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Analysis of immunoglobulin G in multiple sclerosis brain: quantitative and isoelectric focusing studies

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SUMMARY

Immunoglobulin G (IgG) in soluble and particulate fractions of discrete tissue samples from multiple sclerosis (MS) brain was analysed. Supernatant IgG/albumin ratios and particulate-bound IgG levels were highest in samples dissected from MS plaques and adjacent white matter. Acid extracts of particulate fractions from the equivalent of 1 g of MS plaque tissue contained up to 15 μg IgG, an order of magnitude less than the amount in extracts from a subacute sclerosing panencephatitis (SSPE) brain, but 20 times more than those from control brain. By contrast, supernatant fractions from SSPE brain and some MS plaques contained comparable amounts (100–200 μg/g tissue) of IgG, which were 10 times greater than those from control brain.

Samples were subjected to isoelectric focusing (IEF), and IgG was visualized by immunoperoxidase staining. The IEF patterns of IgG from control samples were diffuse but samples from demyelinated MS tissue displayed distinct oligoclonal bands of IgG. A number of common IgG bands were apparent in extracts of supernatant and particulate fractions from the same plaque. The IEF spectra of plaque samples from three MS brains were different. Furthermore, quantitative variations in certain IgG bands were observed in different plaques from the same brain.

INTRODUCTION

An oligoclonal pattern of immunoglobulin G (IgG) is observed on isoelectric focusing (IEF) of cerebrospinal fluid (CSF) from most multiple sclerosis (MS) patients (Kjellin & Vesterberg, 1974; Delmotte & Gonsette, 1977; Siden, 1977). The oligoclonal pattern is unique for each MS patient, and is stable through changes in clinical status or drug treatment (Olsson & Nilsson, 1979). There is much evidence suggesting an intrathecal site of IgG synthesis in MS (Tourtellotte & Ma, 1978; Lowenthal, 1979) and according to the calculations of Tourtellotte *et al.* (1980) the majority of the oligoclonal IgG represents the secretion of plasma cells localized in the vicinity of plaques. Such cells have been observed in sections of MS brain (Esiri, 1980; Prineas & Wright, 1978).

The antigenic specificity of the bulk of the intrathecally synthesized IgG in MS is unknown. Only a small proportion reacts with known viruses (Nordal, Vandvik & Norrby, 1978). Antibody activity to brain antigens in the CSF of MS patients has been reported (Ryberg, 1978) but it is not known what proportion of the oligoclonal IgG this reactivity represents.

Our ignorance of the aetiology of MS and of the significance of the associated oligoclonal IgG response contrasts with the relatively firm understanding of the rare neurological disease, subacute

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sclerosing panencephalitis (SSPE). This disease results from persistent infection of the central nervous system with measles virus, leading to widespread demyelination (Martin, 1979). Oligoclonal IgG bands identified as anti-measles antibody are found in the CSF of SSPE patients (Vandvik & Norrby, 1973). IgG has been eluted at acid pH from particulate fractions of SSPE brain homogenates and shown to contain anti-measles antibody (Weil *et al.*, 1979).

The aim of the present study was to quantify the amounts of IgG present in plaques and apparently normal areas of MS brain, to assess its relative distribution in soluble and particulate bound forms, and to determine whether it is oligoclonal in character.

MATERIALS AND METHODS

Clinical and pathological data. For the present biochemical studies 14 samples of macroscopically normal white matter and 31 plaques with adjacent white matter (up to 1 cm from plaque edge) were dissected from the brains of seven clinically and histologically confirmed cases of MS. Post-mortem times ranged from 11 to 48 hr with interim storage at 4°C. White matter and grey matter were taken from 11 control brains with no neurological involvement and also from three SSPE brains which were generously provided by Dr Gudrin Agnarsdottir, Hammersmith Hospital, London. Material from three MS brains was analysed in more detail and pathological data for these cases are presented in Table 1. Histological evaluations were performed by Professor Ingrid Allen and colleagues, Queens University, Belfast.

Table 1. Clinical and pathological data for MS brains analysed for oligoclonal IgG

				Histological data*		
Brain code	Sex	Age (yr)	Duration of MS (yr)	Inflammation	Astrocytosis	Demyelination
B56	♀	32	13	++	++++	++++
B57	♀	59	24	±	++	++
B66	♂	26	3	+	++++	++++

* At least 20 samples from different areas of each brain were examined. Histological scoring as follows: ±, very little; +, mild; ++, moderate; ++++, very pronounced and widespread.

Preparation of brain fractions. All steps were carried out at 4°C. Brain tissue was dissected and a representative portion of each sample was fixed in 10% formol–saline for histological evaluation of demyelination, and of astroglial and inflammatory cells. The tissue was weighed, minced finely, washed five times with 15 vol of PBS-azide (10 mM Na-phosphate, pH 7·4/150 mM NaCl/0·02% NaN_3) and homogenized in 15 vol of TEP-azide buffer (10 mM Tris–HCl, pH 7·4/1 mM ethylenediamine-tetra-acetate/0·1 mM phenylmethylsulphonyl-fluoride/0·02% NaN_3). All homogenization and resuspension procedures were carried out by repeated passage of the material through a 23-gauge syringe needle. Homogenates were filtered through nylon gauze (60 μ pore size, Henry Simon Ltd) centrifuged at 38,000 ***g*** for 60 min and the supernatants retained. Pellets were washed once by resuspension and centrifugation as above, and were finally resuspended in their original volume of TEP-azide buffer. Aliquots of supernatant and particulate fractions were stored at −30°C until assayed. Total protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Radioimmunoassays. IgG and albumin concentrations in soluble fractions were determined by solid-phase radioimmunoassay (RIA) using ^{125}I-Protein A (Langone, 1978). Protein A (Pharmacia) was labelled to a specific activity of 800–900 Ci/mmol using Na ^{125}I (Radiochemical Centre,

Amersham) and chloramine-T (Hunter & Greenwood, 1962). Free iodide was removed by chromatography on Sephadex G-25.

For both assays, PBS-azide containing 0·25% gelatin was used as the diluent and pooled normal human serum as the standard. The solid-phase reagents used were: (a) Immunobead/latex suspension, which contained rabbit gammaglobulin Immunobeads (0·1 mg/ml; Bio-Rad Labs Ltd) and latex beads (4 mg/ml; Sigma catalogue number SD-6) in diluent; (b) albumin-bead/latex suspension, which contained albumin-beads (0·13 mg beads/ml) and latex beads (4 mg/ml) in diluent. The albumin-beads were prepared by coupling human serum albumin (Sigma) to Matrex Pel 102 activated microbeads (Amicon Corp. Ltd) according to the manufacturer's instructions (under conditions yielding an incorporation of 0·1 mg albumin/mg beads).

For IgG determinations, 0·05 ml of sample was incubated with ^{125}I-Protein A (15,000 c.p.m.) in a final volume of 0·3 ml. After 18 h at 20°C, Immunobead/latex suspension (0·05 ml) was added and the incubation continued for a further 60 min. Diluent (1·5 ml) was added and assay tubes were centrifuged (1500 ***g***; 15 min). Supernatants were aspirated and radioactivity in the pellets was measured in a Wilj 2001 gamma-counter.

For albumin determinations, 0·05 ml of sample was incubated (18 hr; 20°C) with 0·1 ml albumin-bead/latex suspension and 0·1 ml rabbit anti-human albumin serum (diluted 1:10,000; Miles-Yeda Ltd). The incubation was terminated by addition of 1·5 ml diluent and centrifugation (1500 ***g***; 15 min). The supernatant was aspirated and the pellet resuspended with thorough vortex mixing in 0·5 ml ^{125}I-Protein A (30,000 c.p.m.). After 60 min at 20°C, diluent (1·5 ml) was added, the tubes were recentrifuged and pellet-bound radioactivity determinated as above.

^{125}I-Protein A-binding assay for particulate fractions. Particulate fractions (0·67 mg protein) was washed in PBS-azide by centrifugation (150,000 ***g***; 30 min; 4°C). Pellets were resuspended in 0·45 ml PBS-azide and 0·15 ml 2% BSA was added. Aliquots (0·45 ml) of the mixture were added to 0·05 ml 10 nM ^{125}I-Protein A in tubes which were then capped and rotated, end-over-end, for 18 hr at 4°C. Triplicate aliquots (0·125 ml) from each incubation were layered onto 0·2 ml 2% BSA in microfuge tubes which were centrifuged at 38,000 ***g*** for 60 min at 4°C. Supernatants were aspirated and pellet-bound radioactivity determined by gamma-counting. The data presented have been corrected for the amount of ^{125}I-Protein A binding to the tubes in the absence of particulate protein.

Extraction of IgG from particulate fractions and isoelectric focusing. Particulate fractions were washed twice by resuspension in PBS-azide (0·3–0·7 mg protein/ml) followed by centrifugation (150,000 ***g***; 30 min; 4°C). The washed pellet was resuspended to a particulate protein concentration of 1–5 mg/ml in 0·1 M glycine–HCl, pH 2·5/10 μM pepstatin (Sigma)/0·1% BSA/0·2% NaN_3 and transferred to a test-tube which was capped and rotated end-over-end for 30 min at 20°C. The mixture was centrifuged as above and the supernatant neutralized by dialysis at 4°C against PBS-azide.

Supernatant fractions and neutralized extracts from particulate fractions were concentrated by vacuum dialysis in Sartorius membranes and IgG in the concentrated samples was determined by RIA (see above). Aliquots of 25 μl of 50 μl, equivalent to 0·5 g IgG were applied to thin-layer polyacrylamide IEF gels (pH range of ampholines 3·5–9·5; LKB). Proteins were focused and transferred to a sheet of cellulose nitrate on which IgG was visualized by immunoperoxidase staining (Glynn *et al.*, submitted for publication).

RESULTS

In order to compensate for serum contamination in brain supernatant, both IgG and albumin concentrations were measured by standard radioimmunoassays. However, acid extraction of particulate fractions, followed by neutralization and radioimmunoassay of the extract, was a laborious means of assessing relative levels of IgG in these fractions, and so a rapid screening method was devised using ^{125}I-Protein A (see Methods). At 4°C, binding of 1 nM and 200 nM ^{125}I-Protein A to particulate fractions from control, SSPE and MS brain reached maximum values by 16–20 hr (data not shown). The concentration dependence of binding to representative fractions as a function of ^{125}I-Protein A concentration is illustrated in Fig. 1. A SSPE brain sample (SSPE 3)

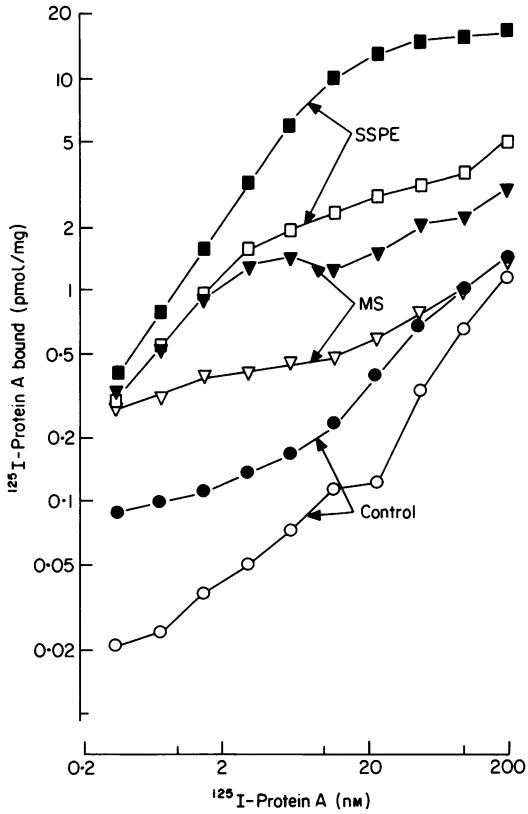

Fig. 1. Concentration dependence of ^{125}I-Protein A binding to particulate fractions from control and SSPE brains and MS plaques. Washed particulate fractions (final protein concentration, 1 mg/ml) were incubated with ^{125}I-Protein A at the concentrations shown in the figure; other conditions and the separation of bound and free ligand were as described in 'Methods'. ○, Control white matter; ●, Control grey matter; ▽, MS plaque (B55, male, 53 years old, 18 year history of MS); ▼, MS plaque (B66); □, SSPE Brain no. 1; ■, SSPE Brain no. 3.

was the only sample to show apparent saturation of binding at 200 nM Protein A, with half-maximal binding at about 8 nM; this value compares with that (5–25 nM) for the K_d of interaction of IgG with Protein A fixed to *Staphylococcus aureus* (Kronvall, Quie & Williams, 1970). The apparent lack of saturable binding in the remaining samples confirms that at high concentrations Protein A binds to material other than IgG (Vidal & Conde, 1980). At Protein A concentrations of 0·78, 12·5, and 200 nM, the ratios of ligand bound to MS plaque (▼) *vs* that bound to control white matter (○) were 22, 11, and 2·6. So as to minimize non-specific binding and to enhance differences between normal and MS brain tissue, 1 nM ^{125}I-Protein A was used in subsequent binding studies.

Relative levels of IgG in particulate fractions from seven MS and 11 control brains were determined using these conditions (Fig. 2 (a)). Mean values (pmol Protein A bound/mg protein ± SD) for plaque and adjacent white matter, normal-appearing white matter from MS brain and control brain white matter and grey matter were: 0·257 ± 0·129, 0·124 ± 0·065, and 0·063 ± 0·021, respectively. These three values were significantly different from each other ($P < 0·001$).

The ratio of concentrations of IgG and albumin in supernatants from five MS and nine control brains is shown in Fig. 2(b). Mean values (± SD) for plaque and adjacent white matter, normal-appearing white matter from MS brains and control brain white matter and grey matter were: 2·53 ± 1·66, 0·92 ± 0·38, and 0·304 ± 0·153, respectively. These three values were significantly

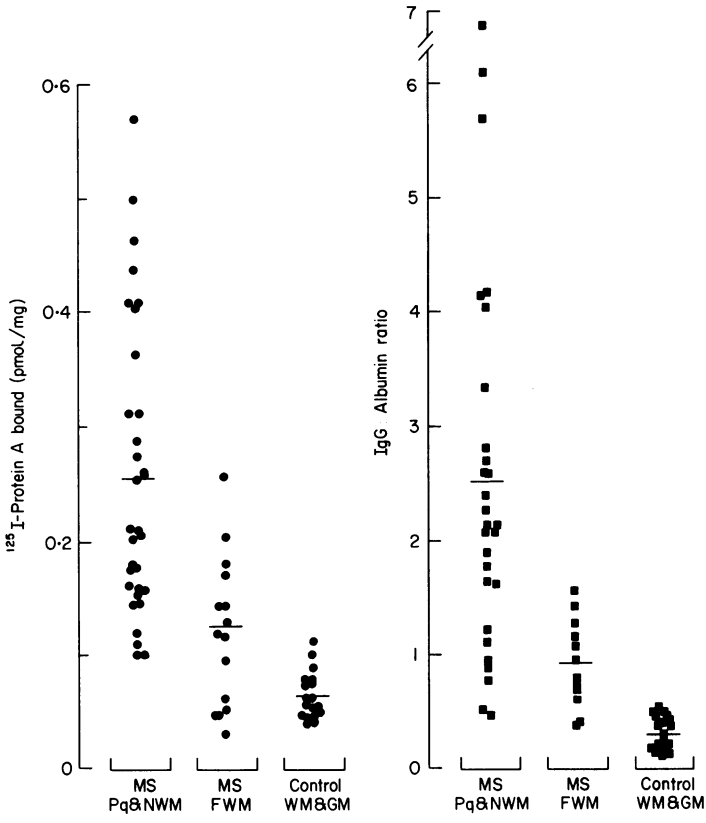

Fig. 2. Particulate and soluble IgG levels in MS brains. (a) ^{125}I-Protein A binding to particulate fractions using 1 nM ^{125}I-Protein A. (b) IgG/albumin ratio in supernatant fractions: Pq & NWM, plaque and adjacent white matter; FWM, macroscopically normal white matter; WM & GM, white matter and grey matter. Horizontal bars show mean values.

different from each other ($P < 0{\cdot}01$). In most samples there was a positive correlation between the amount of IgG in the particulate fraction and that in the supernatant. Elevations of IgG were generally greatest in samples with histologically confirmed astrocytosis and pronounced demyelination.

There was a slight difference ($P < 0{\cdot}05$) between the amount of Protein A binding to particulate fractions of white and grey matter from nine control brains (45 ± 9 and 79 ± 24 femtomol/mg, respectively) although the respective supernatants showed the same IgG/albumin ratios ($0{\cdot}29 \pm 0{\cdot}14$ and $0{\cdot}32 \pm 0{\cdot}16$). The mean value for the ratio in control brain supernatant is close to that found in normal serum (0·307, Tourtellotte, 1970). The ratios in plaque superantant fractions ($2.53 \pm 1{\cdot}66$) may be compared with those determined by identical assays for 24 control and 32 MS CSF samples which were $0{\cdot}15 \pm 0{\cdot}12$ and $0{\cdot}51 \pm 0{\cdot}44$, respectively.

Analysis of particulate and supernatant fractions of mixed white and grey matter samples dissected from three SSPE brains yielded values of 0·68, 0·88 and 1·15 pmol ^{125}I-Protein A bound/mg protein, and IgG/albumin ratios of 0·65, 4·7 and 1·65, respectively. Thus, while the supernatants of some MS plaques displayed IgG/albumin ratios comparable to those in SSPE, their particulate fractions contained substantially less IgG (see also Fig. 1 and Table 2).

IgG in one MS brain (B66) was quantified in more detail (Table 2). The yields of IgG in acid extracts of particulate fractions from these plaques were 12–40 times greater than those from control brain tissue, while that from one SSPE brain (SSPE 3) was 400 times greater than control

Table 2. Yields of IgG in supernatants and acid extracts of particulate fractions from control, MS and SSPE brains

Tissue sample	Tissue weight (g)	Supernatant IgG (μg/mg·protein)	Supernatant IgG (μg/g tissue)	Particulate IgG* (μg/mg·protein)	Particulate IgG (μg/g tissue)
Control white/grey matter	2·92	2·13	18·5	0·012	0·72
†MS pooled macroscopically normal white matter	1·42	7·9	51·3	0·037	1·55
MS white matter near ventricular plaque	0·41	26·3	196·3	0·19	7·05
MS fibrous plaque	0·35	30·5	170·0	0·52	15·6
MS ventricular plaque	0·60	9·0	71·5	0·148	5·13
SSPE brain no. 2 white/grey matter	2·24	31·6	167·4	0·54	26·0
SSPE brain no. 3 white/grey matter	2·13	15·4	147·9	5·2	128·1

* Values in this column represent the amount (μg) of IgG in neutralized acid extract derived from 1 mg of particulate protein.

† All MS samples were from B66 (see Table 1).

levels. IgG represented from 0·2% (control) to 3% of the total protein in the supernatant fraction. The amount of IgG in supernatants from MS plaque tissue was more than an order of magnitude greater than those in extracts from the corresponding particulate fractions. Differences between plaque (or SSPE) and control samples appeared less pronounced when yields were expressed in terms of original tissue weight, rather than per mg of protein, because it was not possible to homogenize the fibrous samples so thoroughly.

IEF patterns of the IgG in supernatant fractions of one control and two MS brains (B57 and B56) are shown in Fig. 3(a). Samples from control brain (a) and from macroscopically normal areas

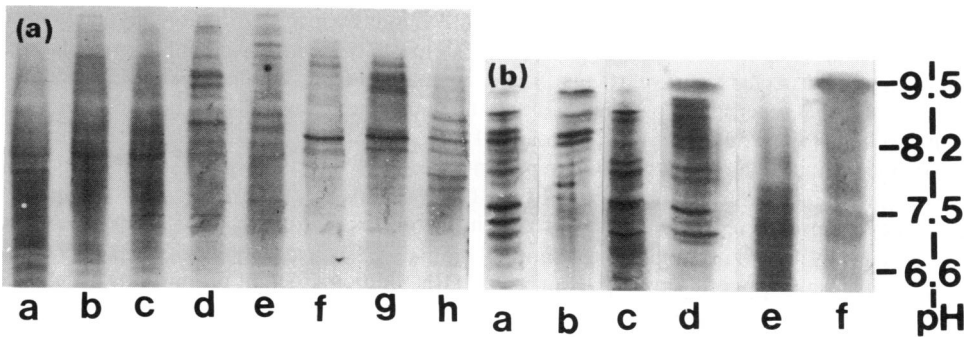

Fig. 3. IEF of IgG in MS brains. **(a)** IEF spectra of IgG in supernatant fractions: (a) Control white/grey matter; (b) B57, macroscopically normal white matter (pooled samples); (c) B57, macroscopically normal white matter (discrete samples); (d) B57, mid-brain plaque; (e) B57, frontal lobe plaque; (f) B56, frontal lobe plaque; (g) B56, mid-brain plaque; (h) B56, occipital lobe plaque. **(b)** IEF of IgG in extracts from particulate fractions and corresponding supernatants: (a) and (b) B66, ventricular plaque (in occipital lobe) supernatant and particulate extract; (c) and (d) B66 mid-brain plaque supernatant and particulate extract respectively; (e) and (f) control brain white/grey matter supernatant and particulate extract. Both Figs 3**(a)** and **(b)** show IgG distribution over pH range 6·5 (bottom) to 9·5 (top).

of MS brain (b, c) showed diffuse staining, while those from plaques and adjacent white matter displayed discrete bands.

A different oligoclonal pattern was observed for each MS brain. Moreover, within any single MS brain, plaques from different areas showed IgG spectra in which certain bands differed in intensity (compare (d) with (e), (f) with (g) with (h)). Control brain supernatant fractions reacted equally with rabbit antisera (1:500 dilution) to human kappa and lambda light chains but the discrete bands in samples from two MS brains (B55, B56) reacted only with anti-kappa serum (data not shown).

IEF was also used to study IgG in plaque samples from seven widely distributed areas of MS brain B66, two of which are shown in Fig. 3(b). Extracts from particulate fractions contained a greater proportion of IgG species with more alkaline isoelectric points than corresponding supernatant fractions, but otherwise the two fractions showed a common band pattern (compare (a) and (b); (c) and (d)). In this brain also different plaques showed quantitatively different IgG spectra.

DISCUSSION

Localized elevations of IgG near plaques in sections of MS brain have been detected by immunohistochemical methods (Simpson *et al.*, 1969; Tavolato, 1975). In the present biochemical study IgG in excess of 100 μg/g tissue has been recovered in supernatant fractions of plaque samples from MS brains with active lesions. When IgG was eluted with acidic buffer from the particulate fractions of the same tissue sample the yields were an order of magnitude less. There was, however, a positive correlation between the quantity of soluble and particulate-bound IgG in MS brain. The IgG extracted from all plaques displayed an oligoclonal pattern on IEF and corresponding supernatant and particulate extracts showed a number of common IgG bands.

Our finding of quantitative differences in the IEF spectra of IgG in different plaques from the same brain confirms the report of Mattson, Roos & Arnason (1980). These authors suggested that the different patterns could represent: (i) a variable response to the same MS antigens in each plaque, (ii) a response to different antigens in each plaque, or (iii) the product of different cell clones drawn to different plaques by a non-specific stimulus.

These three possibilities, or some combination thereof, are all feasible mechanisms for the generation of plaque-to-plaque diversity in IgG content. However, these results are based solely on the observation of molecules of differing isoelectric points which react with anti-human IgG serum and require careful interpretation. While the oligoclonal IgG in MS plaques is originally the product of plasma cells which become localized there, the location of this IgG immediately before its experimental extraction is not clear. In the three MS brains which were most fully analysed we found poor correlation between IgG levels and histologically identified inflammatory cells in the same piece of dissected tissue, particularly in some plaques from B66 in which IgG represented 2–3% of the total supernatant protein (Tables 1 and 2). Extracellular IgG has been observed in MS brain sections (Simpson *et al.*, 1969; Tavolato, 1975), and while some of this results from serum contamination, the major part probably represents the secreted products of localized plasma cells. Using immunohistochemical methods, Esiri (1980) reported IgG staining predominantly in lymphocytes around recent plaques, but also in astrocytes and axons. IgG is known to bind non-specifically to certain components of normal brain tissue such as myelin (Aarli *et al.*, 1975) and oligodendroglia (Traugott, Snyder & Raine, 1979). In addition different proportions of cell types bearing Fc-receptors, such as mononuclear and polymorphonuclear leucocytes and astrocytes, will be present in any given plaque (Tanaka, Iwasaki & Koprowski, 1975) depending on age. Thus the soluble IgG in one plaque homogenate comprises the sum of contributions from several pools, an extracellular one with extrathecal and intrathecal components and an intracellular one derived from plasma cells, astrocytes and other phagocytic cell types. Similarly, the IgG eluted from the particulate fractions represent different pools, that bound non-specifically to tissue components, or through Fc-receptors to glial and inflammatory cells, and possibly some bound to antigen, resulting in the formation of insoluble immune complexes. Thus in different plaques the potential for the binding and phagocytosis of IgG, and for its degradation by hydrolases of glial and inflammatory

cells, would be expected to vary widely. The generation of heterogeneity in immunoglobulin IEF patterns by post-synaptic modification and the problems in analysing complex IEF spectra have been discussed by Williamson (1978).

It is difficult to design *in vitro* experiments to test the above possibilities since many *in vivo* factors will influence the IEF spectra of the intrathecally produced IgG, particularly as the incubation periods may be several years. But it should be emphasized that an initially uniform humoral response within the blood–brain barrier could generate local variations in IEF spectra. Only by identifying the antigenic specificity of oligoclonal IgG can its significance be elucidated. Using the methods described here, large numbers of individual samples from MS brains can be rapidly screened to assess their IgG content both quantitatively (IgG/albumin ratios) and qualitatively (IEF spectra). In the cases where oligoclonal patterns are sufficiently similar, the IgG-containing fractions can then be pooled to analyse reactivity with various putative antigens.

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