

Intrathecal polyspecific immune response to neurotropic viruses in multiple sclerosis: a comparative report from Cuban patients

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Objectives – Intrathecal measles(M)- rubella(R)- and varicella zoster(Z)-antibody synthesis in German and Cuban multiple sclerosis (MS) patients are compared considering the different rubella epidemiology in the tropics. **Patients and methods** – Twenty-three Cuban MS patients with a representative age distribution and gender ratio like the group of 177 German MS patients were analysed for albumin, IgG, IgA IgM, oligoclonal IgG and MRZ- antibodies in cerebrospinal fluid (CSF) and serum. **Results** – Cuban MS patients show similar CSF data patterns like German patients and high frequencies of intrathecal measles- (78/78%) and varicella zoster- (59/55%) antibody synthesis correspondingly. A lower frequency of intrathecal rubella antibody synthesis (rubella-AI \geq 1.5) in Cuban patients (30%, gender ratio of increased rubella - AI m:f = 1:6) compared with German patients (60%, m:f = 1:1.8) is explained by low incidence of rubella infections in Cuba. Only about 10% of the male population (not immunized before 1986, in contrast to females) had rubella antibodies compared to at least 60% in a European male population, representing the relation of increased rubella-AI in male MS patients. **Conclusion** – In MS the frequency of intrathecal antibody synthesis is limited by the fraction of seropositives in the population. Natural infection or vaccination are a necessary and equivalent precondition contributing to the arguments against microorganisms as a cause of MS.

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with a predominant intrathecal IgG class immune reaction. The highly frequent detection of oligoclonal IgG in cerebrospinal fluid (CSF) of MS patients (1) is proposed as an analytical parameter for laboratory-supported diagnosis of MS (2). In the frame of actual quantitative CSF routine analysis (3), it is possible to detect the intrathecal synthesis of specific antibodies by very sensitive methods. An extremely high frequency of intrathecal antibody synthesis against measles (M)-, rubella (R)- and varicella zoster -virus among other neurotropic viruses was observed in MS (1, 4) and autoimmune diseases with involvement of the CNS (5). This so-called MRZ antibody reaction (1, 4), found in

84–94% of MS patients (1), is not detectable with such a frequency in other chronic, subacute or acute neurological diseases (<0.5%). The pathophysiological relevance of this MRZ reaction remains a part of ongoing discussions about the cause of MS. The early discovery of an intrathecal measles antibody synthesis in MS patients (6) nourished the hypothesis of a virus or micro-organism as a cause of MS (7–9). Since then all newly discovered intrathecally synthesized antibody species in CSF of MS patients were suspected, like varicella zoster virus (10) Epstein–Barr virus (11), JC virus (12), HTLV-1 (13), herpes type 6 virus or chlamydia (14). Most recently EBV is favored again. Yet, the detection of an intrathecal antibody synthesis against a micro-organism does not prove that this

micro-organism is a cause of the disease, in particular if the virus is not persisting in brain (15, 16). The quantification of the intrathecal antibody response (17, 18) supports the discrimination between an antibody synthesis against a persisting causative antigen and a concomitant polyspecific synthesis of the antibody. Each of the single M-, R- or Z-antibody fractions are less than 0.5% of the intrathecally synthesized total IgG (1) compared with 20- to 60-fold higher quantities of antibodies in the case of an immune reaction against a causative antigen persisting in CNS, e.g. for measles antibodies in subacute sclerosing panencephalitis (SSPE) compared with measles antibodies in MS or HSV antibodies in HSV encephalitis compared with HSV antibodies in MS (1, 17). An explanation for the outstandingly high frequency of this MRZ reaction in MS different from other chronic diseases of the CNS remains a challenge. It seems to be a reasonable approach to compare MS patients in different countries (1, 19) with different frequencies of neurotropic virus infections (20, 21). We investigated the frequencies of the M-, R-, Z-antibody reaction in a group of MS patients from Cuba (22) as a tropical region with an epidemiology different from Germany. This investigation with patients born and adolescent before the start of the general immunization campaigns is a last chance for such a comparison, not possible in populations with a more or less complete immunization.

Patients and methods

Patients

Multiple sclerosis patients were diagnosed in the clinic of MS, International Center for Neurological Restoration (CIREN), Havana City, Cuba, in the years 2003 and 2004 according to the established diagnostic criteria (23). Of a larger group of patients with definite MS, we selected retrospectively those 23 patients who had a complete analysis of immunoglobulins, oligoclonal IgG and in particular M, R and Z antibodies. In this retrospective study, only 15 patients have been classified according to the course of the disease at the time of lumbar puncture according to Lublin et al (24): seven subjects had a primary or secondary progressive MS (ProgMS) and eight subjects had a relapsing–remitting (RRMS) course of the disease. The residual eight cases were not clinically classified for the type of the MS course, because of a lack of information in the retrospective study. Patients receiving immunomodulatory therapy were not included.

Cerebrospinal fluid and serum samples were taken for routine diagnosis of the disease after

overnight fasting. Residual volumes of paired CSF and serum samples were stored at -70°C and thawed shortly before analysis in the Neurochemistry Laboratory in Goettingen. Bloody or turbid CSF samples were rejected.

The age (median 38 years, range 24–48 years) and gender distribution (m:f = 1:1.9) in the Cuban group were representative of MS patients, as reported for the German group too.

Analytical procedures

Both CSF and diluted serum were quantified for Albumin IgG, IgM and IgA with an automated (particle amplified for IgA and IgM) immunochemical assay on a Dade Behring Nephelometer (Marburg, Germany). The CSF/serum concentration quotients were evaluated in Reibergrams (3) and intrathecal synthesis calculated as intrathecal fractions, IgG_{IF} , IgM_{IF} , IgA_{IF} according to $\text{Ig}_{\text{IF}} = (1 - Q_{\text{lim}}/Q_{\text{Ig}}) \times 100$ in percentages where $Q_{\text{lim}}(\text{IgG}) = (0.93(Q_{\text{alb}}^2 + 6)^{0.5} - 1.7) \times 10^{-3}$, $Q_{\text{lim}}(\text{IgA}) = (0.77(Q_{\text{alb}}^2 + 23)^{0.5} - 3.1) \times 10^{-3}$ or $Q_{\text{lim}}(\text{IgM}) = (0.67(Q_{\text{alb}}^2 + 120)^{0.5} - 7.1) \times 10^{-3}$; Q_{Ig} is the empirically determined quotient of the single patient (3, 25). The blood–CSF barrier function was interpreted with the age-related reference range for the albumin quotient, $Q_{\text{alb}} = (4 + \text{age}(\text{years})/15) \times 10^{-3}$ (26).

Antibody detection in CSF and serum

The intrathecal synthesis of M, R, Z and herpes simplex (H) IgG class antibodies was detected by an ELISA technique (27). Antigen-coated microtiter plates were obtained from Behring for measles virus (Enzygnost OSOK 02/08), rubella virus (Enzygnost OSON 02/08), herpes simplex virus (Enzygnost OSOG 02/08) and varicella zoster virus (Enzygnost OSMK 02/08). The differences of absorbance were measured with a microtiter plate reader (SLT Labinstruments, Crailsheim, Germany) and transferred into arbitrary concentration units calculated with the software ‘easy fit’ of SLT. The antibody index (AI) for $\text{AI} = [Q_{\text{spec}}/Q_{\text{IgG}}]$ or with correction, in the case of $Q_{\text{IgG}} > Q_{\text{lim}}$ with $\text{AI} = [Q_{\text{spec}}/Q_{\text{lim}}]$ was calculated according to Reiber and Lange (27) with reference to the Q_{lim} from the more recent hyperbolic function (25): $Q_{\text{lim}} = (0.93(Q_{\text{alb}}^2 + 6)^{0.5} - 1.7) \times 10^{-3}$.

The normal range was $\text{AI} = 0.7\text{--}1.3$ (27); an intrathecal synthesis was considered for values of $\text{AI} \geq 1.5$.

The MRZ antibody reaction (28) was counted as positive if one, two or three of the M, R, Z antibodies were intrathecally synthesized

(AI ≥ 1.5). Diagnostically, the single value is not sufficient for interpretation as a ‘chronic’ inflammatory process in CNS (autoimmune type) (1.5).

Oligoclonal IgG in CSF was detected by isoelectric focusing in agarose gels followed by an immunoblot technique. The results were expressed in five different types as recommended by the European expert group (26).

Results

General immune reaction

Intrathecal IgG synthesis was observed in 23/23 cases, 22 cases by oligoclonal IgG in CSF (96%) and the residual case by increased VZV- and HSV-AI values, i.e. all 23 cases had an intrathecal immune response. As to be expected, the frequency for detection of an intrathecal IgG fraction, IgG_{IF} > 0, was smaller than the frequency of oligoclonal IgG but with 43% (n = 10/23) still smaller than the value (72%) reported earlier (1) for a larger group. Nevertheless, the ratio of the frequencies of the intrathecal fractions (IgG_{IF}, IgM_{IF}, IgA_{IF} > 0%) between the different immunoglobulin classes IgG_{IF}:IgM_{IF}:IgA_{IF} was 10:4:1 similar to the earlier report with 10:3:1 (1). The age (median:38 years, range 24–48 years) and gender distribution (m:f = 1:1.9) in the Cuban group corresponds well also with age (median 36 years) and gender ratio (m:f = 1:2) in the German MS group (1).

The MRZ antibody reaction was detected in 100% (23/23) of MS patients in this group, i.e. in

all cases at least one of the M-, R- or Z-antibody species was synthesized intrathecally. Table 1 shows the frequencies of the single-antibody species with increased AI (≥ 1.5). The frequencies of normal AI values (0.7–1.3) and of cases without detectable antibodies were 4/23 and 1/23 for measles, 14/23 and 2/23 for rubella and 9/23 and 1/23 for VZV. In addition, the intrathecal herpes simplex antibody response was detected in two of six cases analysed. In both of these cases the VZV-AI was increased as well. This result is in accordance with the earlier report (1).

The main immunological difference between the Cuban group and the German group of MS patients was the 50% lower frequency of intrathecally synthesized rubella antibodies (Table 1) in the Cuban population. This result on frequencies corresponds with the observation of a lower median rubella-AI in the Cuban population (Table 2). The gender-specific evaluation shows that among the seven patients with increased rubella-AI, only one male patient was included, i.e. a ratio of 1:6 compared with the overall male-to-female gender ratio of 1:1.9 in the total group. The corresponding ratio for increased measles-AI was 7:11 and for VZV-AI 6:7.

Frequencies of rubella antibodies

The frequency of rubella antibody concentrations in the Cuban population has not been analysed directly and therefore must be calculated as an approximation to the upper limit value from the data supplied by the National Statistic Department of Cuban Ministry of Health (30) indicating a very low incidence of rubella infections in the Cuban population (Table 4). According to these data in the years 1970–1986 only 2% of the total Cuban population could have gained rubella antibodies by natural infection. As this mild disease is underestimated (20), about 4% of the population could be calculated to have gained rubella antibodies by natural infection between 1970 and 1986 or about 10% between 1955 and 1986, the time range relevant for this study. The patients of the present group were born between 1955 and 1979, i.e. most

Table 1 Frequency of intrathecal measles-, rubella- and varicella zoster-antibody synthesis in a group of Cuban multiple sclerosis patients (n = 23) (frequencies of increased antibody index ≥1.5)

	Antibodies		
	Measles-V	Rubella-V	VZV
AI ≥ 1.5 (n = 23)	18 (78%)	7 (30%)	13 (59%)
Expected ratio from Ref. (1)	18	14	13

Table 2 Intensities of intrathecal antibody response according to size of antibody index values (AI) of measles (M-AI), rubella (R-AI) and varicella zoster (Z-AI) antibodies

	M-AI, median (range)	R-AI, median (range)	Z-AI, median (range)
Cuba (n = 23 patients)	4.3 (1.5–36.1)	1.9 (1.5–9.5)	2.3 (1.5–40)
Germany (n = 177) (1)	3.3 (1.5–46.3)	3.9 (1.5–33.5)	3.6 (1.5–28.9)

Table 3 Frequencies of combinations of one, two, or three simultaneously intrathecally synthesized antibodies in MS patients from Cuba

	Combinations		
	M or R or Z	M + R or M + Z or R + Z	M + R + Z
Cases	12	7	4
Percentage of 23 cases	52	30	18

Table 4 Rubella infections in Cuba

	Age group (years)				Total	
	<1	1–14	15–24	≥25	Cases	%
size of population 1973/1986 (millions)	0. 20/0. 18	3.15/2.36	1.52/2.4	4.14/5.26	9.0/10.2	
1970	109	467	283	202	1061	0.013
1971	103	250	48	34	435	0.005
1972	84	237	35	26	382	0.004
1973	733	10,202	7938	5595	24,468	0.274
1974	2316	25,687	19,908	21,723	60,934	0.767
1975	564	1816	317	239	2936	0.032
1976	405	1642	303	152	2502	0.027
1977	330	1553	316	255	2454	0.026
1978	157	740	89	67	1053	0.011
1979	131	602	288	103	1124	0.012
1980	237	1667	817	312	3033	0.030
1981	758	17,681	10,074	4046	32,559	0.335
1982	286	3364	834	523	5007	0.051
1983	380	3691	582	418	4218	0.043
1984	431	3443	773	525	5172	0.052
1985	485	6436	1921	1418	10,260	0.102
1986	912	12,735	2836	2231	18,714	0.184
1986: start of vaccination campaign						
1991	5	9	0	3	17	0.0002
No cases reported after 1995						

Cases registered by the Cuban Ministry of Health 1970–2003 (30). The data are reported as age- related number of cases and percentage of the total population. The slightly changing size of population is given as representative numbers for the years 1973 and 1986.

(19/23) reached adolescence before 1986, at least the age of 7 years and were not integrated into the later immunization campaign. This figure (10%) is relevant for the male population only, as the male population before 1986 was not immunized at all in contrast to the female population which might have reached a figure of about 50% seropositives (see Discussion). The corresponding data of European populations (20) or in particular for the German population (21) were higher with 50–70% male and 80–90% female rubella positives.

Clinical aspects of MRZ antibody detection

From a diagnostic point of view, it is interesting that a simultaneous antibody synthesis against two or three different antigens (Table 3) represents a strong argument for a *chronic* inflammatory disease of the autoimmune type (1, 5). In the Cuban group, 48% of the MS patients could have been diagnosed by CSF analysis to have a chronic inflammatory process already at time of first clinical symptoms or with a monosymptomatic appearance. This is somewhat less than the 67% in the reference group (1). The different courses of the disease, progMS vs RRMS, did not show any difference in the distribution of the immunoglobulin class pattern or in the MRZ antibody pattern.

Discussion

The detection of an intrathecal antibody synthesis is most sensitive if the calculation of AI ($=Q_{spec}/Q_{IgG}$) involves a correction in cases $Q_{IgG} > Q_{lim}$ (IgG), in which the specific antibody quotient, Q_{spec} , is referred to the upper limit, Q_{lim} , of the reference range of the blood-derived IgG fraction in CSF ($AI = Q_{spec}/Q_{lim}$) (25, 27). In the particular group of this study, 21% of the increased AI values (Table 3) could be detected only by reference of Q_{spec} to Q_{lim} (IgG) instead of Q_{IgG} , the case in which there would have been 21% false-negative interpretations. A still larger loss of sensitivity (40%) was reported for MRZ antibody analysis in aqueous humour of the eyes in another group of MS patients (18).

For the reliability of the interpretation of the different MRZ antibody patterns (Table 1) in the smaller Cuban group of MS patients ($n = 23$) compared with the earlier analysed German reference group ($n = 177$) (1), it is important to recognize that the *relative* frequencies of all immunological data (IgG, IgA, IgM and oligoclonal IgG, M-AI, VZV-AI, HSV-AI) in both groups are very similar and that the data were gained in the same laboratory with the same methods. With these aspects, it seems reasonable to apply the same algorithms for a statistical

evaluation in the Cuban as in the German MS group: the frequency of a single antibody varied in the German group (1) within a range of $\pm 15\%$ depending on the size of the intrathecal IgG fraction. This would mean that the number of cases with an intrathecal rubella antibody synthesis would vary between six and eight in the Cuban group and 12 and 16 in the German group. There is no overlap of the range of frequencies between both groups, i.e. the seven cases found with intrathecal rubella antibodies (Table 1) are significantly smaller than 14, the expected number calculated from the corresponding ratio in the large reference group (Table 1). In particular, the gender ratio (m:f = 1:6) for the group of Cuban patients synthesizing rubella antibodies intrathecally is clearly different from the expected value (1:1.9) according to the overall gender ratio of 1:1.9. In the German group, the rubella positives (1:1.84) correspond well with the total gender ratio (1:1.95).

A reliable interpretation of these findings comes from the different epidemiology in the two countries. As reported (20), the fraction of rubella antibody positives in the age groups <35 years have been as low as 30% in tropical countries like Trinidad or Panama compared with more than 80% in Europe or the USA (20). If in the male population of the tropical countries, only 10% are positive (see below), then the female population must have had a value of about 50% to fit the reported figures (30%). These antibody data from the time before the start of immunization campaigns correspond with the different frequencies of rubella infection in the different countries. As shown in Table 4 (30), the natural infection with rubella in Cuba is very low. According to the reported cases in total only 2.0% of the total population gained antibodies by natural infection in the time range of 16 years between 1970 and 1986, with three main epidemics accounting already for 1.5%. As rubella is a mild disease, it is known to be under-reported (20), even in areas where reporting has been mandatory for years (30). With a corresponding correction (20) we can guess that about 4–5% of the population in Cuba in these 16 years gained rubella antibodies by natural infection. In the relevant time range for the MS patients in this study (1955–1978), this figure must be doubled, i.e. about 8–10%. The selective immunization of females before 1986 allows to conclude that these calculated figures are only representative of the male population in Cuba before 1986. In Germany, before the start of the immunization campaigns (1980), the frequency of rubella antibodies was 50–70% in the age group of

10–15 years increasing to 85% in the age group of 15–20 years (21). This means that in the male population in Germany, the frequency of rubella antibody positives were five- to eightfold higher than in the Cuban population. This explains the 1:6 lower frequency of the intrathecal antibody synthesis in the male Cuban MS population. So, the low frequency of intrathecally synthesized rubella antibodies in the total group of Cuban MS patients (50% of the German MS patients) is primarily a consequence of the low frequency of seropositives in the male Cuban population. Both seronegatives in the rubella analysis were men (2/8), both with oligoclonal IgG, with increased measles-AI and in one case additional with increased VZV-AI. All women (15/15) were seropositive for rubella.

These data are relevant for both groups of patients investigated as they were born and most reached adolescence before the universal immunization campaign in Cuba and Germany. The German MS patients were born before 1977 with a similar median age (36 years) at the time of CSF puncture.

From these results, we also learn that either immunization or native infection is necessary for the intrathecal polyspecific MRZ antibody response. This is another argument refuting a causative role of the virus infection in MS, in concordance with the observation that the incidence of MS did not change with the worldwide, general immunization campaigns and also with the subsequent reports: (a) the viral antigens need not to persist in the brain (15, 16) for this immune response; (b) there are autoimmune diseases with involvement of the brain which also show an MRZ reaction (5); (c) the intrathecally synthesized amount of polyspecific antibodies is very low compared with an acute infection with persistence of the causative antigen (1, 18).

However, it remains an unanswered question, why the corresponding specific MRZ antibody synthesizing B cells after their perivascular infiltration persist with such a high frequency in the CNS of these MS patients, much higher than for any other antigen investigated in MS and also higher than in any other chronic or acute inflammatory disease of the CNS (1, 5, 14, 18). As the MRZ antibodies are gained (by infection or immunization) very early in the childhood, intrathecal synthesis could be seen as a scar from this time. It seems reasonable to investigate this MRZ response in early onset MS (31). In general, the polyspecific immune response which is concomitant in all immune reactions, also in the case of acute diseases with a causative antigen (1, 17, 18, 32), has to be regarded as a consequence

of the complex immune network reactions by anti-idiotypic antibodies, cytokines or T-lymphocyte-mediated cross-reactivity (33, 34). Further research about the MRZ antibody reaction is interesting in this double sense: as part of the complex reactions of the immune system in brain and because of its particularly high frequency for the pathomechanism of MS.

The MRZ antibody analysis is not proposed to replace the usual routine analysis for diagnosis of MS (2) by the much more sensitive (96–98%) albeit unspecific oligoclonal IgG. But this MRZ antibody analysis is very helpful for the investigation of chronic inflammatory processes of the CNS to get a different rather specific information.

In the Cuban MS group, 48% of the patients had a combination of two or three increased AI values of the MRZ antibodies (Table 3) which is somewhat less than in a European population (67%). This means that, already at the time of first clinical symptoms and also if the disease is monosymptomatic like in the case of an optic neuritis, it can be stated with a high probability that it is a *chronic* inflammatory process of the autoimmune type (1, 5). Together with the quantification of the amount of the intrathecal specific antibody synthesis in CSF (17, 18), we also gain a tool for discrimination of antibody synthesis against a causative antigen, e.g. rubella in Ref. (18), and a polyspecific synthesis because of a costimulation of any B-cell clone as part of the immunological network. HSV analysis is not performed in the case of a chronic disease with a low frequency of intrathecal HSV antibodies; only if a HSV or VZV related acute disease is expected (29).

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