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IgG1,3 and 4 oligoclonal bands in multiple sclerosis and other neurological diseases

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Cerebrospinal fluid (CSF) samples from 10 patients with Multiple Sclerosis (MS) and 7 with other neurological diseases (OND) were studied in order to detect oligoclonal restriction of IgG subclasses 1,3 and 4. Agarose isoelectric focusing (AGA-IEF) followed by Western capillary blotting and immunoperoxidase staining with specific monoclonal antibodies were used. All MS samples showed oligoclonal IgG1 and 6 of them also had IgG3 or IgG4 bands. In the OND group only patients with subacute sclerosing panencephalitis (SSPE) and Guillain-Barré disease (GBD) showed CSF oligoclonal patterns for IgG subclasses. Our results demonstrate that in MS CSF other IgG subclasses beside IgG1 may display an oligoclonal pattern. The finding of more than one subclass in the same band indicates a microheterogeneous composition of these oligoclonal bands.

Key-Words: Agarose IEF - monoclonal antibodies - immunoperoxidase staining - MS - IgG subclasses - oligoclonal bands

Introduction

The detection in cerebrospinal fluid (CSF) of immunoglobulins (Ig) forming oligoclonal bands, when electrophoretically separated, is a fundamental diagnostic tool for confirming a clinical suspicion of multiple sclerosis (MS). The presence of CSF banding patterns is generally attributed to a restricted pool of Ig-producing B cell clones confined within the central nervous system (CNS) [1].
Although other Ig classes display quantitative alterations [2,3,4] and oligoclonal features [5], IgG is the most frequent [5] and extensively studied Ig-classes forming oligoclonal bands in MS CSF.
Two different studies have already looked for subclass restriction of the IgG forming oligoclonal bands in MS CSF, one using agarose electrophoresis followed by immunoprecipitation [6] and the other immunoperoxidase staining [7]. Although isoelectric focusing (IEF) gives better visualization of separated proteins, it has never been employed so far to determine whether oligoclonal activation is confined to a single IgG subclass or implies the involvement of several subclasses.
For this reason we performed a qualitative analysis of CSF IgG subclasses 1,3 and 4 in 10 MS and 7 other neurological diseases (OND) patients, using specific monoclonal antibodies after IEF on agarose gels.

Material and method

Patients and samples

Lumbar CSF were obtained from 10 patients with definite MS according to McAlpine et al [8] and 7 patients with OND, that is, 3 with Alzheimer disease, 2 with subacute sclerosing panencephalitis (SSPE), 1 with Guillain-Barré disease (GBD) and 1 with depressive psychosis. Serum from an IgG1 multiple myeloma patient was used as a control. All samples were frozen immediately after collection and stored at $-20^{\circ}C$ until use.

Agarose IEF and immunoperoxidase staining
The isoelectric analysis was performed on ultrathin (0.3 mm) agarose gels, containing 217 mg of Isogel™ Agarose-EF (LKB) and 1.69 ml of Ampholines pH 3.5-9.5 (LKB) in 21 ml of water. 20 µl of unconcentrated CSF or 10 µl of a 1.200 dilution of multiple myeloma serum in phosphate buffer saline (PBS) was absorbed on filter paper strips, which were applied to the gel surface, 1.5 cm from the anodal side. Proteins were focused at constant power for 60 min using a LKB 2117 Multiphore and an LKB 2197 Power Supply. The average final voltage was 1500 V.
Capillary blotting was then carried out on a 0.2 µm nitrocellulose sheet (Schleicher & Schuell). After 30 min the paper was cut into strips and free protein sites were blocked with 3% bovine serum albumin (BSA) in PBS.
Two different staining procedures were followed. In order to detect the whole IgG forming oligoclonal bands, the strips were incubated for 120 min with a 1:200 dilution of rabbit anti-human IgG (γ-chain) peroxidase conjugated antibody (DAKO). In the IgG subclass study, monoclonal mouse anti-human antibody specific for IgG1 (γ1 chain), IgG3 (γ3 chain) and IgG4 (γ4 chain) (Hybritech Inc.) were used. At the time of the experiment no monoclonal IgG2 specific antibody was commercially available.
Visualization was obtained by incubating the strips for 60 min with an antibody dilution of 1:1000 for IgG1, 1:200 for IgG3 and 1:100 for IgG4. After 3 washes in 3% BSA-PBS, strips were incubated for 120 min with a 1:150 dilution of goat anti-mouse IgG,M peroxidase conjugated antibody. After 3 more washes in 3% BSA-PBS, strips were stained by using a solution containing 0,3 mg-ml of 3.3 diaminobenzidine tetrahydrochloride and 0.06% hydrogen peroxide in TRIS buffer (pH 7.4).

Results

The clinical and IEF data from MS and OND patients are listed in Tables I and II. A preliminary IEF whole IgG analysis was carried out on each sample. Myeloma serum displayed three sharp IgG bands localized in a restricted area of the cathodal region. All MS CSF had from 2 to 11 IgG oligoclonal bands from pH 7.7 to pH 9.3. Among the OND patients only SSPE and GBD

TABLE I. *IEF data from MS patients*

Patients	Age (years)	Oligoclonal whole IgG bands	Oligoclonal IgG1 bands	Oligoclonal IgG3 bands	Oligoclonal IgG4 bands
1.	28	3	3	1	—
2.	31	2	2	2	—
3.	34	6	4	—	2
4.	26	3	2	—	—
5.	33	3	3	—	—
6.	26	4	2	—	—
7.	36	11	11	—	2
8.	39	7	4	3	—
9.	45	8	2	—	2
10.	30	5	3	—	—

TABLE II. *IEF data from OND patients*

Patients	Diagnosis	Age (years)	Oligoclonal whole IgG bands	Oligoclonal IgG1 bands	Oligoclonal IgG3 bands	Oligoclonal IgG4 bands
1.	Myeloma	63	3	3	—	—
2.	SSPE	14	7	4	1	2
3.	SSPE	11	4	3	—	—
4.	GBD	40	6	3	—	1
5.	Depression	39	—	—	—	—
6.	Alzheimer	67	—	—	—	—
7.	Alzheimer	58	—	—	—	—
8.	Alzheimer	62	—	—	—	—

CSF showed IgG bands.
IgG subclasses were then analyzed in all samples (Fig. 1). Each subclass focuses in a specific pH range, namely from 7.7 to 9.3 for IgG1 (Fig. 2), from 7.4 to 9.2 for IgG3 (Fig. 3), and from 6.9 to 8.3 for IgG4 (Fig. 4). (We decided to consider as oligoclonal even single bands as long as they contrasted sharply against the background staining).
IgG bands from myeloma serum were confirmed as exclusively formed by IgG1. All 10 MS CSF demonstrated oligoclonal bands specific for IgG1; 3 had IgG3 and 3 had IgG4 bands. In all these samples IgG1 bands were always more numerous then the other subclass bands and also showed a more intense staining. Moreover each subclass displayed oligoclonal patterns differing from other subclasses and from patient to patient. None of these MS patients showed at the same time oligoclonality for all the IgG subclasses studied: in 6 of them, IgG1 bands were found along with IgG3 bands (3 patients) or IgG4 bands (3 patients), while in the remaining 4 patientsIgG1 was the only oligoclonal subclass to be found.
In 2 patients with SSPE and one with GBD - i.e. the only two inflammatory diseases included in the OND group - IgG subclass oligoclonal bands were also detected: one of the 2 SSPE patients had 4 IgG1, one IgG3 and 2 IgG4 bands, while the other one had only 3 IgG1 bands. In the single GBD CSF analyzed, 3 evident IgG1 bands were found along with one IgG4 specifif band.
The IgG4 staining revealed, in all the MS, SSPE, and GBD samples, a recurrent pattern made up of 4 broad bands in the pH range 7.3-7. 9(Fig. 4). This feature, owing to its constant occurrence and low variability, was considered aspecific, while 4

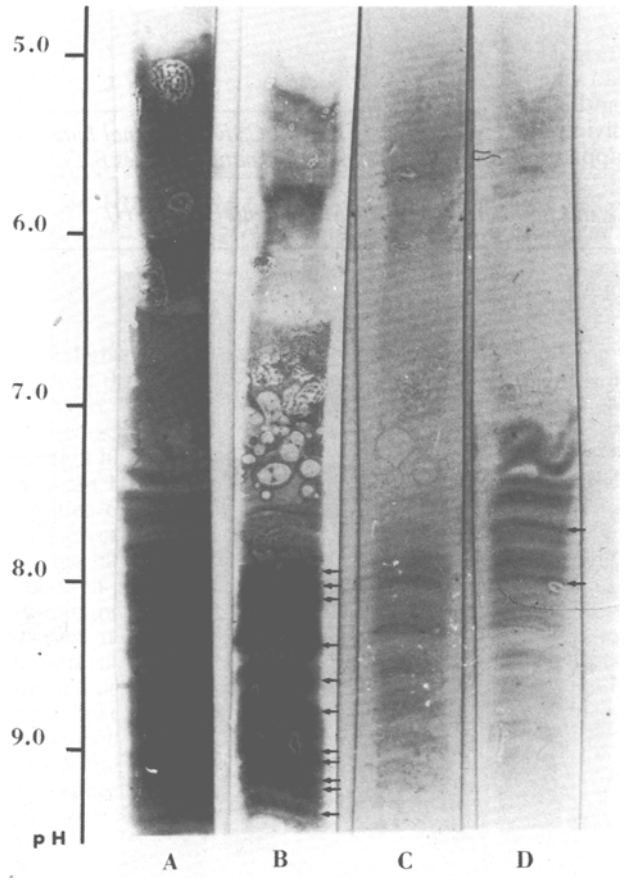


Fig. 1. *AGA-IEF distribution of whole IgG (A), IgG1 (B), IgG3 (C), and IgG4 (D), in the CSF of a MS patient: only IgG1 and IgG4 show oligoclonal bands (indicated by arrows).*

of these patients showed IgG4 bands (Fig. 4, lane A,C,D,F) that matched our criteria of oligoclonality.

Discussion

The first aim of our study was to define whether one or more of the IgG subclasses were involved in the formation of the oligoclonal bands in MS CSF. Our results show that all 10 MS patients had IgG1 oligoclonal bands and 6 of them had IgG3 or IgG4 bands. Individual IgG1 bands always matched oligoclonal bands recognized by a whole IgG antibody. IgG3 and IgG4 bands were found more anodally than the pH region (8.0-9.5) where oligoclonal bands are usually observed [9]. They matched bands that could be overlooked when detected by a whole IgG specific staining. In fact this technique fails to resolve minimal amount of oligoclonal IgG subclasses from an intense background of multiclonal IgG.
The IgG subclass specific staining allowed us to recognize in 4 MS and one SSPE patients (Fig. 5) single IgG bands composed of more than one subclass. Metha et al [10] employing IEF and immunofixation claimed that many of the individual bands in MS CSF are formed by either kappa or

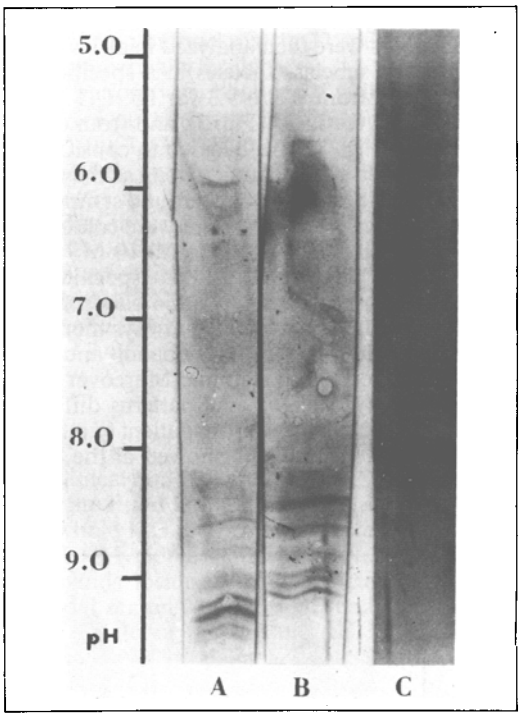
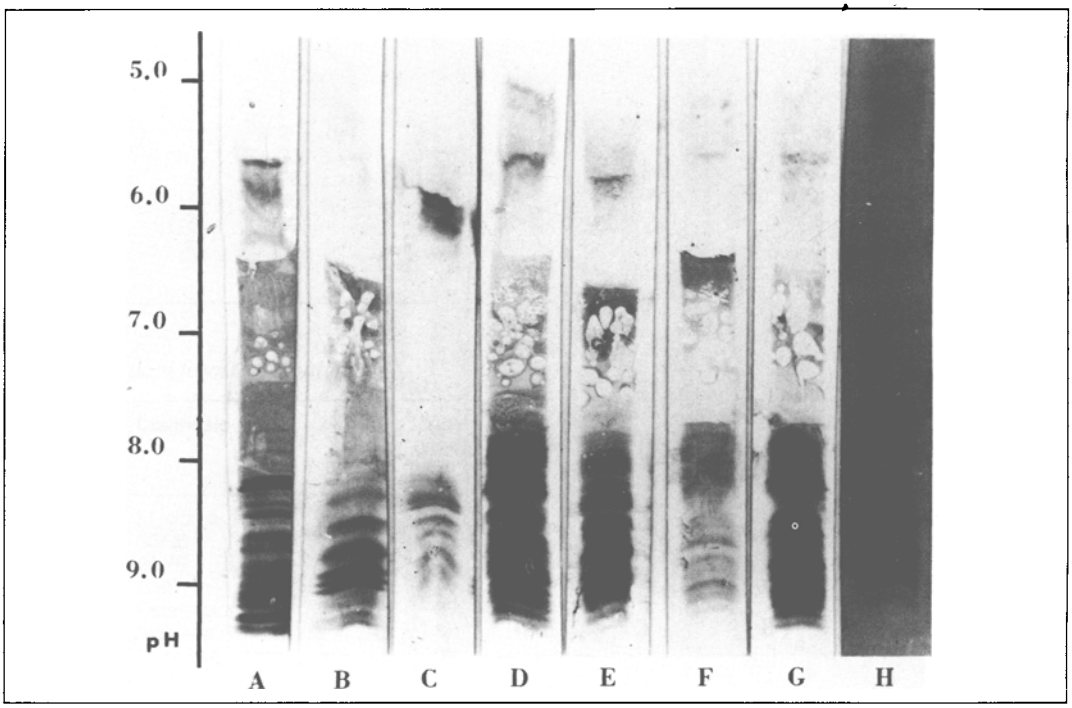


Fig. 3. *AGA-IEF IgG3 CSF oligoclonal bands in 1 SSPE (lane a) and 2 MS patients (lanes B,C).*

Fig. 2. *AGA-IEF IgG1 CSF oligoclonal bands in 1 SSPE (lane A) and 7 MS patients (lanes B-H).*



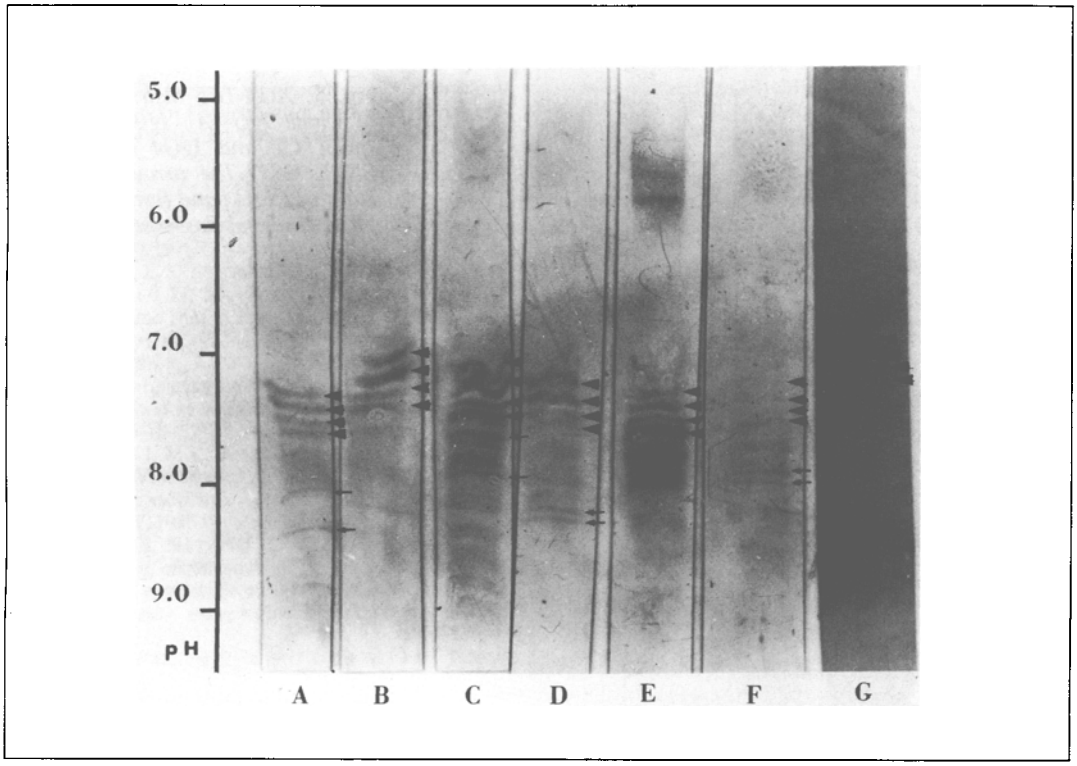


Fig. 4. *AGA-IEF of CSF IgG4 in 1 SSPE (lane A) and 6 MS patients (lanes B-G). IgG4 oligoclonal bands (arrows) and aspecific IgG4 banding patterns (arrow heads) are indicated.*

lambda light chain type, thus suggesting a monoclonality of those IgG bands. Laurenzi [11], on the other hand, found that several IgG bands were simultaneously composed of both light chain types. Further, it has been proved that single oligoclonal bands can be formed by antibodies directed to more than one antigen (12) showing the contemporary presence of different immunoglobulins in the same band. The detection of more than one subclass in a single band is an additional proof that immunoglobulins, although differing in subclass type and/or antigen specificity, may share a common isoelectric point. We think that this evidence denotes, at least in some cases, a microheterogeneity of single IEF IgG bands rather than a true monoclonality.

A few authors have studied CSF IgG subclasses in MS either for quantitative purposes or for evaluation of their electrophoretic features. Vandvik et al [6] found that 9 out of 16 MS CSF, when electrophoretically studied, showed precipitate arcs of oligoclonal type exclusively formed by IgG1. Eickoff et al [13], using RIA, gave a further demonstration that in MS CSF the IgG response is mainly represented by IgG, with a marked increase of its absolute and relative values over control CSF. More recently, Keir et al [7] by virtue of electrophoresis followed by immunofixation, revealed in MS CSF oligoclonal patterns specific for other IgG subclasses, although IgG1 was always the most evident one.

Our data, obtained by using IEF and monoclonal antibodies, support these later findings. Moreover, our separation procedure provides optimal visualization of and selective information on immunoglobulin bands that could not be achieved by traditional electrophoretic methods.

Although we are not able to evaluate yet the real incidence of each subclass in the formation of IgG bands, we can confirm that other subclasses beside IgG1 are oligoclonal in MS. The relatively low incidence of IgG3 and IgG4 bands probably reflects their low CSF levels and/or the still insufficient sensitivity of our detection method. Only the coupling of quantitative and qualitative studies of CSF IgG subclasses, including the obtaining of data on IgG2, will satisfactorily define this aspect of the immune response in MS.

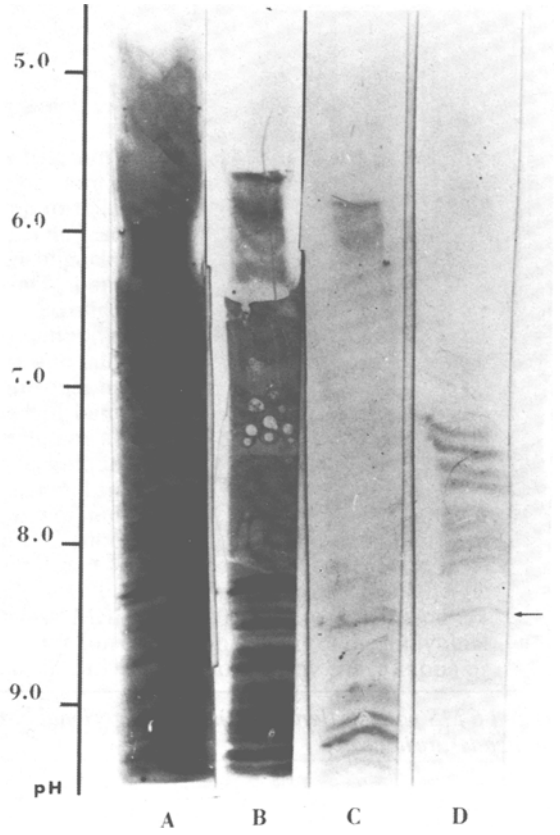


Fig. 5. *AGA-IEF distribution of whole IgG (A), IgG1 (B), IgG3(C), and IgG4 (D) in SSPE CSF. The arrow indicates one IgG band formed by all the three subclasses studied.*

Sommario

Allo scopo di rilevare un'eventuale restrizione oligoclonale per le sottoclassi IgG1,3 e 4 sono stati studiati campioni di liquido cerebrospinale (LCS) ottenuti da 10 pazienti con Sclerosi Multipla (SM) definita e da 7 pazienti con altre malattie neurologiche (AMN). La metodica utilizzata è stata l'isoelectric focusing in agarosio seguita da Western capillary blotting su nitrocellulosa e colorazione con immunoperossidasi mediante anticorpi monoclonali. Tutti i campioni di SM mostrarono IgG1 oligoclonali e sei di essi presentarono contemporaneamente bande di IgG3 o di IgG4. Tra le AMN solo i pazienti con Panencefalite Sclerosante Subacuta e sindrome di Guillain-Barré mostrarono bande oligoclonali formate da sottoclassi di IgG. I nostri dati dimostrano che nel LCS di SM altre sottoclassi di IgG, oltre alle IgG1, possono essere oligoclonali. La presenza di più di una sottoclasse nella stessa banda depone per una microeterogeneità di queste bande oligoclonali.

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