TEMPORAL INVARIANCE AND CLONAL UNIFORMITY OF BRAIN AND CEREBROSPINAL IgG, IgA, AND IgM IN MULTIPLE SCLEROSIS

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Multiple sclerosis (MS)[1] is associated with Ig synthesis inside the blood brain barrier (BBB). The Ig is usually IgG1 κ, and is allotype- and idiotype-restricted (reviewed in 1 and 2). Two-dimensional electrophoresis (2DE) (3) of the L chains of cerebrospinal fluid (CSF) IgG, IgA, and IgM, as well as of IgA and IgM H chains, has established that, in many MS patients, the immune response is truly paucicIonal; the IgG response that is quantitatively dominant commonly expresses <20 major L chains, but there are instances where most of the L chain protein in MS CSF is comprised of one or a few major L chains (4). In some central nervous system disorders, high levels of specific antibody are produced. In subacute sclerosing panencephalitis (SSPE), as much as 80% of the total intra-BBB–produced IgG may be viral-specific (5), and most or all the oligoclonal IgG bands react with viral antigen (6). The Ig-synthesized intra-BBB in MS may also be antigen specific (7). Furthermore, persistent multifocal and multiphasic inflammation in the MS brain may indicate persistent antigen. The restrictions of MS CSF Ig are also considered indicative of a specific immune response (2). An alternative hypothesis, based on IEF spectrotype differences in IgG eluted from different regions of a single MS brain, asserts that IgG production in MS is nonsense antibody (8). Nonsense antibody proposals have been influential in hypotheses implicating MS as a disorder of immunoregulation (9, 10) and are made more credible by the failure to identify major antigen targets for the immune response in MS. Whether MS CSF IgG is a nonnsense or specific antibody is pivotal in MS research, since intra-BBB–synthesized IgG, if antigen specific, may constitute the powerful probe required to isolate and identify antigen(s) relevant to MS.

IEF has limitations as a procedure for the determination of the clonal complexity of immune responses. First, monoclonal Ig may fractionate into multiple bands by IEF (11). Second, a single band may be clonally heterogenous and represent structurally diverse Ig molecules that cofocus because of shared pI

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[1] *Abbreviations used in this paper:* BBR, blood brain barrier; CSF, cerebrospinal fluid; 2DE, two-dimensional electrophoresis; MS, multiple sclerosis; PAS, protein A–Sepharose; SSPE, subacute sclerosing panencephalitis.

(12). Third, human IgG, and particularly MS CSF IgG is neutral-to-basic in its range of pI, and the resolving power of IEF for basic proteins is limited. Another series of problems arises when IEF spectrotype changes are attributed to changes in Ig clonal expression; IEF spectrotype differences may be the result of a loss of particular B cell clones or the emergence of new clones, or Ig may be stable at a clonal level while differences in IEF mobility could result from posttranslational protein modification, including deamidation, differential glycosylation, or proteolysis. 2DE has recently (4, 13, 14) been applied to the analysis of Ig from several species. This method is suitable for the analysis of restricted immune responses and is uniquely useful for L chain analysis, since pI gradients that encompass the entire pI range of human L chains are obtainable. Also, L chains demonstrate considerable M_r heterogeneity by 2DE, further enhancing the ability to resolve structurally disparate L chains. Because of the unique M_r and pI mobilities of H chains of IgM, IgA, and IgG subclasses, Ig H chains are also readily resolved from each other (14). By immunoblotting, 2DE spots representing proteolytic fragments of Ig can also be identified (4). For these reasons, we supplement IEF analysis with 2DE and show here that the humoral, intra-BBB immune response in MS, at a clonal level, is profoundly stable over long periods. Also, we show that IgG, IgA, and IgM isolated from different regions of MS brains and matched CSF are similar. These results conflict with nonsense antibody proposals but are consistent with allotypic (15) and idiotypic (16–18) analyses, and indicate extraordinary molecular uniformity and temporal persistence of the Ig response in MS.

Materials and Methods

Patients. CSF from normal persons and patients with MS, and normal and MS brain tissue were obtained from the National Neurological Research Bank, Los Angeles, CA. Tissues and body fluids were stored at −70°C, and in most cases were thawed once before analysis. Patients were chosen for analysis on the basis of availability of CSF (and sera) from individual patients over long intervals of disease, and included patients in various stages of disease with varying severity of disability; the CSF IgG concentrations ranged from 2.0 to 25 mg/dl.

Isoelectric Focusing. Native IEF of IgG and IgA was performed using polyacrylamide-IEF gels (LKB Instruments, Inc., Gaithersburg, MD) encompassing the pI range 3–9.5. An interelectrode distance of 13 cm was chosen for IgG analysis, and 11 cm for analysis of IgA. IEF was carried out for 2,400 V·h for IgG and 3,600 V·h for IgA. After completion of IEF, the electrode wicks were removed and the portions of the gels where IgG and IgA migrated were overlayed with cellulose acetate strips (Sepraphor) containing rabbit anti–human IgG H chain or anti–human IgA H chain serum (Dako Corp., Santa Barbara, CA) at a 1:10 dilution in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. The overlay was performed on the cooling plate of the electrophoresis chamber thermostatically set to 4°C. After 2 h, the cellulose acetate strips were removed and the gel was washed in a total volume of 6 liters of 150 mM NaCl, with several changes over 24–48 h to remove non-Ig protein. The gel was subsequently processed for silver staining using the procedure of Oakley et al. (19). This method permits the detection of IgG and IgA in CSF without prior CSF concentration (20). Slab gels with the same ingredients as the tube gels used for 2DE in the O'Farrell procedure (3), were used to analyze IgG L chains and IgA and IgM H chains. After IEF, the proteins were transferred from the IEF slab gels to nitrocellulose using the procedure of Bowen et al. (21) as described (4). Specific L and H chains were then detected by the peroxidase procedure.

Purification of CSF, Serum, and Brain IgG, IgA, and IgM. This was done exactly as

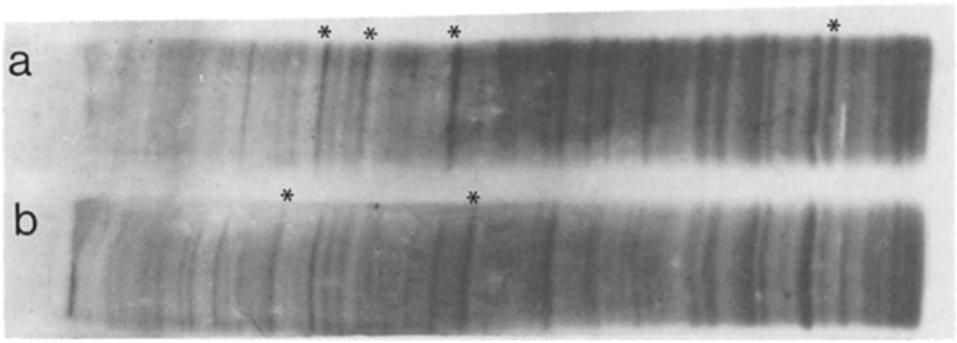

FIGURE 1. IEF gels of CSF obtained from one patient with MS. CSF were obtained in 1974 and 1982 (*a* and *b*, respectively). The procedure of IEF and immunofixation with antibody to human IgG H chains followed by silver staining is described in the text. Location of bands (*) that are more prominent in lane *a* or *b*.

described (4). Brain Ig were isolated using a modification of the procedure of Hall and Choppin (22) for the isolation of measles viral polypeptides from SSPE brain; briefly, tissue was homogenized in 50 mM Tris, 150 mM NaCl buffer, pH 7.5, containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, with 5 mM EDTA, 1 mM EGTA, 4 mM PMSF, 5 mM iodoacetamide, and 20 μM pepstatin A added to inhibit proteolysis. The supernatants from a high speed centrifugation of the brain homogenate were incubated with 120 μl of a 10% suspension of protein A–Sepharose (PAS) for 12 h on a rotator. All procedures were carried out at 0–4°C. The PAS beads were washed extensively with large volumes of solubilization buffer and the bound proteins were eluted with SDS buffer (3) containing 2% DTT; 2DE was carried out as described (3, 4).

Results

IEF of IgG and IgA. Native IEF of IgG was performed on serial CSF obtained from 19 patients with clinically definite MS. The intervals over which serial CSF were available from individual patients ranged from 7 to 12 yr (mean of 8 yr). In most of these 19 patients, the native IgG IEF spectrotype was similar or identical when the first and last available specimens were compared for pattern of IgG bands. As shown in Fig. 1, *a* and *b*, however, there were some changes in spectrotype, although the changes usually represented changes in band intensity in serial analysis rather than loss of major bands or acquisition of major new bands. In some of the 19 MS patients there were considerable temporal changes in CSF IgG levels and in levels of intra-BBB IgG synthesis (7). These changes were not reflected in changes in the IgG banding pattern by IEF, nor did changes seem more prominent in patients with the greatest quantitative changes in CSF IgG. Some of the 19 patients had received chemotherapies that caused a reduction or normalization in MS CSF IgG levels, as reported elsewhere (2). These variations in CSF IgG were not reflected in major spectrotype alterations when equal amounts of IgG protein were applied to the IEF gels. To further analyze the IgG response intra-BBB, CSF IgG was isolated using PAS affinity chromatography; the IgG was denatured and reduced using lysis buffer (3) and separated on slab gels; the constituent L chains were then identified, after blotting to nitrocellulose, using the peroxidase technique. It was shown that the stability of the intact IgG spectrotype is parallelled by stability at the level of the constituent

L chains. This form of CSF IgG analysis was performed on 6 of the 19 patients. Unlike IgM, IgA readily enters the matrix of polyacrylamide-IEF gels (LKB Instruments, Inc.); therefore, the procedure used to analyze native IgA was identical to that used for IgG analysis except that the CSF sample was applied 2 cm from the catholyte and the IEF was extended to 3,600 V·h. The IgA fractionated into ~12 discrete bands; the spectrotypes were constant in five of the six MS patients studied except for minor changes in band intensity (data not shown). For native IgA analysis, only the earliest and latest available CSF samples were studied; for IgG spectrotype analysis, yearly CSF, when available from individual patients, were studied. Five MS patients had CSF spectrotypes compared yearly over as many as 11 yr. Finally, IgA and IgM were isolated by affinity chromatography, and the constituent H chains were separated according to the range of pI on slab gels containing 9.4 M urea. Separate immune precipitations and slab gel IEF were performed for each Ig class. The patterns of IgA and IgM H chains were stable in all five patients studied serially by this technique (data not shown).

2DE of Ig Isolated from MS CSF. Fig. 2 shows the results of analysis of CSF L chains by 2DE from the same MS patient shown in Fig. 1. At least 60 spots can be detected in the original gel. Although there are changes in intensity of some spots in these gels, in this patient, an additional patient (Fig. 2, *c* and *d*), and in seven other patients studied serially, almost the entire L chain complex of spots remained essentially identical or very similar. By visual inspection, at least 70% of the major spots were not altered even in patients studied over >10 yr. In addition to spots on 2DE gels corresponding to intact H and L chains, spots of lower M_r (~35,000–40,000 and ~33,000) reactive with anti-H chain antisera were usually detected (4). These spots were at times very prominent and, since they were seen on 2DE gels of Ig purified by PAS, they probably contain at least the Fc portions of IgG required for binding to protein A. These fragments were detected even when CSF was denatured in SDS without prior Ig isolation, and their intensity was not reduced with the use of proteolytic inhibitors, suggesting that Ig fragmentation occurred in vivo (4). Using the same procedure used for IgG, we purified IgA and IgM from CSF using immunoabsorbents. Serial CSF from three patients shown to have intra-BBB IgA and IgM synthesis (4) were studied this way. As with IgG, the IgA and IgM H chains were fairly similar over time (Fig. 3).

2DE of Ig Isolated from Brain. Finally, IgG was isolated from matched CSF, sera, and different regions of autologous MS brains. A total of 11 regions from three MS brains were studied; in two cases, the autologous CSF and sera were also available for analysis and, in the third patient, only the autologuous serum was studied. We used a solubilization procedure (22) efficient at extracting free IgG, IgG fragments, and bound IgG, including immune complexes, from the brain. Figs. 4 and 5 show the results of 2DE analysis of the purified Ig from one MS patient. First, IgG and IgM H chain degradation fragments were prominent (data not shown). Second, there were dramatic differences, in both the L chain and in the μ chain region, between serum and both autologous CSF and brain-extracted Ig, further evidence for a clonally unique immune response intra-BBB in MS (Figs. 4 and 5). Third, the L chain pattern of CSF IgG obtained 5 yr

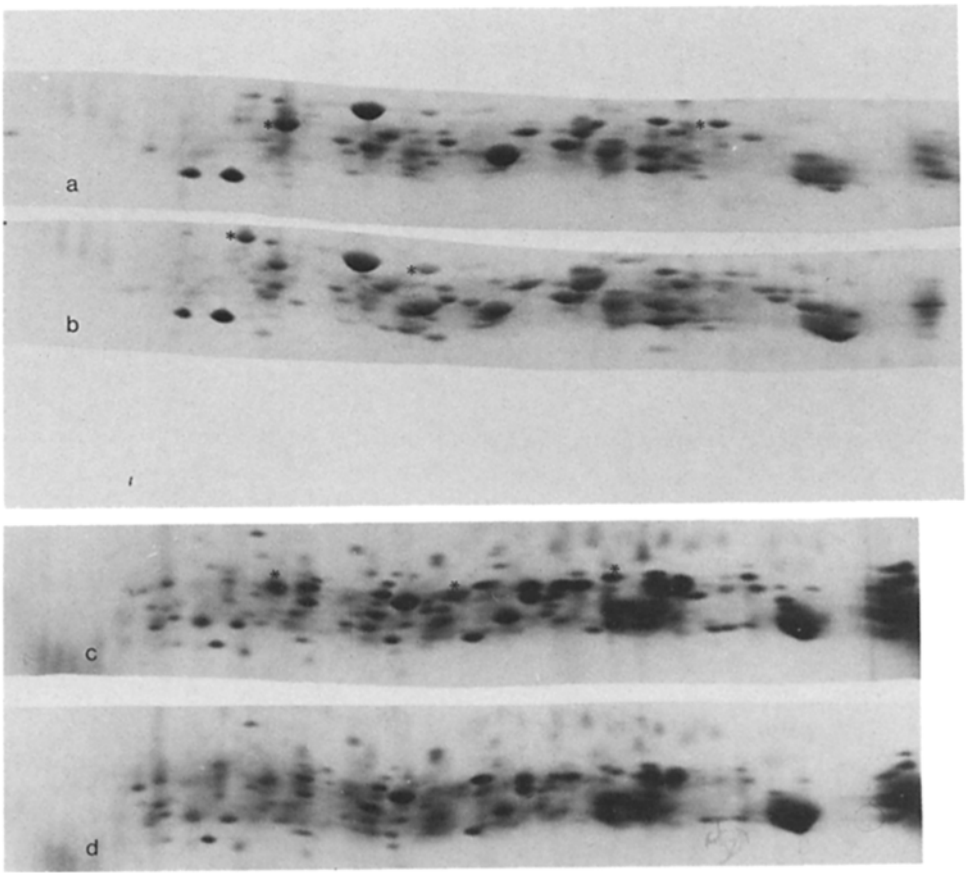

FIGURE 2. 2DE of PAS-isolated CSF Ig from the same patient shown in Fig. 1. The L chain region is shown. The patterns are nearly identical. Some spots differing in intensity between CSF *a* and *b* are indicated (*). Specimens *a* and *b* were obtained from this patient in 1974 and 1982, respectively. 2DE of IgG L chains from an MS patient (*c* and *d*). Spots that differed in intensity between *c* and *d*, and unique spots, are indicated (*). Specimens *c* and *d* were obtained from this patient in 1975 and 1981, respectively.

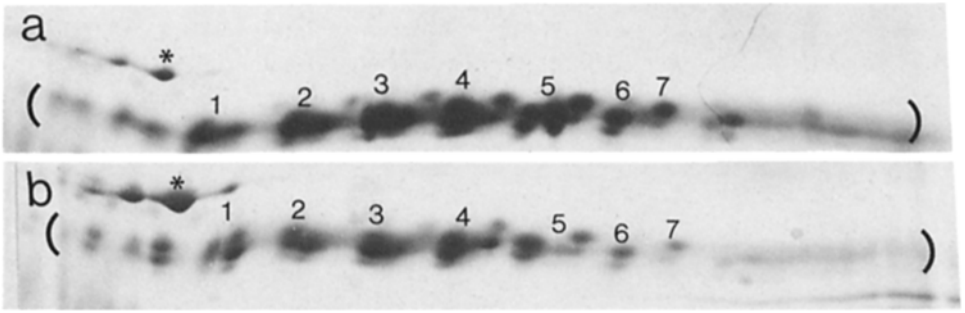

FIGURE 3. 2DE of IgM μ chains isolated from two samples of CSF from a single patient with MS, obtained 9 yr apart. A spot that is probably a component of the complement system (4) and is prominent in 2DE gels of purified MS CSF Ig, particularly IgM, is shown (*).

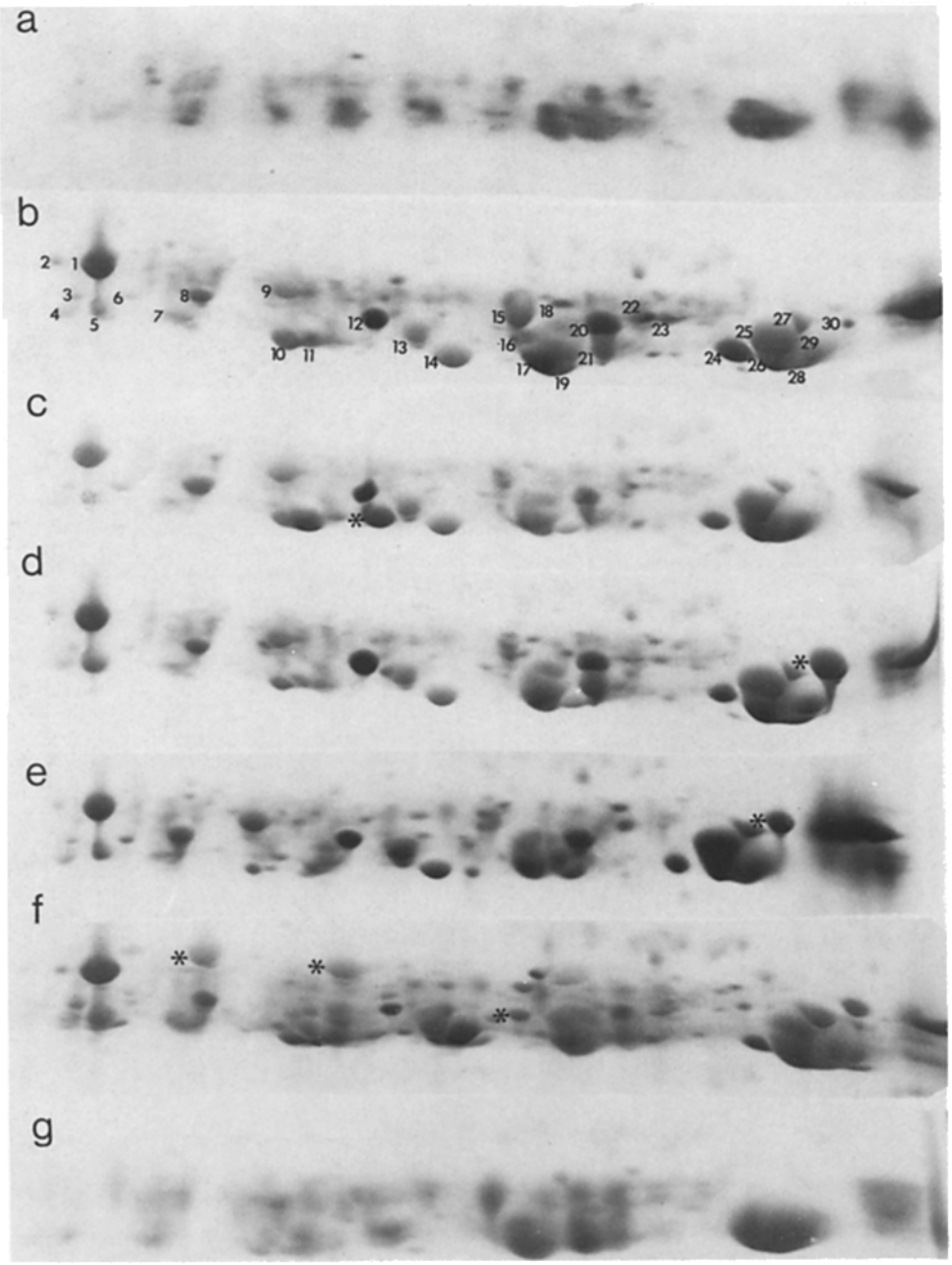

FIGURE 4. 2DE of Ig isolated from control brain (*a*), Ig isolated from four regions of an MS brain (two sections from each cerebral hemisphere containing one or several plaques of demyelination) (*b–e*), and of autologous CSF (*f*) and serum (*g*). CSF and serum Ig was isolated as described (4). For Ig isolation from brain, tissue was solubilized by a modification of the procedure of Hall and Choppin (22) as described in the text. ~3 g of tissue from each brain region was used and the Ig was isolated using 120 μl PAS for each affinity purification. Only the L chain regions of the 2DE gels are shown. The L chain region in *b* was chosen as prototype for comparison with the L chains of CSF and the other three brain regions from which IgG was isolated (*c–e*). The numbers in *b* indicate prominent L chain spots. The asterisks in *c–f* indicate spots that differ substantially in intensity from the prototype (*b*). The CSF L chain complex shown in *f* is CSF obtained from this patient 5 yr before death. Autolysis time for the control brain (*a*) was 18 h, and the autolysis time for the MS brain (*c–e*) was 12 h.

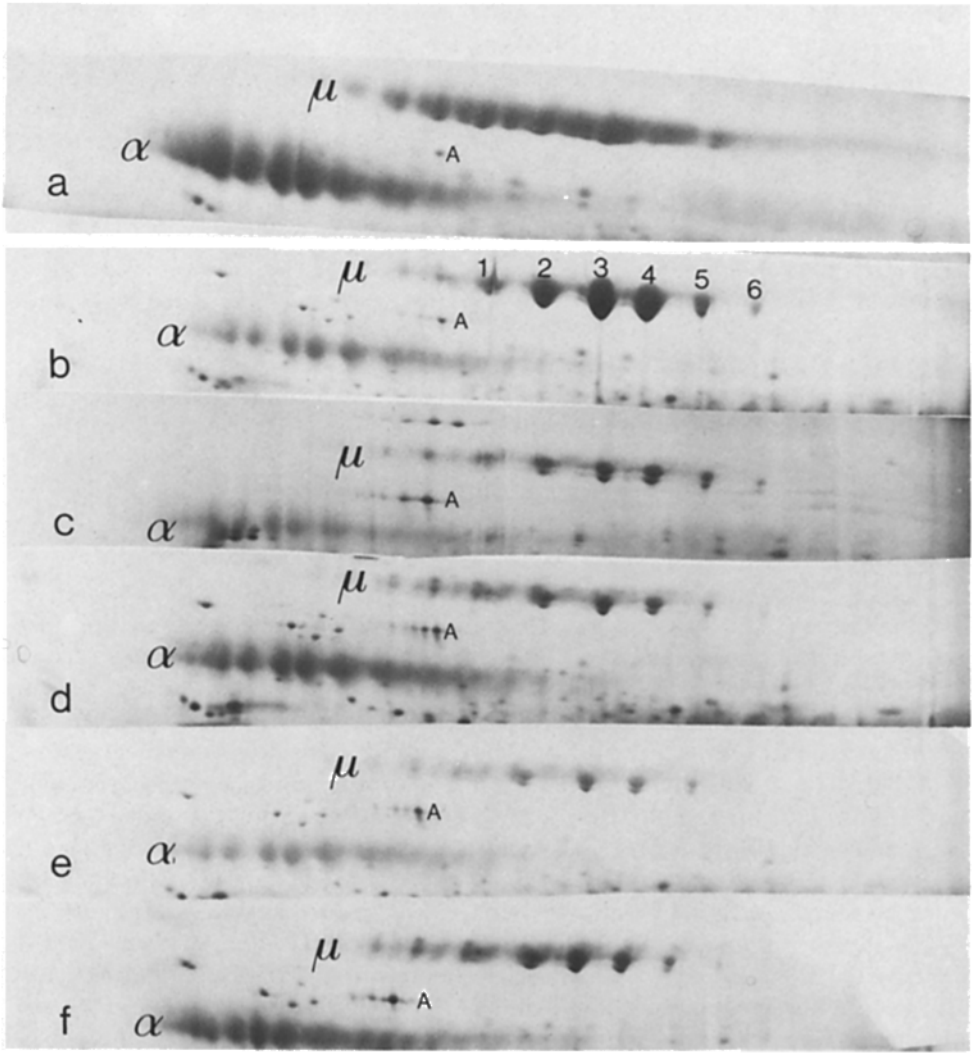

FIGURE 5. μ chain and α chain region of the 2DE gels (*a–f*), the L chain portions of which are shown in Fig. 4. The location of the IgM and IgA H chains was established by nitrocellulose blotting (21). *a* and *f* are, respectively, the matched serum and CSF; (*b–e*) are the μ and α chain portions of the 2DE gels of Ig isolated from four regions of autologous brain; (*b*) prototype for comparison of the spots in the μ chain region (numbered 1–6) in the brain and CSF extracts compared with serum. Despite the differences in intensity, the major μ chain and α chain spots are identically located in *b–f*. The μ chain region is remarkably different in *b–f* compared with autologous serum μ chains.

before the patient's death and that of IgG extracted from four regions of autologous brain, are similar, reinforcing the view that oligoclonal CSF IgG derives from in situ synthesis by plasma cells in brain, which then diffuses through the extracellular spaces of the brain into CSF (2, 7). The greater complexity of the brain L chains compared with autologous CSF, and of both compared with most other patients with MS, may be due to the contamination of the brain-

extracted IgG with serum IgG derived from cerebral blood vessels, as well as to IgG present in situ due to focal breaks in the BBB in this patient. Fourth, the μ chain complex of CSF and brain is identical except for quantitative differences, and these discrete spots in the μ chain region contrast with the normal dispersed pI pattern of the autologous serum μ chains (Fig. 5). In this patient, no differences were seen in the IgA H chains of CSF and brain compared with those of serum, although appreciable numbers of IgA-positive plasma cells have been identified by immunocytochemistry in the MS brain (23) and specific IgA intra-BBB responses are sometimes found in MS by 2DE (4). A similar 2DE pattern was observed in the two other MS patients studied in this manner. Finally, because it has been reported (8) that IEF patterns of IgG isolated from different regions of SSPE brain are more similar to each other than those found in MS, we also eluted Ig from three regions of one SSPE brain. A pattern of stability comparable to that obtained in the MS brain elution study was seen, although differences in the surfaces area occupied by particular spots and in the stain intensity of some spots are prominent (Fig. 6), as illustrated for some L chain spots of one MS patient (Fig. 4).

Discussion

The sensitive analytical procedures used in this study collectively establish the temporal persistence of clonally homogenous Ig in MS CSF in patients studied longitudinally, and establish a relatively uniform distribution of homogenous Ig populations throughout individual MS brains. 2DE is the most vivid method to demonstrate this uniformity and temporal stability, since discrete silver-stained L and H chain spots are defined not only by pI and M_r but by contour and color, and are easily compared in different 2DE gels. One important advantage of 2DE compared with IEF is its ability to identify spurious pI shifts of homogenous Ig that could result from posttranslational protein modifications. Allotype- and idiotype-specific antisera can also be used to measure the presence and persistence of structurally unique Ig populations in body fluids and tissues. Measurement of allotype levels has indicated (15) a relatively uniform distribution of specific IgG allotypes in different regions of individual MS brains. Similarly, idiotype analyses, although failing to show significant crossidiotypy among IgG obtained from different MS patients, indicate (16) a relatively uniform distribution of specific idiotypes in different regions of individual MS brains. Idiotype analyses also indicate that IgG populations defined by a variety of antiidiotypic reagents persist over long periods in MS CSF despite flutuations over time (16–18). It is not surprising that there are some regional spectrotype differences, however, since there are focal breaks in the BBB in MS that contaminate intra-BBB–synthesized IgG with serum IgG, as well as regional differences in T cell populations (24, 25) that may modulate Ig synthesis by some B cells. Furthermore, when spectrotype differences occur as a result of changes in Ig expression at the clonal level, such changes should not be construed as indicative of change in antigenic specificity, since it has previously been shown (12, 26), for example, that the antigen-specific SSPE CSF spectrotype is variable in sequential analysis of individual patients. Such changes may merely be a manifestation of factors such as cell selection by antigen (27), maturation of the immune response (27), cyclic

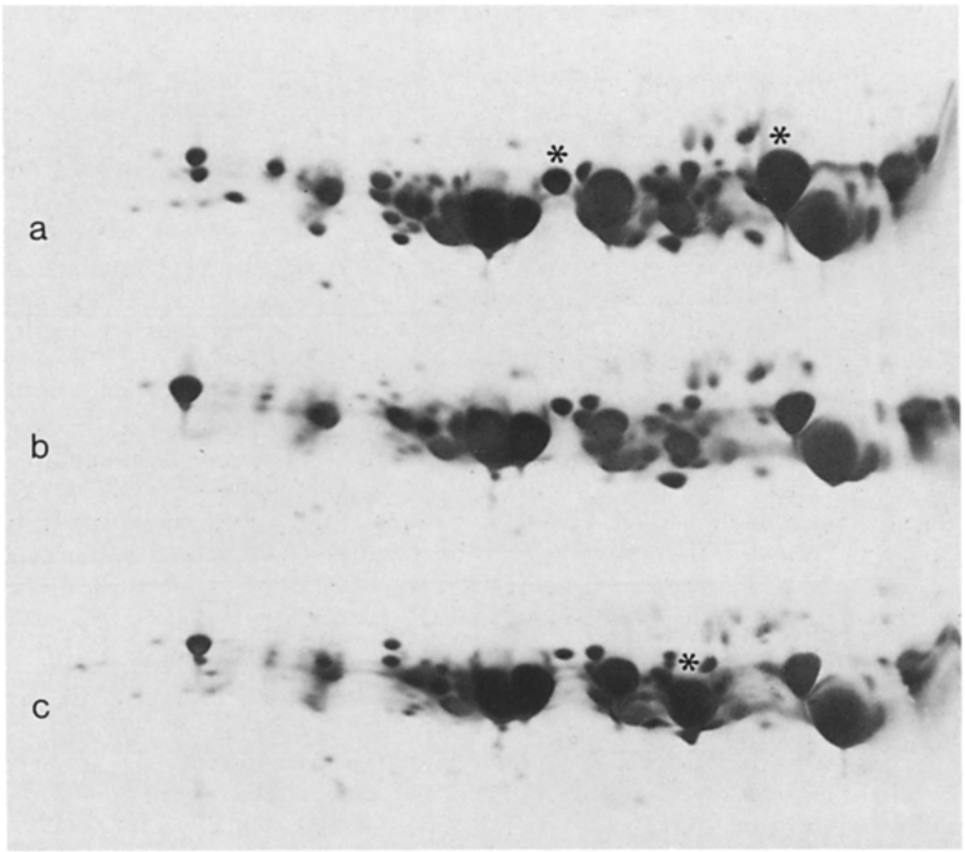

FIGURE 6. L chain regions of 2DE gels (*a–c*) of Ig isolated from three regions of one SSPE brain; the principal spots correspond in *a–c*, but there are differences in intensity of some spots (*), and some of the minor spots are region specific.

flux in levels of specific antibody production (28), or modulation of Ig synthesis by immunoregulatory T cells (24, 25). In this context, it is extraordinary that the changes we have documented are not greater. Thus, although MS CSF and brain Ig may represent nonsense antibody, this view is now strongly at variance with 2DE, idiotypic, and allotypic analysis. Furthermore, our failure to find a major antigen for intra-BBB–synthesized Ig in MS may not relate to nonsense antibody production but instead may reflect the molecular complexity of the central nervous system and the presumed antigenic target, the myelin membrane (4).

There are other implications of our results. Since MS CSF and brain IgG is pauciclonal but not monoclonal, suppression of humoral immune response using an antiidiotypic route is not feasible. However, it has never been clearly established whether MS CSF Ig are truly demyelinating or cytotoxic to the myelin membrane. In contrast, it has been established (reviewed in 29) that T cell lines are capable of causing experimental allergic encephalomyelitis when adoptively transferred into syngeneic animals; that T cells are present at early stages of lesion development, and that T cellular competence is required for the devel-

opment of the experimental disease. Recently (30), antibodies to and cDNA probes for the antigen-specific T cell receptor have become available. cDNA probes for Ig genes have already been used to characterize B cell clonal heterogeneity in lymphoproliferative disorders. Cleary et al. (31) have estimated that Southern blot analysis is capable of detecting unique B cell rearrangements in B cell populations if such unique rearrangements constitute 1% or more of the total B cell population under study. This is less than the resolving power of 2DE for clonal analysis of Ig, but is informative when B cell rearrangements do not result in the production of measurable Ig protein. If MS PBL contain relatively high levels of T cells that have undergone unique rearrangements, this should now be detectable with cDNA probes and Southern blot hybridization. Similarly, the availability of antibodies for the constant regions of the subunits of human T cell receptors should facilitate 2DE analysis of the receptor proteins after biosynthetic labeling. In this way, a clonal characterization of the immune response at the T cell level could be accomplished, to determine if immunosuppressive therapy using clonotype-specific anti-T cell antibodies is feasible in MS. T cell clonotype suppression is preferable to pan T cell suppression, since it is likely that prolonged therapy with antibodies for the entire T helper lymphocyte subset would be deleterious. Since we have shown MS CSF and brain Ig to be restricted in complexity, at least at the B cell level, the approaches that have resulted in spectacular progress in the molecular characterization of restricted mammalian immune responses (e.g., the murine anti-PC response [32]) can now be applied to MS. Furthermore, the methods described here should be useful in the analysis of other human immune responses characterized by limited diversity Ig production.

Summary

Elevated cerebrospinal fluid (CSF) IgG and oligoclonal IgG bands on electrophoresis are valuable clinical markers for B cell proliferation in the brains of patients with multiple sclerosis (MS). Using two-dimensional electrophoresis, (2DE) we have established that the humoral immune response in MS brain is characterized by finite clonal complexity for the major Ig classes. An important question is whether this immune response is clonally stable or varies with time, related to the development of new lesions and random entry of B cells into the MS brain. To investigate this, we performed serial electrophoretic studies on CSF obtained from 19 patients with MS; the intervals ranged from 7 to 12 yr, with a mean of 8 yr. These analyses included studies of IgG, IgA, and IgM, and revealed that the humoral immune response in MS is clonally stable over long periods. Spontaneous fluctuations or reduction in CSF IgG levels by drugs did not qualitatively affect B cell clonal proliferation in MS brain, in that dominant bands and spots were not obliterated. It has been asserted that IgG synthesis in MS is nonsense antibody because the spectotypes of IgG isolated from different regions of MS brains differ. Factors other than clonal heterogeneity could account for differences found using one-dimensional analysis. B cell clonal products resolve into unique and well-resolved spots by 2DE; the method is uniquely suitable for analysis of restricted immune responses. Therefore, Ig were isolated from 11 regions of three MS brains and the 2DE patterns were compared.

The similarity of the 2DE patterns indicate unequivocally that major clones are distributed uniformly although some clones are more prominent in some brain areas. IgA and IgM isolated from the same areas also showed similar patterns. Furthermore, the patterns of light and heavy chains in brain regions differed from serum but were similar to the autologous CSF, providing new evidence that CSF IgG in MS derives from synthesis in situ. Our results indicate that, once initiated, B cell clonal proliferation persists indefinitely and is little altered qualitatively at a clonal level over time, even when CSF IgG levels change or are altered by drugs. Our results are consistent with allotype and idiotype analysis of Ig production in MS and conflict with nonsense antibody proposals of the origin and nature of in situ synthesized Ig in MS.

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