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VIRUS-SPECIFIC IMMUNOGLOBULINS IN MULTIPLE SCLEROSIS

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SUMMARY

When the specificity and titre of IgG and IgM towards six viruses was compared by immunofluorescence in sera and CSF of patients with multiple sclerosis (MS) and control subjects, the MS specimens differed from controls in three properties.

1. A proportion, probably 22/56, of MS sera contained IgM that was weakly specific for measles virus-infected cells only. The antigen remains unidentified.
2. The titre of IgG specific for measles virus and that against herpes simplex virus is slightly increased in the serum of MS patients but the titre of IgG specific for mumps, rubella and varicella zoster is not significantly altered. The altered titre against vaccinia is of doubtful significance.
3. A proportion, 16/25, of CSF from MS patients contain measles virus-specific IgG and of these eight contained herpes virus-specific IgG. One contained herpes virus-specific IgG alone. Rubella virus IgG, of comparable titre in serum to measles- and herpes-specific IgG, was not found in the CSF.

INTRODUCTION

A slightly increased average titre of antibody in serum against measles virus was first described in multiple sclerosis (MS) by Adams & Imagawa (1962) and has been confirmed since by several workers (Brody *et al.*, 1972). However, as specific responses to viruses by different classes of immunoglobulins have not been reported in either sera or cerebrospinal fluids (CSF) from patients with MS, we decided to investigate these using the indirect immunofluorescent technique. The main advantage of this method is that all functional types of antibody are detected.

As persistent virus-specific immunoglobulin M (IgM) may be presumptive evidence of the presence of virus antigen (Haire & Hadden, 1970; Connolly, Haire & Hadden, 1971) we made a preliminary survey and found that IgM specific for measles and for mumps virus was present in a small proportion of sera of patients with MS, but anti-cellular IgM was present in many (Millar *et al.*, 1971). The purpose of the present study was to investigate

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these findings more closely using six different viruses, and to relate them, if possible, to the levels of virus-specific immunoglobulin G (IgG) in the same sera.

Relative amounts of virus-specific antibody in serum and CSF have been used to postulate local production of antibody within the central nervous system (Clarke, Dane & Dick, 1965; Connolly *et al.*, 1967; Millar, 1971), therefore the role of virus-specific IgM and IgG in this relationship was also studied in a smaller number of patients with MS and control subjects.

MATERIALS AND METHODS

Patients

The diagnosis of MS was confirmed by one of us (J.H.D.M.).

Series A consisted of fifty-seven patients, twenty-eight males and twenty-nine females, and fifty-seven control subjects who were healthy blood donors, matched for sex and age to within 2 years.

Series B consisted of twenty-five patients and seventeen control subjects from whom CSF and blood were obtained at the same time. Control subjects were patients in a similar age group requiring myelography for possible disc lesions.

Sera

All sera were stored in aliquots of 1·0 ml at −20°C. Before immunofluorescent tests they were absorbed overnight at +4°C with acetone-treated HEp_2 cells, except for rubella virus tests when acetone-treated mouse liver powder was used for absorption. Absorbed sera were inactivated at 56°C for 30 min. Sera which gave viral IgM staining or anti-cellular IgM staining were also absorbed with heat-aggregated Cohn Fraction II human γ-globulin (HGG) and human brain powder. The absorption of sera with HGG removes anti-globulins of the IgM class, not always detected by standard agglutination tests for RF, and avoids false-positive IgM staining (Shirodaria, Fraser & Stanford, 1973). Several sera were absorbed with high titre crude measles virus or high titre crude herpes simplex virus made by concentrating cell-free virus in the ultracentrifuge.

CSF

CSF specimens were checked for the absence of blood contamination and were stored at −20°C. After inactivation they were tested unabsorbed. Later they were retested after absorption with human brain powder.

Virus-specific IgG and IgM staining

(i) *Antigen.* Sera and CSF were tested on both uninfected and virus-infected cells.

Cell cultures. HEp_2 cells, used for measles, mumps and vaccinia viruses and BHK21 cells, used for rubella virus were seeded thinly on coverslips so that cells were well spread out and were non-confluent. Confluent monolayers of human embryo fibroblasts were employed for varicella zoster virus.

Virus seeds. Measles virus (Edmonston strain adapted to HEp_2 cells), mumps virus (Ender's strain grown in allantoic fluid), herpes simplex virus (H.F.E.M. strain grown in HEp_2 cells), varicella zoster virus (Zoster-infected cells stored in 25% foetal calf serum and 10% glycerine) and rubella virus (Judith strain grown in BHK21 cells) were all inoculated so as to infect 40–50% of the cells.

Temperature and time of incubation. Indirect staining of virus antigen by IgM is dependent on both temperature and time of incubation (Haire, Adair & Fraser, 1973). These were different for each virus-cell system and were selected to give bright characteristic staining. Incubation at 35°C for 48 hr was the optimum in most cases, but incubation at 32°C for 24 hr was most suitable for herpes simplex virus. Fixation was carried out in fresh acetone for 10 min at room temperature.

(ii) *Fluorescein conjugates.* Sheep anti-human IgM conjugated with fluorescein isothiocyanate (FITC) (Wellcome Reagents, Ltd), tested and evaluated for specificity on an indirect viral system (Chantler & Haire, 1972), was used. Sheep anti-human IgG (Wellcome Reagents, Ltd) was conjugated with FITC (Sigma Chemical Company) in our own laboratory. The conjugates were absorbed with acetone-treated mouse liver powder, HEp_2 cells and BHK21 cells, before their optimum staining titres were assessed.

(iii) *Fluorescent staining.* The sera and CSF were coded before testing. Dilutions of sera were applied to virus-infected and non-infected preparations for 45 min at 35°C, and after washing, the conjugate was applied for 45 min at 35°C. Sera were tested against six viruses at a dilution of 1 in 10 for IgM, and titrated for IgG whenever possible. CSF were tested undiluted for both virus-specific IgM and IgG against selected viruses, and specific IgG was titrated in some. Stained preparations were read before de-coding, and all sera showing IgM staining were retested again before de-coding.

RESULTS

Anti-cellular IgM staining was found to be of two different specificities:

(i) *Against uninfected cells*

Even after removal of conventional non-specific staining by absorption with cultured human cells seventeen/fifty-seven patients with multiple sclerosis had sera which caused fibrillar staining of HEp_2 cells (Haire, 1972), while none of the sera from the control group gave this reaction. Sera of eight/fifty-seven patients with MS caused diffuse staining of the cytoplasm of the HEp_2 cells compared with four/fifty-seven of the control group. Neither type of anti-cellular staining was removed by absorption of sera by aggregated HGG nor with human brain powder. This IgM did not stain intracellular aggregates of virus nor, in a few instances tested, was it removed by absorption of sera by semi-purified high titre virus.

(ii) *Against measles virus-infected cells*

In the MS group twenty-two/fifty-six sera gave IgM staining of measles virus-infected cells, while nine/fifty-six still gave staining after absorption with heat-aggregated HGG; in the control group one/fifty-six gave staining before the absorption and none after. This staining was not present in adjacent non-infected cells. One test was unreadable in each group. The fluorescence was characteristic and differed from IgM staining produced by serum during the acute and early convalescent stage of measles infection in that cytoplasmic aggregates of virus antigen were unstained. The staining was weak and brighter at the cell periphery, and its intensity did not vary with dilution within the staining titre, which is low, ranging from 5 to 20. These facts suggest that a surface antigen was stained and that the amount of antigen present was a limiting factor in obtaining fluorescent brightness. The reaction was not removed by absorption by aggregated HGG, cultured human cells, human

brain powder or high titre herpes simplex virus, but it was removed by absorption with high titre measles virus. None of the sera gave true IgM staining of cells infected with the viruses of mumps, herpes simplex, vaccinia, varicella zoster or rubella. Two sera showed IgM staining of intra-cellular aggregates of mumps virus, which was removed by absorption by aggregated γ-globulin.

Virus-specific IgG in serum (Series A)

The geometric mean titres (GMT) obtained and the significant differences between titres in MS patients and healthy control subjects are shown in Table 1; the range of end-points

TABLE 1. Geometric mean titre (GMT) of virus-specific IgG antibody in multiple sclerosis patients (MS) and normal control subjects (C) and significant differences between the two groups (Series A)

IgG antibody to:	GMT* of antibody in		χ^2 (d.f. = 1)	Number of MS and C pairs
	MS	C	†	
Measles	31·4	18·3	9·03‡ (40) 4·03§ (80)	57
Mumps	1·8	1·5	0·19 (5)	56
Herpes simplex	40·8	17·5	7·19‡ (40) 7·32‡ (80)	57
Vaccinia	4·0	2·3	5·07§ (5)	57
Varicella zoster	9·2	8·5	0·04 (10)	55
Rubella	25·9	25·7	0·55 (20) 0·32 (40)	57

* Antibody titre is expressed as a reciprocal.
† Reciprocal of titre at which significance tests are performed.
‡ Significant at $P < 0{\cdot}01$.
§ Significant at $P < 0{\cdot}05$.

is shown in Fig. 1. Only one serum lacked measles antibody (at a dilution) of 1 in 5 when tested by this method, but sera with no detectable antibody against the viruses of mumps and vaccinia at the same dilution were found frequently. A significant increase of IgG was found against both measles and herpes simplex viruses. There was no such difference of rubella-specific IgG between the groups, as has been shown by another method (Panelius *et al.*, 1971), although the titres may be seen to be roughly equivalent to those for measles and herpes simplex antibody.

The slight increase of IgG against vaccinia virus is also significant, but less so than the increase against measles and herpes simplex and only a small number of sera contained vaccinia virus-specific antibody. No increase of IgG against varicella zoster antibody was found.

Virus-specific IgM and IgG in CSF (Series B)

While the MS patients and control subjects in Series B are unequal in number and are not matched for sex and age, the difference between the groups in the GMT of IgG against

measles and herpes simplex viruses are significant here as in Series A (MS group, 33·63 and 27·20; control group, 11·77 and 12·44, respectively).

IgM specific for the viruses of measles, herpes simplex and mumps was not found in the twenty-five CSF from MS and seventeen CSF from the control group.

Measles virus-specific IgG was present in sixteen/twenty-five CSF of the MS group, eight of which also had herpes simplex virus-specific IgG; one CSF had herpes IgG only. The presence of virus-specific IgG in the CSF is related to its titre (in serum), but not

Reciprocal of titre	Measles		Mumps		Herpes simplex		Vaccinia		Varicella zoster		Rubella	
	MS	C	MS	C	MS	C	MS	C	MS	C	MS	C
320												
160												
80												
40												
20												
10												
5												
< 5												
No. of pairs	57		56		57		57		55		57	

FIG. 1. Titres of virus-specific IgG antibody in (●) multiple sclerosis patients (MS) and (○) normal control subjects (C) (Series A).

absolutely as Table 2 shows. The GMTs of virus-specific IgG in the serum were slightly for measles specific IgG was present in the CSF than when it was not (43·2 versus 33·63 higher when antibody; 33·41 versus 27·20 for herpes simplex antibody).

The distribution shown in Table 2 suggests that the presence of measles virus antibody is independent of the presence of herpes simplex virus antibody; whereas herpes simplex virus antibody reaches the CSF mostly when measles virus antibody is already present. The numbers are small and our technique is not sensitive enough to decide whether the difference is quantitative or qualitative.

Absorption, by each of the two viruses separately, of two CSF which stained both measles and herpes simplex virus antigens removed only the specific IgG, which suggested that two separate antibodies are present and not a single antibody which co-incidentally reacts with both measles and herpes simplex viruses.

Cross-reactions with normal brain tissues were tested by absorbing thirty of the CSF (nineteen of the MS group and eleven of the control group) and no alteration of specific fluorescent staining resulted. Brain tissue from a case of MS was not available to carry out a further test.

TABLE 2. Incidence of measles and herpes simplex virus-specific IgG antibody in CSF of twenty-four MS patients in relation to titre of serum antibody* (Series B)

Measles virus-specific IgG antibody		Herpes simplex virus-specific IgG antibody	
Serum antibody titre	Present in CSF†	Serum antibody titre	Present in CSF
160	+ (N)	160	–
80	+ (N)	80	–
80	+ (N)	80	–
80	+ (10)	80	+
80	+ (10)	80	+
80	+ (N)	5	–
80	+ (10)	40	+
80	+ (5)	20	+
40	+ (10)	20	–
40	–	80	–
40	+ (N)	5	–
40	+ (N)	20	+
40	+ NT	—	–
40	–	10	–
40	–	80	–
20	–	40	+
20	+ (5)	20	+
20	+ (20)	20	+
20	–	20	–
10	+ (10)	80	–
10	–	40	–
10	–	80	–
10	–	N	–
5	+ (N)	20	+

* Antibody titre is expressed as a reciprocal.

† Figures in parentheses indicate dilutions at which measles virus-specific IgG was present in CSF.

N = Neat. NT = Not titrated.

Mumps virus-specific IgG was not detected in the CSF of the series nor was rubella virus-specific IgG found in the fluids tested (eleven of the MS group and sixteen of the control group).

Ratios between virus-specific IgG titres in serum and CSF in our series of fifteen patients range from 1:1 to 160:1 (Table 2).

DISCUSSION

The slight rise in the titre of antibody against measles virus, and occasionally against other viruses, which has been noted by many observers in MS (Brody *et al.*, 1972), we have found

to be true of the IgG response against both measles and herpes simplex viruses, but not against zoster virus (Ross, Lenman & Rutter, 1965). This rise could be explained as a non-specific over-activity of the immune system or by a specific stimulus involving a serological relationship between the two viruses and antibody to some undiscovered antigen. Our results rather strengthen the case for the latter idea.

The finding that a reasonable proportion of sera from MS patients contain antibody which combines with an antigen at the surface of measles virus-infected cells, but not at the surface of uninfected cells or cells infected with other viruses which we have tested, seems specially significant. Most of the antibody seems to be IgM. Even if some of the staining, which is technically difficult because of faintness, is not true primary IgM staining (Shirodaria, Fraser & Stanford, 1973), it is still specific to the measles virus-infected cell and is characteristic in twenty-two/fifty-six patients with MS. We consider that it is mostly specific IgM and that its presence probably indicates continuous stimulation by a minor antigen of measles or by some new antigen which cross-reacts with measles virus. Differential antibody response to different components of measles virus has recently been shown in MS by Salmi, Norrby & Panelius (1972).

The incidence of measles virus-specific IgG in the CSF of MS patients compared with normal subjects is not so high as that found by the measles haemadsorbing antibody test of Brown *et al.* (1972) but together with the lesser serum: CSF ratios, it points again to some measles-related antigen in the vicinity of the brain; this is supported by the fact that anti-rubella IgG was not detected in CSF of individual patients in whom circulating anti-measles and anti-rubella IgG were of equal titre. For the same reason passive transudation or leakage is unlikely to be the sole mechanism by which anti-measles IgG reaches the CSF. On the other hand, although herpes simplex virus may lodge in cerebral ganglia and may, like measles, cause encephalitis, we cannot suggest why some patients do have anti-herpes simplex IgG in the CSF while again anti-rubella IgG is absent. We are not confident that reduced serum: CSF ratios of virus-specific IgG necessarily indicates synthesis of antibody in the CSF although there is no doubt that both IgG synthesis (Cohen & Bannister, 1967) and cells synthesizing IgG have been demonstrated previously in MS (Sandberg-Wollheim, 1969). Both leakage and synthesis may occur (Webb *et al.*, 1968). The significance of measles antibody in MS will be further strengthened if we can substantiate our former observations (Millar *et al.*, 1971) that IgM specific for measles virus-infected cells is not found often in other diseases of the central nervous system and if measles virus-specific IgG in CSF can be shown to occur more frequently in MS than in other diseases of the central nervous system.

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