Loc) = [QIgG3 - 0.77 √ + 1.7 × 10-3] × IgGSERUM (QAlb) 2 + 23 × 10-6

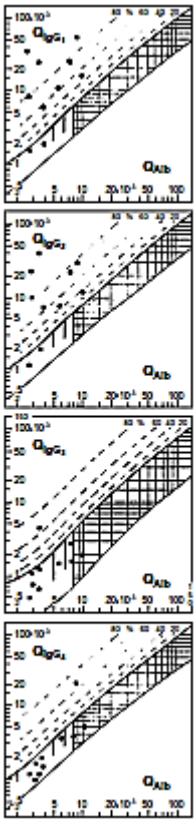

Figure 3. Reibergrams for IgG subclasses. Reibergram obtained from patients with Angiostrongylus cantonensis meningoencephalitis. [10] Note that the points above the darkness hyperbolic curve mean that there is intrathecal synthesis of the IgG subclass under study. Patients who maintain blood / CSF barrier dysfunction have albumin Q (QAlb) values greater than 5 × 10-3 because they are pediatric patients. The reibergram for IgG3 is different compared to the other subclasses due to its molecular weight.

Reibergrams for IgG subclasses were applied in neuroinfective diseases [10] and in other neurodegenerative disorders [11]

6-Reibergrams for IgE

IgE is an immunoglobulin characteristic of type 1 hypersensitivity and this protein has a leading role in allergic diseases and in infectious diseases caused by parasites.

The study of the major immunoglobulins was performed in terms of their molecular characteristics and were compared with IgE. As can be seen, the IgE is very similar to the IgG3 and the IgA, so the values of the constants of the IgA are adopted in order to prepare the reibergram for IgE [12]

TABLE 2. Molecular characteristics of IgE compared to other immunoglobulins [10]

	IgG1	**IgA**	**IgG3**	**IgE**
Molecular mass (KDa)	150	160	170	180
Heavy chain molecular mass (KDa)	51	56	60	72
Mass-associated carbohydrates (%)	2-3	7-11	6-10	12
Hydrodynamic radius (Å)	52	58	58	59

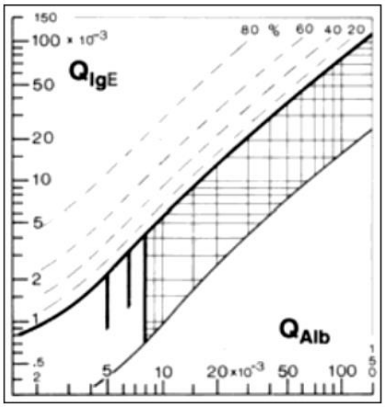

Figure 4. Reibergram for IgE 7 [12] with permission of Elsevier.

Reibergram of IgE is a very useful tool to make the auxiliary diagnosis of Angiostrongylus cantonensis meningoencephalitis [13, 14].

7. Reibergrams for the Complement system. The complement pathways.

In a neuroimmunological process, some components of the intrathecal response complement system were produced.

This intrathecal response due to the complement system varies according to the activation pathway and the possibility that these components can be synthesized in the central nervous system or not.

Thus, it is possible to find immune response patterns linked with the complement system in some diseases. [15]

Intrathecal immune response in several disorders allows the discovery of different patterns of complement components, according to the biological agents of the infectious disease.

With these patterns an auxiliary diagnosis can be performed and it can also be applied in neuroepidemiological studies.

In order to study the participation of the different components of the complement system in the intrathecal immune response it is important to measure the complement system components in the central nervous system.

For these reasons, different reibergrams of the complement components were made when the nature of those components made it possible.

8. Reibergram for C3C

The C3c fraction is a product of the degradation of the C3 factor of the complement system and its synthesis in the cerebrospinal fluid is a sample of the biological action of the complement against exogenous immunogens such as different biological agents or autoimmunogens by hypersensitivity cytotoxic type II mechanisms.

Molecular characteristics of different immunoglobulins and C3c are observed in Table 3

TABLE 3. Molecular characteristics of some immunoglobulins and of the C3c. [10]

	IgG1	IgA	IgG3	C3c
Molecular mass (KDa)	150	160	170	145
Carbohydrates associated to the molecule (%)	2-3	7.11	6-10	2-3
Hydrodynamic radius (Å)	51	58	58	54

The constants used for the preparation of the formula for the intrathecal detection of C3c are the constants of IgG because this protein is the one that has more similarity with the C3c according to Fick's diffusion laws.

The formula for the manual calculation of intrathecal synthesis is the following:

C3c (loc) = QC3c - (0.93 Qalb2 + 6 × 10-6 −1.7 × 103) C3 serum

The reibergram for the proposed C3c appears in Figure 5.

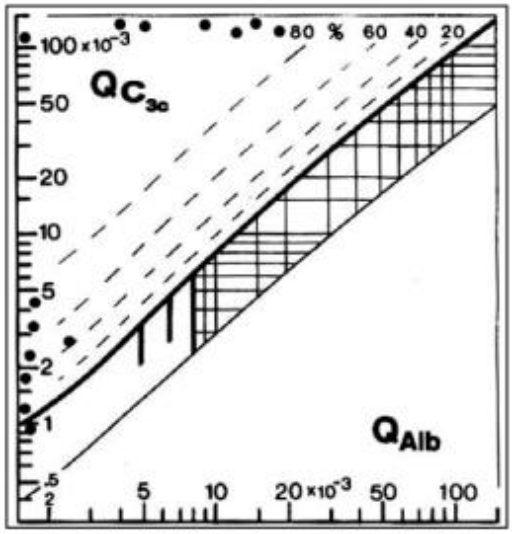

Figure 5. Reibergram to determine the intrathecal synthesis of C3c [16].

C3c reibergram is employed to characterize the immune intrathecal response in different infectious neurological disorders [17, 18] and other neurological disorders as multiple sclerosis [19].

9. Reibergram for C4

One of the components of the classical pathway corresponding to the complement system is C4. It is very useful in the clinic and it is also interesting because it is through C4 that the lectin pathway is linked to the classical one from which a common pathway is followed. Therefore, it is used to discriminate whether the classical pathway or the other complement activation pathways are activated [15].

The reibergram of C4 was made taking into account the similar characteristics that this protein has with those of IgA. In **Figure 6A** nine of the patients with neurological infectious meningoencephalitis (Black circle) had values above the upper solid line which indicated intrathecal synthesis of C4. Ten controls (Black triangle) without infectious neurological disorders had values in the normal range, indicating no intrathecal C4 synthesis. Also plotted are QIgA (White triangle) from 10 patients suffering from meningoencephalitis and five of these had IgA intrathecal synthesis. Two patients had no detectable IgA in the CSF.

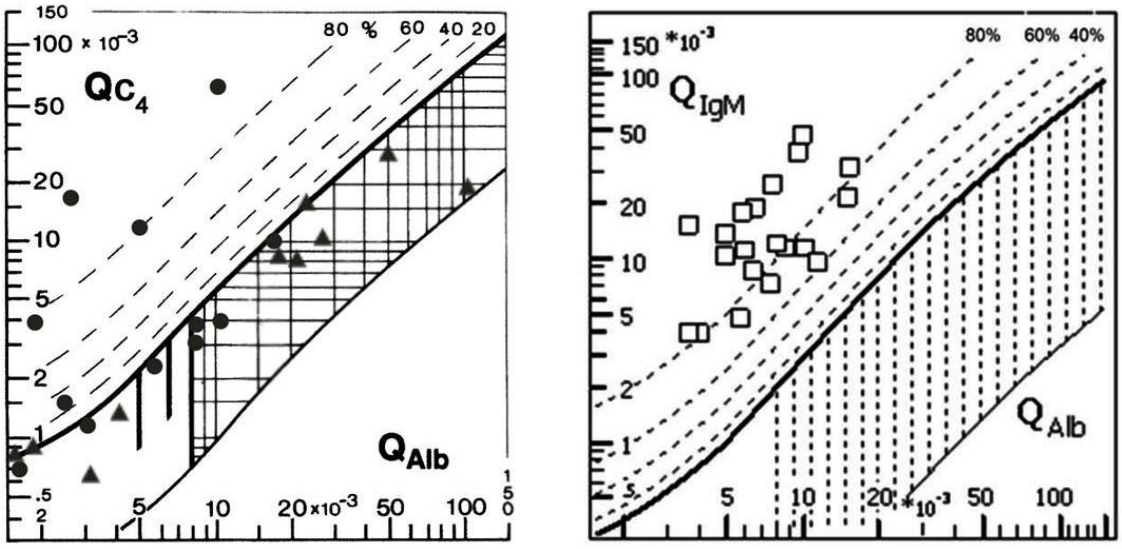

Figure 6. Left Reibergram for C4 [20]. Right Reibergram for the soluble complex C5-c9. Taken from [21] with permission of Elsevier

10. Reibergram for C5-9 complex

In 2018 a study that employs the Reibergram for IgM in order to use it for the evaluation of the intrathecal synthesis of the soluble complex C5-9 was published. Figure 7 shows in white squares the intrathecal synthesis of this complex using the IgM reibergram, considering that the soluble attack complex C5-9 has a molecular weight similar to that of IgM. This strategy to determine the intrathecal synthesis of this component is similar to the other reibergrams already done.

11. Reibergram for MBL (Mannose- binding lectin)

The lectin pathway is the third pathway for complement activation. It is a route that does not need the presence of immunoglobulins to be activated and is therefore part of the innate immunity. It is possibly the oldest route in phylogeny but the most recent to be discovered, so it is currently not fully detected because the concentrations of its components are in the order of nanograms per liter and could only be identified and quantified when very sensitive methods were created.

It has five initiators, one of which is the MBL that was known since the early twentieth century and was considered an acute phase protein until it was linked to this pathway of complement activation.

The MBL reibergram was taken from the similar molecular weights existing between IgM and MBL.

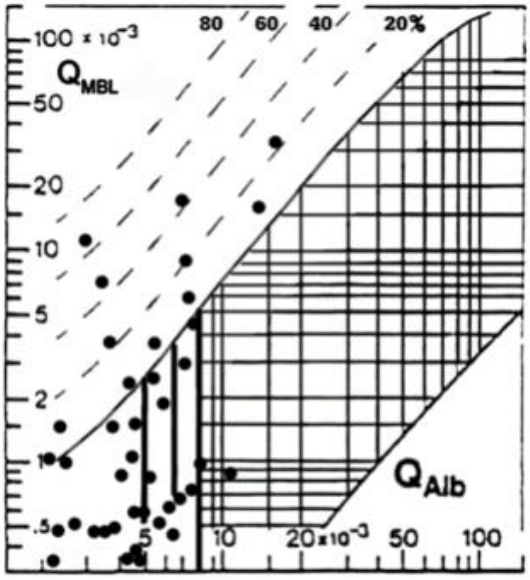

Figure 7. Reibergram for MBL. Taken from [22]

MBL participates in infectious diseases that affect the central nervous system [23] and its dynamics and intrathecal synthesis have been reported [24], as well as MBL deficiency in some patients with meningoencephalitis due to Angiostrongylus cantonensis. [25] Recently, intrathecal MBL synthesis has been described in Guillain Barré patients. [26]

Conclusions

Reibergrams are graphs of vital importance to study the intrathecal synthesis of the different components of the immune response, and its practical application has been increasing as it has become essential to discriminate the intrathecal immunity in order to explain neurological diseases.

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