

## Quantification of Virus-Specific Antibodies in Cerebrospinal Fluid and Serum: Sensitive and Specific Detection of Antibody Synthesis in Brain

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Specific antibody synthesis in brain could be detected with maximal sensitivity by combining an advanced enzyme immunoassay with a sophisticated evaluation method that involves calculating the ratio between the cerebrospinal fluid (CSF)/serum quotients for specific antibodies ( $Q_{\text{spec}}$ ) and total IgG ( $Q_{\text{IgG}}$ ). This Antibody Index ( $AI = Q_{\text{spec}}/Q_{\text{IgG}}$ ) discriminates between a blood-derived and a pathological, brain-derived specific antibody fraction in CSF and takes into account individual changes in blood/CSF barrier function. For local synthesis of polyclonal IgG in the central nervous system ( $Q_{\text{IgG}} > Q_{\text{Lim}}$ ), we propose the correction  $AI = Q_{\text{spec}}/Q_{\text{Lim}}$  ( $Q_{\text{Lim}}$  represents that IgG fraction in CSF originating only from blood, calculated from the individual albumin quotient of a single patient). The normal reference range for the AI was between 0.7 and 1.3 ( $n = 250$  control patients for each antibody species). Values of  $AI \geq 1.5$  indicated a local specific antibody synthesis in the central nervous system. Sensitivity and precision were greatest if we analyzed the virus-specific antibodies in CSF and serum simultaneously with an enzyme immunoassay in continuous concentrations (arbitrary units) instead of titer steps. We have applied the method successfully to antibodies to measles, rubella, herpes simplex, varicella-zoster, human immunodeficiency virus (HIV), and cytomegalovirus, and to anti-*Toxoplasma* or *Borrelia* antibodies. Clinical relevance is demonstrated for an acute zoster virus infection (monospecific response), chronic diseases such as HIV encephalitis with acute opportunistic *Toxoplasma* infection, and multiple sclerosis (secondary polyspecific response).

**Additional Keyphrases:** enzyme immunoassay · CSF/serum quotients · Antibody Index · multiple sclerosis · HIV encephalitis · zoster ganglionitis · measles · rubella · herpes simplex · *Toxoplasma* · *Borrelia burgdorferi*

Several methods have been proposed for quantitative discrimination between a pathological, brain-derived fraction and the blood-derived fraction of cerebrospinal fluid (CSF) immunoglobulins (1-6).<sup>1</sup> The use of the albumin CSF/serum quotient ( $Q_{\text{Alb}}$ ) is well established

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<sup>1</sup> Nonstandard abbreviations: CSF, cerebrospinal fluid;  $Q_{\text{Alb}}$ , albumin CSF/serum concentration quotient; ELA, enzyme immunoassay; CNS, central nervous system;  $Q_{\text{IgG}}$ , total IgG CSF/serum concentration quotient;  $Q_{\text{spec}}$ , specific antibody CSF/serum concentration quotient; AI, Antibody Index; PBS, phosphate-buffered saline; HIV, human immunodeficiency virus; HSV, herpes simplex virus; VZV, varicella-zoster virus;  $Q_{\text{Lim}}$ , calculated upper limit of the normal range of  $Q_{\text{IgG}}$ .

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as a reference for the individual blood/CSF barrier function (1-7). Because, in a pathologically increased CSF protein content, the ratios between the immunoglobulin CSF/serum quotient and the albumin quotient are changed, one of us developed an evaluation diagram (5) for assessing local IgG synthesis in brain with a biphasic discrimination line. With the use of supporting methods (e.g., identification of oligoclonal IgG fractions by isoelectric focusing of CSF) (8), it was possible to develop an improved quotient diagram with a hyperbolic discrimination line (6). This concept could be extended to other serum-derived proteins by modifying the molecular size-dependent constants in the hyperbolic function (6).

Moreover, strong evidence indicates that the pathologically increased protein content in CSF in neurological diseases is mainly a function of a decreased CSF flow (9), increasing the time for influx of the blood-derived proteins into CSF (10).

These improved evaluation methods eliminated many faults in interpretation of CSF data. The introduction of the IgA and IgM quotient diagrams increased the clinical relevance of CSF data in differential diagnosis of inflammatory neurological diseases (6).

The introduction of sensitive methods like enzyme immunoassays (EIAs) and qualitative methods like immunoblot (11), Western blot (12), or immunospot assay (13, 14) to identify an antigen-specific response offered the potential of improved sensitivity and specificity of diagnoses of neurological diseases. Again, identification of a local synthesis of antibody in the central nervous system (CNS) by CSF analysis requires a sensitive, quantitative method to discriminate between the CSF antibody fraction originating from blood and that from an intrathecal synthesis. As shown for the immunoglobulin classes IgG, IgA, or IgM (6),  $Q_{\text{Alb}}$  can be a generally applicable marker for the individual blood/CSF barrier function. To evaluate a local synthesis of specific antibodies, e.g., zoster virus antibodies, the total immunoglobulin CSF/serum quotient ( $Q_{\text{IgG}}$ ) of the individual patient would be an ideal marker of barrier function. Because both the specific antibody and total immunoglobulins of the same immunoglobulin class have the same molecular size, they must have the same barrier permeability. Consequently, the ratio between the specific antibody CSF/serum quotient ( $Q_{\text{spec}}$ ) and  $Q_{\text{IgG}}$  should equal 1.0 in normal conditions and should increase in cases of additional synthesis of a specific antibody in the brain.

This linear ratio has been introduced (15) as the specific Antibody Index:  $AI = Q_{\text{spec}}/Q_{\text{IgG}}$ . The ratio takes

into consideration changes in the barrier function but does not correct for a large local synthesis of polyspecific IgG in the CNS, which increases the IgG quotient and leads to falsely low Antibody Index values. This aspect was overlooked in earlier publications, resulting in a low sensitivity for identifying a local specific antibody response in the CNS (15, 16). In particular, all electrophoretic methods that involve applications of similar amounts of IgG for paired analysis of CSF and serum samples are unable to take into account the brain-derived IgG contribution. In the case of a large local synthesis of IgG in the brain, the Antibody Index can be corrected by using the generally valid ratio between  $Q_{\text{spec}}$  and  $Q_{\text{Alb}}$ . Therefore, we introduced a barrier-related correction of the IgG quotient.

The sensitive identification of a specific antibody response in the brain by CSF analysis requires a highly sensitive method to quantify the low concentrations of the antibody species in CSF. These low concentrations in normal CSF result from the known blood-to-CSF protein gradients of as much as 1000:1 for IgG or 10 000:1 for IgM in normal cases. Based on such purely theoretical reasons, therefore, the usual methods for analysis of antibody titers in serum obviously are not sensitive enough for CSF analysis and not precise enough for calculating CSF/serum quotients. The use of CSF/serum quotients to identify an immune process in the brain minimizes certain problems of titer-value analysis, e.g., specificity and borderline values between normal and pathological titers.

Here we present an advanced ELA technique for quantifying the specific antibody concentration in CSF and serum and use it in combination with an advanced evaluation method for the specific Antibody Index, independent of changes in barrier function and local synthesis of IgG in the brain. This concept is very successful with respect to early or safer diagnosis of various neurological diseases (16-18). Clinically, it is important to discriminate between two different classes of neurological diseases with a local specific antibody response: (a) acute infections with a monospecific immune reaction that indicates the causative antigen (virus, bacteria, or plasmodium) (16, 19), and (b) chronic diseases such as multiple sclerosis, where virus-specific antibodies have been synthesized as a secondary polyspecific immune reaction (14, 15, 17) that has been interpreted as an unspecific stimulation of virus-specific B-cell lines.

## Materials and Methods

### Reagents

Antigen-coated microtiter plates were obtained from Behring (Marburg, F.R.G.): human immunodeficiency virus (Enzygnost Anti-HIV micro OUVA 10/11), cytomegalovirus (Enzygnost Cytomegalie OSDL 02/03), *Toxoplasma* (Enzygnost Toxoplasmosis/IgG, OUND), measles virus (Enzygnost Masern, OSOK 02/03), rubella virus (Enzygnost Rubella OSDN 02/03), herpes simplex virus (Enzygnost Anti-HSV OSOG 02/03), and

varicella-zoster virus (VZV; Enzygnost Varicella-Zoster OSMK 02/03). Test plates for cytomegalovirus, measles, rubella, HSV, and VZV were coated with antigen and control preparation (uninfected cell cultures). Additional reagents were obtained from the following companies: peroxidase-conjugated antibodies (goat anti-human IgG), *o*-phenylenediamine dihydrochloride, bovine serum albumin, and Antifoam A emulsion from Sigma Chemical Co., St. Louis, MO; phosphate-buffered saline, pH 7.2 (PBS; Dulbecco), from Biochrom, Berlin, F.R.G.; Tween 20 from Serva Feinbiochemica, Heidelberg, F.R.G.;  $\text{H}_2\text{O}_2$  (300 g/L, Perhydrol) and bromphenol blue indicator from Merck, Darmstadt, F.R.G. Condensed milk (~300 g/kg dry weight) was from various commercial sources.

### Preparations

**Dilution buffer:** Per liter of water, 9.55 g of PBS, 2 g of bovine serum albumin, 2 g of Tween 20, 10 g of condensed milk or 3 g of milk powder, 200  $\mu\text{L}$  of Antifoam A, 10 mg of bromphenol blue indicator, and 100 mg of thimerosal preservative (Serva).

**Washing solution:** Per liter of water, 9.55 g of PBS, 2 g of Tween 20, and 200  $\mu\text{L}$  of Antifoam A.

**Sample preparation:** Dilute 80  $\mu\text{L}$  of CSF and 40  $\mu\text{L}$  of serum 40- to 200-fold and 8000- to 40 000-fold, respectively, with dilution buffer (we used an automated diluter: Abimed, Langenfeld, F.R.G.). For 8000-fold dilutions, use a two-step dilution.

**Standard serum:** Antibody-positive sera from various patients were pooled, suitably prediluted (1000- to 8000-fold) with dilution buffer, and stored in 0.5-mL aliquots at  $-30^\circ\text{C}$ . A single serum could be used as well. The prediluted serum pool, which represents the upper standard value, was diluted with six serial twofold dilution steps. Standard dilutions were chosen so that the corresponding absorbances were between 2.0 and 0.05 A.

**Control serum:** Pooled positive sera, diluted (to yield an absorbance between 0.3 and 0.9 A) and stored at  $-30^\circ\text{C}$  in 2-mL aliquots.

**Antibody solution:** Conjugated antibody solution (IgG-class antibodies), diluted 1000-fold with dilution buffer.

**Substrate solution:** Prepared freshly just before use. Mix 1 mL of *o*-phenylenediamine (1 mol/L, 1-mL aliquots stored at  $-20^\circ\text{C}$ ), 49 mL of 0.1 mol/L citrate buffer (pH 5.5), and 35  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (300 g/L).

**Stopping solution:**  $\text{H}_2\text{SO}_4$ , 1 mol/L.

### Assay

Microtiter plates coated with antigen and control preparation (cytomegalovirus, measles, rubella, VZV, and HSV) contained seven standards plus one positive control. In addition, CSF samples (each diluted 40- and 200-fold) and serum samples (diluted 8000- and 40 000-fold) from 10 patients were pipetted into the antigen-coated wells and into the wells coated with the control preparation. For each patient, therefore,  $2 \times 4$  (8) wells were necessary.

The microtiter plates coated only with antigen, with-

out the control preparation (HIV and Toxoplasma), contained a negative control, six standards, and a positive control. CSF and serum samples from 20 patients, each diluted two ways as described above, were placed on the plate. Because we used only antigen-coated wells on these plates, only four wells per patient were necessary.

We incubated the foil-covered plates, 150  $\mu\text{L}$  of sample/well, overnight at room temperature on a microplate shaker, and then performed two wash cycles with a washer (EAW II; SLT Labinstruments, Crailsheim, F.R.G.). After the second incubation, with 150  $\mu\text{L}$  of antibody solution (IgG-class antibodies) for 3 h at 37  $^{\circ}\text{C}$  (in covered plates in a water bath), we performed four wash cycles. The third incubation (on the microplate shaker), with 150  $\mu\text{L}$  of substrate solution, was stopped with 100  $\mu\text{L}$  of stopping solution after 20 min at room temperature in the dark. The differences of absorbance,  $\Delta A = A_{450} - A_{570}$  or  $\Delta A = A_{490} - A_{570}$ , were measured with a microtiter plate reader (SLT Labinstruments).

The  $\Delta A$  for the control preparation or for the negative control was subtracted from the sample  $\Delta A$  values by use of a suitable PC program (SLT "easy fit"). The standard values were evaluated in a log/log diagram. The greatest measurable standard concentration ( $\Delta A \sim 2.0$ ) was defined to be 100 arbitrary concentration units (Figure 1). The PC program calculated arbitrary concentration values for CSF and serum (with multiplication by the dilution factor). Under normal conditions, both dilutions of the samples fit into the standard curve, resulting in two concentrations that differ by a factor of 5 ( $\pm 10\%$ ). For larger variations, the analysis should be repeated. For variations within the 10% limits, we chose those values in the range of the best performance in the

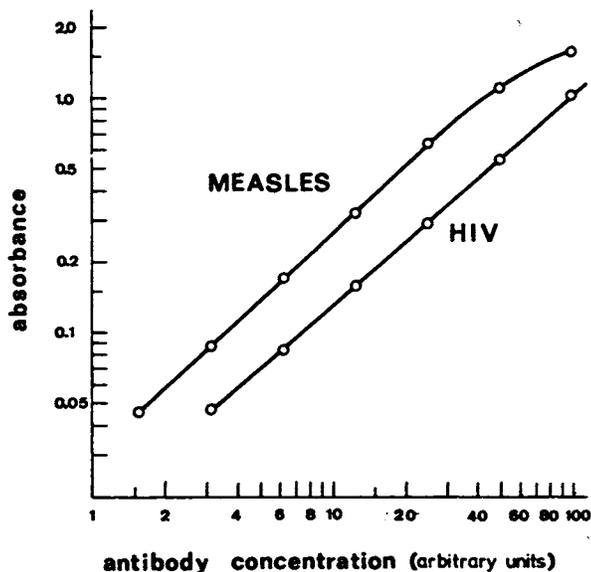


Fig. 1. Standard curves for antibody concentration in the EIA. Serum pools containing antibodies to HIV or measles virus were suitably diluted (1000- to 8000-fold) to yield an absorbance at 450 nm of  $\sim 1.5$ – $2.0 A$  in the assay. The upper standard value of each series was defined to be 100 arbitrary concentration units. The prediluted serum pool was diluted with six serial twofold dilution steps. The simultaneous analysis of CSF and serum samples with the standard dilutions and the subsequent calculation of a CSF/serum quotient eliminates the arbitrary variables from the unknown standard concentration

standard curve for calculating the final CSF/serum quotient.

#### Patients

The data originated from patients of the Neurological University Clinic, Göttingen, routinely analyzed during 1989. The final diagnoses of the diseases studied in this paper were based on clinical and neurochemical data together with usual electrophysiological methods and neuroimaging (18). The control group resembled those patients for whom a suggested neurological base of disease could be excluded (e.g., headache without an organic origin).

#### Results

##### Method

The representative standard curves in Figure 1 for concentrations of antibodies to measles virus and HIV were generated from results for two serum pools, which were diluted suitably to obtain an absorbance in the assay between 0.05 and 2.0  $A$ . The upper standard value of each series was defined to be 100 arbitrary concentration units. Detection at 450 nm (instead of 490 nm, the wavelength of maximal absorptivity) was preferred so that we could use less-dilute CSF and serum samples. The presence of bromphenol blue in the prediluted sample and in the antibody solution helped us avoid faulty pipetting of samples into the test plate. The presence of milk in the sample incubation step reduced the blank values by two- to 10-fold. For maximal sensitivity of the EIA, we had to separate the different incubation steps. After a prolonged incubation time (overnight) for antibody binding from patients' samples, we used a shorter second incubation with the antibody-conjugated peroxidase, which kept the enzyme reagent more stable.

Table 1 presents the day-to-day imprecision of single concentration values (arbitrary concentrations) in CSF and serum, together with the subsequent imprecision for  $Q_{\text{spec}}$  or Antibody Index values for the same patient, analyzed in eight different series. We used the same standard serum in these different analytical series. The CV values refer to determinations of concentrations of samples with absorbances between 0.1 and 0.6  $A$  in the assay.

The imprecision (CV) in a series of assays of a predi-

Table 1. Day-to-Day Imprecision of Virus-Specific Antibody Analysis in CSF and Serum

Virus	CV, %			
	CSF	Serum	$Q_{\text{spec}}$	Antibody Index <sup>a</sup>
Measles	7.9	3.1	10.0	11.2
Rubella	11.8	4.8	9.4	10.5
Varicella-zoster	10.0	8.4	7.8	9.1
Herpes simplex	7.2	14.7	14.8	18.3

<sup>a</sup>  $n = 8$  series each.  $Q_{\text{spec}}$ : CSF/serum quotient of specific antibodies.

<sup>b</sup>  $Q_{\text{spec}}/Q_{\text{IgG}}$ .  $Q_{\text{IgG}}$  was also analyzed as for day-to-day imprecision: CV = 3.6% ( $n = 8$ ). The Antibody Index was calculated on the basis of the actual  $Q_{\text{IgG}}$  of the daily analysis.

luted control serum was 2.0% to 2.8% for the various species in Table 1 as well as for antibodies to HIV, cytomegalovirus, and toxoplasmosis.

The simultaneous analysis of two dilutions at a five-fold difference of each sample (CSF and serum) was sufficient in most cases. Fewer than 5% of the samples needed reassay with greater dilutions. Also, if the absorbance of a diluted sample (8000-fold for serum, 40-fold for CSF) was  $<0.05 A$ , we did not evaluate the values. Where both dilutions from a single sample could be evaluated, we used these two values as an internal control of precision by calculating the concentration ratio (theoretical value = 5).

In principle, the day-to-day imprecisions (Table 1) of a single patient's  $Q_{\text{spec}}$  were less than those of the single CSF or serum values, a consequence of the simultaneous analysis of the corresponding CSF and serum samples on the same microtiter plate. Because of an additional variation from the  $Q_{\text{IgG}}$  (CV = 3.6%), the Antibody Index shows a still larger CV (Table 1).

In single cases, we obtained a maximal variation for the Antibody Index of CV = 30%, the sum of the variances from four different determinations.

#### Evaluation of Specific Antibody Quotients

Figure 2a represents the  $Q_{\text{IgG}}$  diagram for a patient with clinically confirmed zoster ganglionitis.  $Q_{\text{IgG}}$  was in the normal range ( $1.7 \cdot 10^{-3}$ ), and the VZV quotient ( $Q_{\text{VZV}} = 3.3 \cdot 10^{-3}$ ) was below the discrimination line but above the patient's empirical IgG quotient. In this case, the Antibody Index ( $\text{VZV-AI} = Q_{\text{VZV}}/Q_{\text{IgG}}$ ) was 1.9, indicating a local synthesis of VZV antibodies in the brain, despite a normal  $Q_{\text{IgG}}$  and a lack of oligoclonal IgG fractions in CSF.

The  $Q_{\text{IgG}}$  diagram in Figure 2b represents the data for a multiple sclerosis patient with an intrathecally syn-

thesized immunoglobulin fraction, IgG(Loc), of 64.2 mg/L, corresponding to 72% of total IgG in CSF (6). These values are calculated from  $\text{IgG}(\text{CSF}) = 89.3 \text{ mg/L}$ ,  $\text{IgG}(\text{S}) = 7.7 \text{ g/L}$ ,  $Q_{\text{Alb}} = 5 \cdot 10^{-3}$  by the following equation:

$$\text{IgG(Loc)} = [Q_{\text{IgG}} - 0.8\sqrt{Q_{\text{Alb}}^2 + 15 \cdot 10^{-6}} + 1.8 \cdot 10^{-3}] \cdot \text{IgG(S)} \quad (1)$$

Equation 1 refers to the hyperbolic discrimination line (6) as the empirical upper limit for an IgG fraction in CSF that originates completely from blood without any contribution from intrathecally synthesized antibodies; i.e.,  $\text{IgG(Loc)} = 0$ . All  $Q_{\text{IgG}}$  values located on this discrimination line are called  $Q_{\text{Lim}}$  (Figure 2b). The actual value of  $Q_{\text{Lim}}$  depends on the individual  $Q_{\text{Alb}}$ . In the above case,  $Q_{\text{Lim}}$  can be calculated by equation 2:

$$Q_{\text{Lim}} = 0.8\sqrt{Q_{\text{Alb}}^2 + 15 \cdot 10^{-6}} - 1.8 \cdot 10^{-3} = 3.3 \cdot 10^{-3} \quad (2)$$

Equation 2 characterizes the discrimination line for a decreasing ratio between  $Q_{\text{IgG}}$  and  $Q_{\text{Alb}}$  found with increasing blood/CSF barrier dysfunction, i.e., with increasing  $Q_{\text{Alb}}$  (Figure 2). As shown in Figure 3, this discrimination line varies and requires different constants in equations 1 and 2 for IgA or IgM determinations (6).

The specific antibody quotients for antibodies to measles virus ( $Q_{\text{measles}}$ ), rubella virus ( $Q_{\text{rubella}}$ ), varicella-zoster virus ( $Q_{\text{VZV}}$ ), and herpes simplex virus ( $Q_{\text{HSV}}$ ) represent IgG-class immunoglobulins, which can also be evaluated in this IgG diagram. Values exceeding the

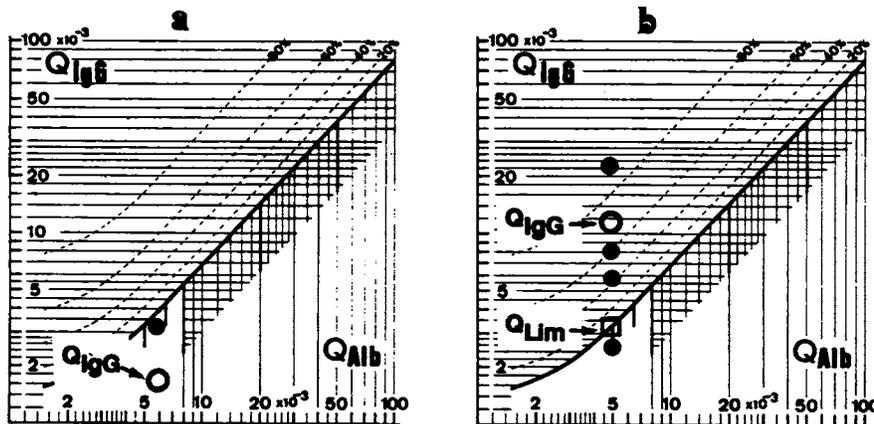


Fig. 2.  $Q_{\text{spec}}$  and  $Q_{\text{IgG}}$  of patients with zoster ganglionitis (a) and multiple sclerosis (b)

In the quotient diagrams,  $Q_{\text{IgG}}$  is shown as a function of  $Q_{\text{Alb}}$ , representing the blood/brain and blood/CSF barrier function (6). Below the discrimination line (representing  $Q_{\text{Lim}}$  values, fitted by a hyperbolic curve), the fraction in CSF is primarily blood-derived. Above the discrimination line, an increasing contribution of the locally (CNS) synthesized IgG or IgG-class antibodies can be demonstrated (given as the percent of locally synthesized IgG or antibodies of the corresponding total CSF concentration). (a) The CSF/serum data for this patient with zoster ganglionitis were  $Q_{\text{Alb}} = 6.0 \cdot 10^{-3}$ ,  $Q_{\text{IgG}} = 1.7 \cdot 10^{-3}$ , and  $Q_{\text{VZV}} = 3.3 \cdot 10^{-3}$ ; the corresponding Antibody Index was  $\text{VZV-AI} = 1.9$ . With  $Q_{\text{IgG}} < Q_{\text{Lim}}$ , we used the equation  $\text{AI} = Q_{\text{spec}}/Q_{\text{IgG}}$ . (b) The CSF/serum data for this multiple sclerosis patient were  $Q_{\text{Alb}} = 5.0 \cdot 10^{-3}$ ,  $Q_{\text{IgG}} = 11.6 \cdot 10^{-3}$ ,  $Q_{\text{Lim}} = 3.3 \cdot 10^{-3}$ ,  $Q_{\text{measles}} = 22.4 \cdot 10^{-3}$ ,  $Q_{\text{rubella}} = 8.2 \cdot 10^{-3}$ ,  $Q_{\text{VZV}} = 5.9 \cdot 10^{-3}$ , and  $Q_{\text{HSV}} = 2.6 \cdot 10^{-3}$ . The corresponding Antibody Index values are as follows: measles-AI = 6.8; rubella-AI = 2.5; VZV-AI = 1.8; and HSV-AI = 0.8. With a local IgG synthesis ( $Q_{\text{IgG}} > Q_{\text{Lim}}$ ), we used the equation  $\text{AI} = Q_{\text{spec}}/Q_{\text{Lim}}$ . In general, a  $Q_{\text{spec}}$  above a 30% line in the diagram ( $Q_{\text{spec}} \geq 1.5 \cdot Q_{\text{Lim}}$ ) represents a pathological Antibody Index value ( $\geq 1.5$ ).

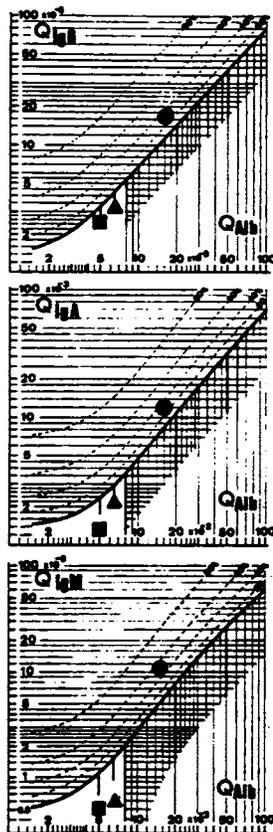


Fig. 3. Quotient diagrams of two patients with HIV encephalitis. The quotient diagrams are different for the different immunoglobulin classes (6), with corresponding characteristic areas as described in legend of Fig. 2.  $\Delta$ , First puncture of patient 1 (1a in Table 3) with  $Q_{HIV} = 2.6 \cdot 10^{-3}$ .  $\blacksquare$ , Second puncture of patient 1 (1b in Table 3) with  $Q_{HIV} = 6.7 \cdot 10^{-3}$  ( $\geq 1.5 \cdot Q_{Lim}$ ).  $\bullet$ , Puncture of patient 2 with an opportunistic *Toxoplasma* infection (2 in Table 3) with  $Q_{HIV} = 25.2 \cdot 10^{-3}$ ,  $Q_{Toxoplasma} = 25 \cdot 10^{-3}$ ,  $Q_{Cytomegalovirus} = 10.6 \cdot 10^{-3}$ , and  $Q_{HSV} = 11.8 \cdot 10^{-3}$  ( $Q_{Lim} = 11.8 \cdot 10^{-3}$ ). Patient 1 shows in both punctures normal  $Q_{AB}$  ( $Q_{AB} < 6.5 \cdot 10^{-3}$ , age-dependent limit), i.e., no blood/CSF barrier dysfunction, in contrast to patient 2, who has a blood/CSF barrier dysfunction and an additional intrathecal synthesis of IgG, IgA, and IgM. The antibody values given above (and in Table 3) refer to IgG-class immunoglobulins.

discrimination line ( $1.5 \cdot Q_{Lim}$ ) by  $>30\%$  are indicative of a local antibody synthesis in brain: e.g., measles, rubella, and VZV in Figure 2b (HSV antibodies in CSF obviously originate from blood only in this illustration).

Figure 2b makes it obvious that the application of the Antibody Index calculation according to  $AI = Q_{spec}/Q_{IgG}$  in the case of an increased  $Q_{IgG}$  would lead to a false-negative result: e.g.,  $VZV-AI = Q_{VZV}/Q_{IgG} = 0.5$ . In such a case we must refer to the corrected quotient value on the discrimination line,  $Q_{Lim}$ , with (e.g.)  $VZV-AI = Q_{VZV}/Q_{Lim} = 1.8$ . When a numerical evaluation is called for, we need two different formulas for maximal sensitivity of the Antibody Index:

$$AI = Q_{spec}/Q_{IgG} \quad (\text{if } Q_{IgG} < Q_{Lim}) \quad (3)$$

$$AI = Q_{spec}/Q_{Lim} \quad (\text{if } Q_{IgG} > Q_{Lim}) \quad (4)$$

Equation 3 is used if  $Q_{IgG}$  represents the barrier conditions referring to a predominantly blood-derived CSF protein fraction without a significant local IgG synthe-

sis in the CNS, i.e., if  $Q_{IgG} < Q_{Lim}$ . Equation 4 is used if  $Q_{IgG} > Q_{Lim}$ .

### Reference Range

With these evaluation conditions for the Antibody Index, we calculated the reference range as 0.7–1.3 for all antigens evaluated in 150 control patients (control group 1 in Table 2). Only in 1% of these control patients were values of 1.4 detected. Values  $\geq 1.5$  for the Antibody Index indicated a local antibody synthesis in the CNS.

The normal range of the Antibody Index was not influenced by severe blood/CSF barrier impairment or large local synthesis of IgG in the CNS, as shown by a separate evaluation of a heterogeneous group with various neurological diseases (control group 2 in Table 2). These data for control group 2 confirm that the evaluation according to equation 4 is correct. This group included several cases of polyradiculitis Guillain-Barré syndrome, bacterial meningitis, meningeal carcinosis, neurosyphilis, and Lyme disease—in none of which has a polyspecific antibody synthesis in brain been observed. A virus-specific response appeared only in those cases where the virus was the causative agent or in cases of a chronic disease with a secondary stimulation of antibody synthesis against the neurotropic viruses. Table 4 presents examples of the antibody response in such cases.

### Examples of Neurological Diseases

With the following three cases we demonstrate the clinical relevance of Antibody Index values in comparison with other CSF data.

**HIV encephalitis with an opportunistic *Toxoplasma* infection:** Figure 3 shows the immunoglobulin quotients for IgG, IgA, and IgM (6) in different phases of HIV encephalitis. Table 3 reports the corresponding changes in the Antibody Index values together with other CSF data. At the time of the first puncture, only a small pleocytosis was detectable. Neither an increased HIV-AI nor a general humoral immune reaction could be observed; there were no oligoclonal bands (8) or activated B-lymphocytes (20). The second puncture in a

Table 2. Calculations of the Antibody Index from Two Different Control Groups

Virus	Antibody Index (and % SD)	
	Control 1, normal <sup>a</sup>	Control 2, diseases <sup>b</sup>
Measles	0.93 (15)	0.93 (19)
Rubella	0.95 (16)	0.95 (15)
Varicella-zoster	1.00 (17)	0.99 (16)
Herpes simplex	1.00 (19)	1.01 (20)

<sup>a</sup> Patients without neurological diseases ( $n = 150$ ); values calculated according to equation  $AI = Q_{spec}/Q_{IgG}$ .

<sup>b</sup> Patients with various neurological diseases including severe blood/CSF barrier impairments and (or) local IgG synthesis but without a polyspecific local antibody synthesis ( $n = 100$ ); values calculated according to the  $AI = Q_{spec}/Q_{IgG}$  or  $AI = Q_{spec}/Q_{Lim}$ , respectively.

**Table 3. Data for Two Patients with HIV Encephalitis**

	1a <sup>a</sup>	1b <sup>a</sup>	2 <sup>b</sup>
Q <sub>Ab</sub> ( $\cdot 10^3$ )	6.5	5.0	16.5
Q <sub>IgG</sub> ( $\cdot 10^3$ )	3.3	2.9	17.0
Q <sub>Lim</sub> ( $\cdot 10^3$ )	4.2	3.3	11.8
Oligoclonal IgG	0	(+)	+
CSF cell count/ $\mu$ L	11	7	7
Activated B cells, % of total lymphocytes	0	0.3 (G)	1.7 (G+A+M)
Antibody Index values			
HIV	0.8	2.3	6.9
Cytomegalovirus	0.9	0.9	0.9
Toxoplasma	0.9	1.0	2.1
HSV	0.8	1.0	1.0

Complementary data of the patients are shown in Fig. 3.

<sup>a</sup> CSF punctures of the same patient about two months (1a) and eight months (1b) after infection.

<sup>b</sup> Patient 2 also had an opportunistic acute Toxoplasma infection.

later phase of HIV encephalitis indicated clearly the humoral immune response (oligoclonal bands in isoelectric focusing) with a local HIV antibody synthesis in the CNS (HIV-AI = 2.3), together with a cellular IgG-specific response. The third set of data, from another patient (see Figure 3), was indicative of an opportunistic Toxoplasma infection in the CNS (Toxoplasma-AI = 2.1). The blood/CSF barrier impairment, together with an additional intrathecal synthesis of IgA- and IgM-class immunoglobulins, is typical of the opportunistic infection, which would not be seen in a pure HIV encephalitis. The detection of IgA- and IgM-synthesizing B-lymphocytes in CSF is consistent with the observed humoral immune response. The Antibody Index values in the first and second puncture were calculated by equation 3. For the third value (patient 2) the Antibody Index value was calculated by equations 4 and 2:  $\text{Toxoplasma-AI} = Q_{\text{Tox}}/Q_{\text{Lim}} = 24.5/11.8 = 2.1$ . In addition, the herpes simplex Antibody Index was determined to be in a late stage of HIV encephalitis [designated a 6 on the Walter Reed clinical scale of acquired immune deficiency syndrome (AIDS)]. Occasionally an increased HSV Antibody Index could be observed (16), despite the absence of a herpes simplex encephalitis, indicating a secondary polyspecific immune reaction (as in Table 4).

**Zoster oticus:** The sensitivity of the method is demonstrated in Figure 2a by the case of a patient with an isolated facial nerve paresis. The patient had neither additional clinical signs nor pathological values in CSF analysis, i.e., a normal blood/CSF barrier function, no oligoclonal IgG-synthesis, and no cellular immune response. Only the analysis of virus-specific antibodies showed an increased Antibody Index for VZV (VZV-AI = 1.8). Without the VZV analysis, an idiopathic facial paresis would have been diagnosed. The later appearance of vesicular exanthema of the ear confirmed the diagnosis of a zoster ganglionitis, in this case a zoster oticus.

**Multiple sclerosis:** The patient with multiple sclerosis (Figure 2b) shows a typical set of data: a normal blood/CSF barrier function, but a large humoral immune response, which could be identified easily by an increased IgG quotient, the small pleocytosis (11 cells/ $\mu$ L of CSF), and a few activated B-lymphocytes (IgG class). These data alone would not be sufficient to confirm the diagnosis, but vice versa, a high barrier dysfunction or the lack of humoral immune response would contradict the diagnosis (18). Only the appearance of antibodies synthesized locally in the CNS against measles, rubella, or zoster virus could give a high plausibility for this diagnosis. Because these viruses are not the etiologic agent in multiple sclerosis, one must look for other cases of such a secondary immune response. But there are very few chronic diseases, with a very small incidence, that show these phenomena. The Antibody Index data from a second multiple sclerosis patient are shown in Table 4, indicating the variability of the individual antibody response.

**Other inflammatory diseases:** In Table 4, we present several cases to demonstrate the antibody response in various neurological diseases. In two cases of a subacute sclerotic panencephalitis, we observed a measles Antibody Index of 37 and 76, but the Antibody Index values for rubella virus, VZV, and HSV were normal.

HSV encephalitis and zoster ganglionitis (Table 4) are additional examples of an increased Antibody Index against the etiologic agent. One of these cases shows the well-known biological cross-reactivity of HSV and VZV. None of the patients investigated with HSV or zoster encephalitis or zoster ganglionitis (18) had an increased measles or rubella Antibody Index.

The case of a late HIV encephalitis (Walter Reed Scale 6) with an increased HSV Antibody Index represents one of the 30% of HIV patients in a late phase of the chronic disease with an etiologically irrelevant local synthesis of antibodies. This is also mainly the case in the multiple sclerosis patients but also in the rare cases of chronic neuroborreliosis (Table 4). In the acute cases of Lyme disease (*Borrelia*, Table 4), we observed no polyspecific antibody response against neurotropic viruses (19).

## Discussion

Here we have combined a sensitive method for quantifying CSF antibody concentrations with a blood/CSF barrier-related evaluation method. Many earlier publications report evaluations of isolated absolute CSF values disregarding an increased blood concentration of an antibody or a blood/CSF barrier impairment as the possible origin of an increased CSF value. As a false consequence, the authors suggested a local antibody synthesis in brain. Among these misleading evaluations are reports about virus antibody synthesis in "cerebrovascular diseases" or polyspecific immune response in subacute sclerotic panencephalitis.

The highly sensitive and specific techniques like Western blot (12), immunoblot after isoelectric focusing of CSF (11), or immunospot assay (13, 14) still have the

**Table 4. Antibody Index Values in Inflammatory Neurological Diseases**

Virus or bacteria	SSPE	Zoster gangl.	HSV Enc.	HIV encephalitis		MS	Neuroborreliosis	
				WR2	WR6		Acute	Chronic
Measles virus	<b>76.0</b>	0.9	1.0	0.9	1.1	<b>7.7</b>	0.9	<b>3.2</b>
Rubella virus	1.0	1.0	0.9	1.0	0.9	<b>14.3</b>	1.0	<b>7.9</b>
Varicella-zoster virus	0.9	<b>7.4</b>	1.0	1.1	1.0	<b>5.6</b>	0.9	0.9
Herpes simplex virus	1.1	<b>3.3</b>	<b>46.7</b>	0.9	<b>2.3</b>	1.0	1.1	<b>2.6</b>
Human immunodeficiency virus	—	—	—	<b>5.4</b>	<b>4.2</b>	—	—	—
<i>Borrelia b.</i> (IgG)	—	1.0	—	—	—	0.9	<b>25.3</b>	<b>19.3</b>
<i>Borrelia b.</i> (IgM)	—	1.1	—	—	—	1.1	<b>124.0</b>	<b>10.1</b>

SSPE, subacute sclerotic panencephalitis; gangl., ganglionitis; Enc., encephalitis; WR2/WR6, clinical grade of AIDS, according to Walter Reed scale; MS, multiple sclerosis. Bold numbers indicate pathologically increased AI values.

same problem—the barrier-related evaluation. Of course, most authors apply the same amounts of total IgG for CSF and serum samples in the paired analysis. This is not optimal only if locally synthesized, brain-derived IgG increases the CSF content of IgG. These qualitative methods are highly sensitive if the very small brain-derived portion consists of qualitatively different bands in the CSF that are not present in serum. This is the case with oligoclonal immunoglobulins of different electrophoretic mobility (11) or with antibodies specific against different parts of the antigen (12). But the application of a falsely low amount of CSF IgG from a local IgG synthesis in brain would reduce the sensitivity of the qualitative methods. Again, the application of a quantitative EIA method for specific parts of the antigen could improve the sensitivity (18).

The earliest reports of CSF/serum quotients for specific virus or bacteria antibodies used titer values for quotient formation (21). Because the faults were propagated from two titer values (CSF and serum), a large imprecision in the CSF/serum quotient (with a maximal factor of 4) must be taken into consideration; i.e., Antibody Index values are defined to be pathological only if  $AI > 4$ . The introduction of a method for the evaluation of continuous concentration values (arbitrary units) was possible only in CSF diagnosis because with formation of the CSF/serum quotients, the arbitrary variables could be eliminated. By this method any positive serum sample or serum pool can be used as a standard if the patients' samples (serum and CSF) are analyzed together on the same test plate or in the same test series simultaneously with the standard dilutions. Because of the high accuracy of this method, we recommend replacing the analysis of antibody titers in CSF diagnosis with this method in all cases where quotients must be calculated.

Additionally, we report an advantageous evaluation method. The Antibody Index represents the ratio between  $Q_{spec}$  and  $Q_{IgG}$ . In a pure blood-derived IgG and antibody fraction in CSF, this ratio has the theoretical value of 1. This linear correlation of both CSF/serum quotients is of great advantage, compared with the hyperbolic correlation function between  $Q_{spec}$  and  $Q_{AIB}$ . But the linear function, referring to  $Q_{IgG}$ , can be used only under certain limitations. In an intrathecal IgG

synthesis, the proposed correction ( $Q_{Lim}$ ) must be used. Finally, with this correction the hyperbolic correlation with  $Q_{AIB}$  is used.

As an example, we report in Figure 2b the data from a multiple sclerosis patient with  $Q_{rubella} = 8.2 \cdot 10^{-3}$  and  $Q_{IgG} = 11.6 \cdot 10^{-3}$ . We found the rubella Antibody Index to be increased with respect to the theoretical limit  $Q_{Lim} = 3.3 \cdot 10^{-3}$  and we calculated it as  $AI = Q_{rubella}/Q_{Lim} = 2.5$ . Referring the  $Q_{rubella}$  to the empirical  $Q_{IgG}$  would have yielded a falsely low  $AI = 0.7$ . The same problem would occur for the Toxoplasma-AI in the reported case of HIV encephalitis (Table 3).

The normal range for the Antibody Index (0.7–1.3) represents mainly the propagation of the imprecisions from four determinations (Table 1). Comparing the Antibody Index values from control patients without neurological disease in Table 2 (control group 1) with the Antibody Index values from patients with inflammatory or other neurological diseases (control group 2) shows a very convincing accuracy for the mean and the CV. These data confirm the accuracy of the evaluation method with both equations 3 and 4 for the absence or presence of a local humoral immune response. Given these methodological developments, it was possible to improve the clinical relevance, as has been shown with clinical details for HIV encephalitis (16), multiple sclerosis (17, 18), HSV encephalitis, and zoster ganglionitis (18). In multiple sclerosis, the humoral immune response is a constant neurochemical sign. As shown earlier (8), in 98% of the multiple sclerosis cases, the humoral immune response could be identified as oligoclonal IgG fractions in isoelectric focusing (8, 14). That means the absence of oligoclonal IgG would give a high plausibility (98%) to discarding the diagnosis of multiple sclerosis. With the identification and quantification of a polyspecific immune response (14, 15, 17), for the first time the diagnosis of multiple sclerosis can be confirmed from neurochemical data. By the correction for polyclonal IgG synthesis with equation 4, the sensitivity of the method has been drastically increased from an earlier reported prevalence of 70% (15) to 93% (18).

In correspondence with clinical data, and eventually with magnetic resonance imaging (22), the identification of formation in the CNS of a polyspecific antibody against one or several of the three virus species (mea-

sles, rubella, varicella-zoster) allows the diagnosis of multiple sclerosis in 93% of the patients as early as the appearance of the first clinical symptoms (18).

As shown in detail for multiple sclerosis, zoster ganglionitis, and HSV encephalitis (18), the diagnostic sensitivity of the Antibody Index is greater than the sensitivity of the Western Blot (specific antibodies), isoelectric focusing of oligoclonal immunoglobulins, or the statistical  $Q_{1gG}$  evaluation (in order of decreasing sensitivity).

In principle, one must differentiate between two cases of pathological Antibody Index values: the first case, in which the antigen was the etiologic agent for the disease, and a second case, where secondary polyspecific immune reactions took place without a persistence of antigen or corresponding clinical signs of a specific virus infection. Other than multiple sclerosis, a few other chronic neurological diseases show this phenomenon, e.g., in the late stage of HIV encephalitis (16) or in chronic Lyme disease (19). For all clinical interpretations of these locally synthesized antibodies, one must keep in mind that local synthesis in the brain is a long process, often occurring over many years (18, 21), and does not indicate the acuity of the process. This was shown for HSV encephalitis (18), neurosyphilis (21), and Lyme disease (19). The relations of IgM-class to IgG-class antibody synthesis, as shown in the case of acute Lyme disease (Table 3), cannot be taken as a sign of acuity. In other cases of acute Lyme disease, the IgG-class *Borrelia*-AI (214!) was much larger than the IgM-class *Borrelia*-AI (33.6). With these examples of a neurological disease of bacterial etiology (19), we have documented another successful application of this evaluation method. This evaluation of CSF antibody data by an Antibody Index represents a further expansion of the CSF/serum quotient concept (6, 7).

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