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IMMUNE STUDIES IN MULTIPLE SCLEROSIS

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SUMMARY

The serum and cerebrospinal fluid of patients with multiple sclerosis, in presence of human complement, caused destruction of glial cells and myelin in cultured nervous tissue, particularly in the acute stage of the disease. Cytotoxicity of the cerebrospinal fluid (CSF) was not wholly specific for multiple sclerosis, being observed also in cases of encephalitis and certain viral diseases. The degree of cytotoxicity of the CSF correlated with the increase in γ-globulin (IgG) in the cerebrospinal fluid. Immunoelectrophoresis studies of the CSF showed the presence in 50% of cases of an abnormal fast γ-globulin component.

These findings have two important implications. Firstly they support the concept that demyelination in multiple sclerosis and related diseases is the result of an autoimmune process. Secondly they are useful for the laboratory diagnosis of multiple sclerosis and its differentiation from non-demyelinating disorders of the nervous system.

Multiple sclerosis was first considered to be caused by an abnormal immune reaction by Pette (1942) and Ferraro (1944) and this concept is still current (Schrader, 1961), whilst experimental allergic encephalomyelitis (EAE), the experimental model of multiple sclerosis, is an established immunological disease (Kabat, Wolf & Bezer, 1947). Kabat *et al.* (1947), Uchimura & Shiraki (1957), Shiraki & Otani (1959) and Wolf (1963) have made histopathological comparisons between the two diseases, but there have been few attempts at immunological comparisons.

Lamoureux, Boulay & Borduas (1966) described in rhesus monkeys with EAE a complement dependent myelinocytotoxic antibody which appeared in the cerebrospinal fluid (CSF) and serum soon after immunization with nervous tissue, and which was associated with a progressive increase in γ-globulin in the CSF. This paper describes the presence of a similar myelinocytotoxic antibody in the serum and CSF of patients with acute and chronic multiple sclerosis. The degree of cytotoxic activity of the CSF correlated well with the increase in γ-globulin in the CSF, and with the presence of an abnormal fast γ-globulin in the CSF.

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MATERIALS AND METHODS

Patients studied

The patients were divided into groups according to the certainty of the diagnosis and the stage of the disease. Group 1 included fifteen patients with definite multiple sclerosis (MS) who were in an acute exacerbation and had not been treated with ACTH. The term 'definite', as used in this paper, refers to the combination of characteristic symptoms of multiple sclerosis and at least three phases of exacerbation and remission. Group 2 included seventeen patients with definite MS who were in remission or in an inactive stage of the disease. Group 3 included seven patients with definite MS who were in an exacerbation of the disease and had been treated with ACTH. Group 4 included seven patients with an organic neurological disease wherein the diagnosis of MS was suspected but not established Group 5 included twenty-eight patients, these being fifteen with organic neurological disease other than MS, five with organic neurological disease of uncertain diagnosis, and eight with acute psychosis. Group 6, the controls, were twenty-four patients from whom CSF was obtained during myelography or pneumoencephalography, and who were not thought to have organic intrinsic nervous disease; the diagnoses included neurosis, headache and herniated disc. We obtained serum from twenty-four healthy subjects.

Blood and CSF samples

Serum and CSF from patients in groups 1 and 3 were taken soon after admission to hospital and in no case later than 1 week after the exacerbation. Samples from patients in group 2 were obtained at least 1 week after an exacerbation of MS, and from patients with long-standing and inactive disease. CSF contaminated with blood was discarded.

Sterile vacuum 15 ml disposable B-D tubes (British Drug House Co. Ltd, U.S.A.) were used for the collection and processing of both serum and CSF. This glassware was found to be particularly suitable for tissue culture procedures.

Laboratory techniques

Our methods for the concentration of CSF, cellulose acetate electrophoresis, immuno-electrophoresis and measurement of cytotoxicity for cultured nervous tissue were as described by Lamoureux *et al.* (1966).

Studies on cytotoxicity were performed by exposing cultured nervous tissue with 1% fresh human serum as a source of complement to the test sample of serum or CSF. Damage to glial cells, myelin and neurons was assessed visually after 18–24 hr of contact, using the criteria of damage described by Lamoureux *et al.* (1966). The degree of damage is expressed by a semi-quantitative *cytotoxic index* (Lamoureux *et al.*, 1966) which is derived from a summation of degrees of damage, graded from 0 to 4+, to glial cells, to myelin, and to neurons, and from the amount of debris present, also graded from 0 to 4+. The cytotoxic index could therefore range from 0 to a maximum value of 16.

In order to minimize non-specific and often reversible damage to cultures, due probably to the re-adaptation of the cells to new culture conditions or to non-specific antibodies present in some normal sera (Fedoroff & Webb, 1962; Fedoroff & Doerr, 1962) and probably present also in CSF, all test samples of serum and CSF were decomplemented, millipore filtered, diluted with nutritional media, and readings were made only after the test sample

was in contact with the cultured nervous tissue for 18–24 hr. A cytotoxic index above 4 despite these precautions was considered to be a positive result for cytotoxicity, whilst an index below 4 was considered negative. Cultured nervous tissue was the only tissue culture system used to assess cytotoxicity.

RESULTS

Electrophoresis: increased γ-globulin in CSF in MS

Values for the various proteins in the CSF for groups 1–6 are shown in Table 1. The values are expressed as mean percentages of the total protein of the CSF. All fifteen patients

TABLE 1. Proteins in CSF in multiple sclerosis and other neurological diseases

Group and diagnosis	No. of cases	Component (mean percentage of total CSF protein)						
		Pre-albumin	Albumin	α_1	α_2	β_1	τ*	γ†
1. Acute MS	15	4·1	51·2	4·1	6·8	8·7	3·6	20·1 (2·1)
2. Remission and chronic MS	17	3·9	55·4	6·2	8·8	8·7	3·5	14·4 (1·8)
3. Acute ACTH treated MS	7	4·5	63·4	3·5	6·7	11·6	3·7	6·6 (0·68)
4. Doubtful MS	7	4·2	57·3	5·6	8·4	9·8	3·3	10·6 (2·5)
5. Other neurological diseases	28	4·4	60·8	5·4	9·0	8·9	3·7	7·3 (0·81)
6. Controls	24	6·5	54·8	7·9	10·4	9·3	3·8	7·1 (1·9)

The mean percentage of γ-globulin in acute multiple sclerosis was nearly three times that of the controls, this difference being highly significant ($P<0·001$).

* = τ-Globulin.

† Figures in parenthesis are standard deviations.

in group 1, acute MS, showed an increased γ-globulin ranging from 15·4% to 30·8% of total CSF protein, with a mean of 20·1%. The difference between the mean level of γ-globulin for group 1, acute MS, and for controls (7·1%, SD 2·8), was highly significant ($P<0·001$). For group 2, patients in remission or with inactive MS, the γ-globulin was increased and ranged from 8·8% to 19·3% of total CSF protein, with a mean of 14·4%; the difference between groups 2 and 6 was also significant ($P<0·05$). For all other groups including group 3, patients with MS treated with ACTH, the mean CSF γ-globulin level did not differ significantly from that of the controls. It may be noted that in groups 1 and 3 the mean percentage of the α-globulins, particularly α_1-globulin, was lower than in the controls but this difference was not significant ($P>0·05$).

Immunoelectrophoresis: fast IgG and increased IgA in CSF in MS

Antiserum to whole serum protein and antisera to some specific serum proteins were used for the immunoelectrophoresis studies. The immunoelectrophoresis patterns obtained with this system revealed that abnormalities can be demonstrated in the CSF of patients with multiple sclerosis while such abnormalities were not present in the serum. The pattern of normal CSF (Fig. 1) showed that the γ-globulin (IgG) line was shorter than that found in normal serum.

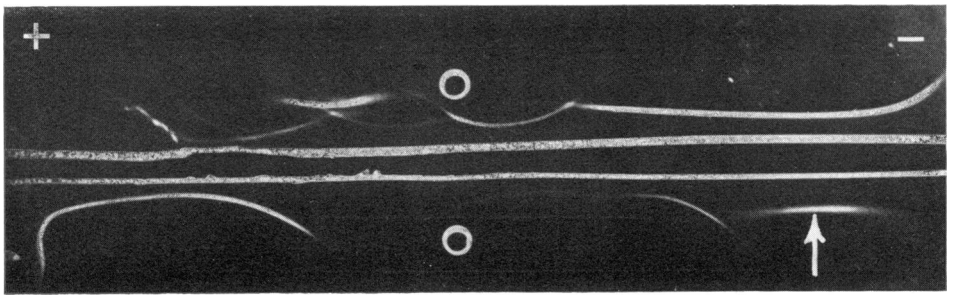

FIG. 1. Immunoelectrophoresis of normal CSF. The upper well contained normal serum and the lower well normal CSF, and the trough contained a rabbit antiserum to whole human serum protein. The IgG line of normal CSF (arrow) is shorter than that of serum.

TABLE 2. Percentage of cases in which various protein components of the CSF were demonstrable in neurological diseases (Fast IgG was demonstrated only in multiple sclerosis whilst IgA appeared mainly in multiple sclerosis but was also present in 4% of controls)

Group and diagnosis	No. of cases	Component (percentage of cases in which component was present in CSF)						
		CRP	IgA	IgM	IgG		β-Lipo	α_2M
					Fast	Slow		
1. Acute MS	15	0	33	16	49	100	0	17
2. Remission and chronic MS	17	0	50	0	50	100	0	6
3. Acute ACTH treated MS	7	n.t.	20	0	0	100	0	40
4. Doubtful MS	7	0	42	0	14	100	0	14
5. Other neurological diseases	28	0	0	11	0	77	0	11
6. Controls	24	0	4	0	0	93	0	4

CRP = C-reactive protein; β-Lipo = β-lipoprotein; α_2M = α_2-macroglobulin; n.t. = Not tested.

The CSF and serum from all cases were studied by analysing their reaction with antisera specific for serum α_1-glycoprotein, α_2-macroglobulin, C-reactive protein, β-lipoprotein, fraction II, IgG, IgA, IgM and finally with an antiserum to whole human serum protein. The percentage of cases having various of the above components in the CSF is shown in

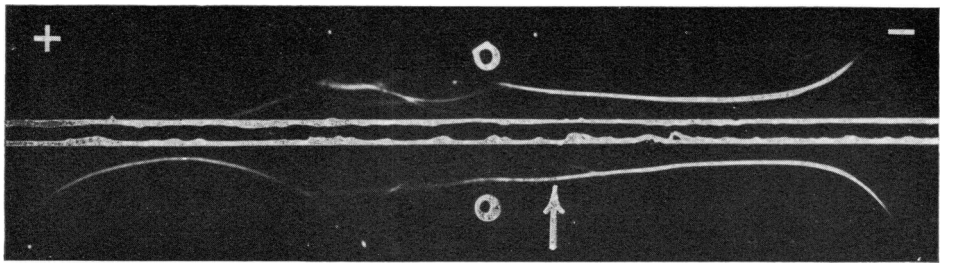

FIG. 2. Immunoelectrophoresis of CSF in acute multiple sclerosis (MS). The conditions were as for Fig. 1 except that the lower well contained CSF from a patient with MS. The IgG line (arrow) is extended toward the anode and is much longer than the IgG line of normal serum and normal CSF.

Table 2; consistent changes occurred only in relation to immunoglobulins G and A. In 50% of patients in groups 1 and 2 the CSF showed an IgG line which extended well towards the anode and, compared with the controls, was longer and less well defined, often being doubled or tripled at each end. It is referred to as fast IgG. In group 3, MS treated with

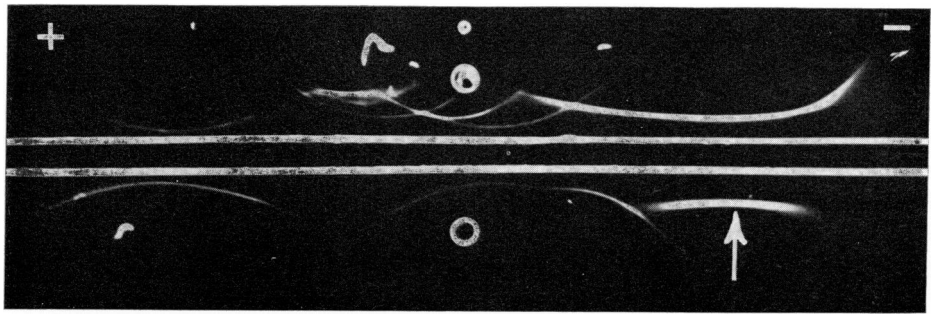

FIG. 3. Immunoelectrophoresis of CSF in MS after treatment with ACTH. The conditions were as for Fig. 1 except that the lower well contained CSF from a patient with MS treated with ACTH. The IgG line (arrow) has a normal appearance.

ACTH, the IgG line was much shorter and showed only minor changes (Fig. 3). From 20% to 50% of patients with MS (all groups) showed an increased density of the IgA line as compared with 4% of the controls. IgM was present in 16% of CSF samples from patients in group 1 but was absent from the controls. Consistent differences were not found between the groups in regard to α_1-glycoprotein, α_2-macroglobulin and transferrin, and neither C-reactive protein nor β-lipoprotein were found in any CSF sample (Table 2).

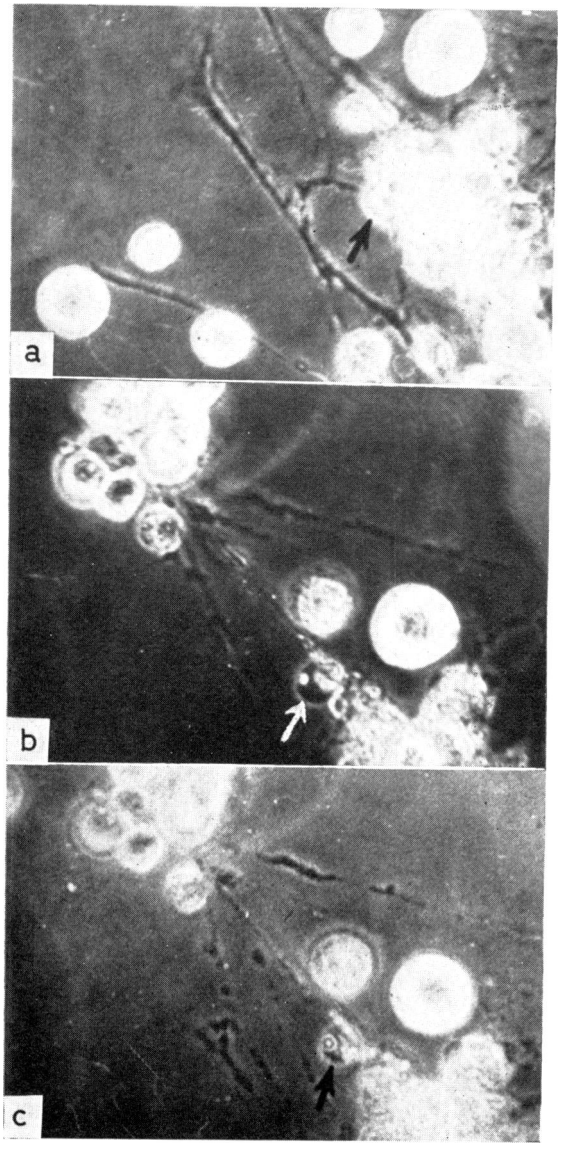

FIG. 4

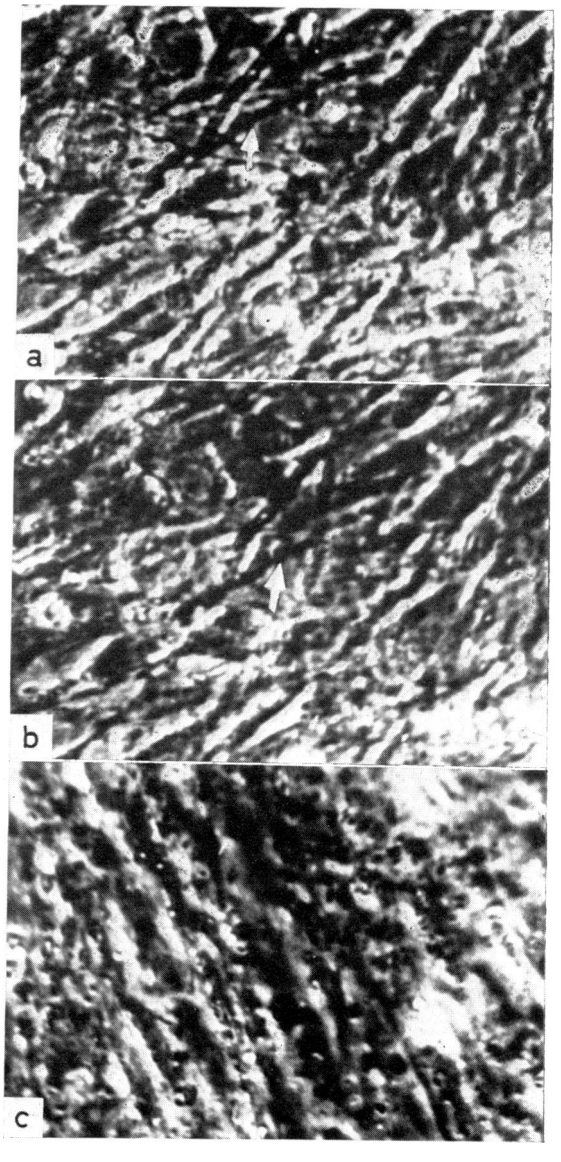

FIG. 5

FIGS. 4–8 are photographs of cerebellar cells derived from living newborn rats after 24–93 days of culture in Leighton tubes. Phase contrast microscopy ×100–450.

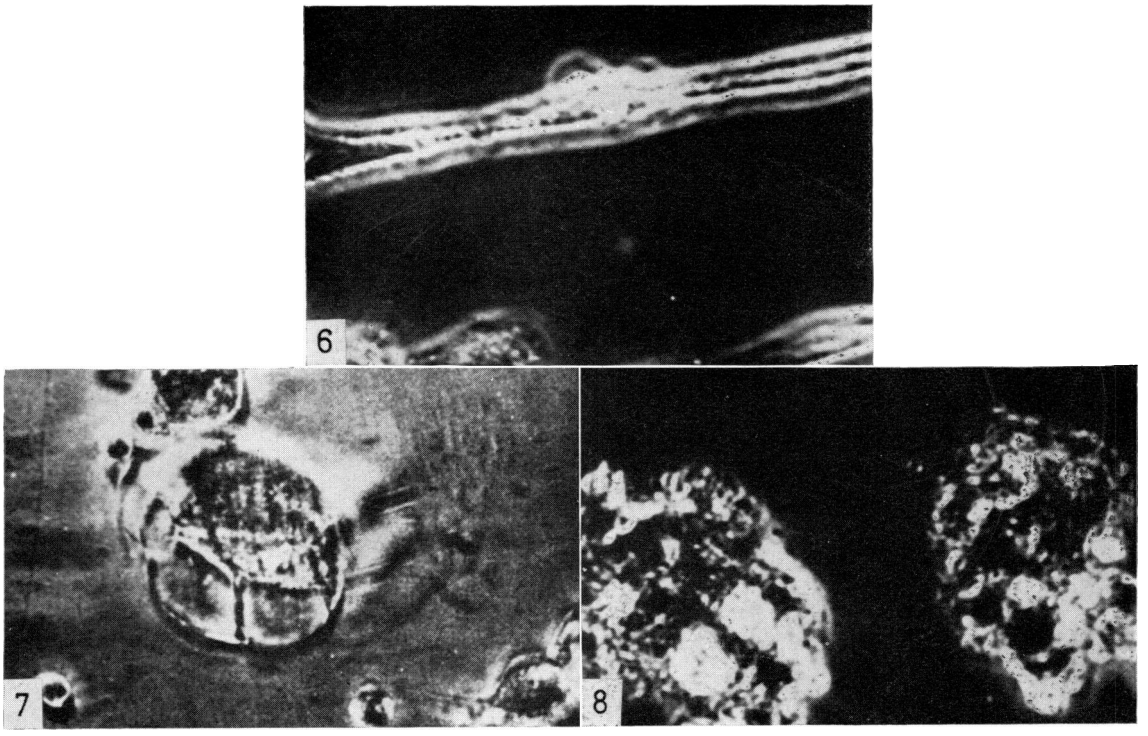

FIG. 4. A peripheral field of a 22-day culture after exposure to a toxic serum from a patient with MS. The arrow indicates a glial cell which is undergoing progressive damage. (a) Immediately after addition of serum showing that the glial cells and myelin are intact; the arrow points to a glial cell which will be damaged. (b) Same field after 27 min showing that the glial cells and myelin sheaths are swollen and irregular: during the damaging process the glial cells have changed position. (c) Same field after 35 min showing that some swollen areas of myelin have disrupted leaving the axis intact.

FIG. 5. A field of a 93-day-old culture of nerve cells after exposure to a non-diluted toxic CSF from a patient with acute MS. (a) After 160 min showing swollen myelin fibres (arrows). (b) Same field after 190 min showing further swelling of myelin fibres. (c) Field of the same culture after 24 hr showing complete destruction of myelin fibres and debris.

FIG. 6. Disruption of a myelin fibre after 4 hr exposure to CSF from a patient with acute MS.

FIG. 7. A single swollen oligodendrocyte in a damaged culture showing the nucleus and the cytoplasm compressed onto the wall of the cell membrane: disruption of the cell leaves a round intact nucleus in the medium, seen above and below the damaged cell.

FIG. 8. Agglomeration of damaged and dead cells in a culture after 24 hr exposure to a toxic CSF from a patient with acute MS.

Myelinocytotoxic effect of serum and CSF on cultured nervous tissue

Serum and CSF from patients with multiple sclerosis damaged glial cells, myelin and some neurons of cultured nervous tissue, as shown in Figs. 4–8, and damage occasionally occurred within 30 min. The damage to cultured nervous tissue was similar to that previously observed by Lamoureux *et al.* (1966) with serum and CSF from monkeys with EAE.

The mean values of the cytotoxic index of serum and CSF for groups 1–6 are shown in Table 3. The mean cytotoxic index for serum was 11·5 for group 1 patients and 7·2 for

TABLE 3. Cytotoxic effect of serum and CSF in multiple sclerosis and other neurological diseases

Group and diagnosis	No. of toxic sera	Mean cytotoxic index of serum*	No. of toxic CSF	Mean cytotoxic index of CSF*
1. Acute MS	14/15	11·5	14/15	10·4
2. Remission and chronic MS	12/16	7·2	11/14	9·2
3. Acute ACTH treated MS	2/7	3·3	5/7	8·3
4. Doubtful MS	3/6	6·1	5/7	7·0
5. Other neurological diseases	7/28	4·4	8/28	3·8
6. Controls	4/24	2·1	3/23	1·6

The cytotoxic index obtained with the serum was much higher in group 1, acute MS, than in the other groups, but the cytotoxic index obtained with CSF showed less difference. In all groups of patients with MS the mean cytotoxic index is well above that obtained with other neurological diseases and with other controls

* Derived from a summation of the degree of damage (0 to + + + +) to glial cells, myelin, neurons, and the amount of debris present (0 to + + + +): thus the range of the cytotoxic index was 0–16.

group 2 patients, and for CSF was 10·4 and 9·2 respectively. These means were well above those obtained for the controls which were 2·1 for serum and 1·6 for CSF. The mean cytotoxic index of the CSF for group 3, patients treated with ACTH, was 8·3 which was considerably higher than that of the serum, 3·3. Thus treatment with ACTH reduced the cytotoxic activity of the serum but had little effect on that of the CSF. It may be noted that none of the patients in group 3 received more than three injections of 40 units of ACTH.

The mean cytotoxic index of serum and CSF for patients in group 5 with neurological diseases other than MS was 4·4 and 3·8. However, this group contained certain patients

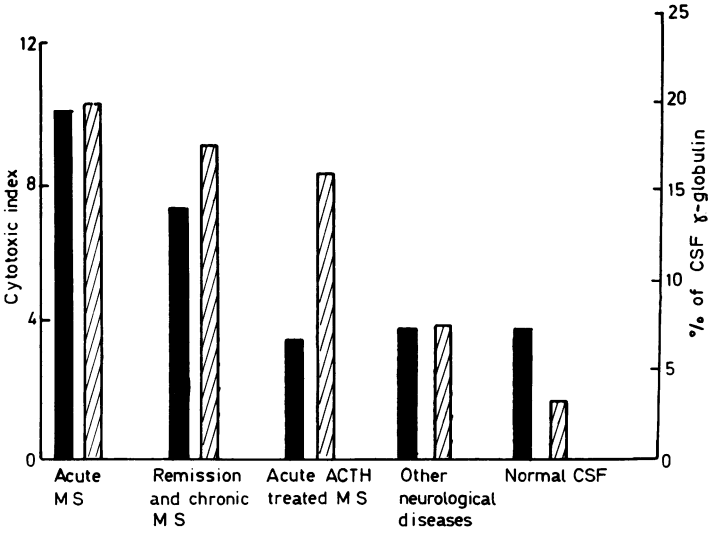

FIG. 9. Comparison between the level of γ-globulin in the CSF and the cytotoxic index of the CSF for groups 1, 2, 3, 5 and 6. Both the cytotoxic index (cross-hatched) and γ-globulin (black shading) were increased in patients with multiple sclerosis, but in MS treated with ACTH, the mean cytotoxic index of the CSF was raised despite a normal level of γ-globulin. The column called 'normal CSF' refers to group 6, the controls.

TABLE 4. Cytotoxic effect of serum and CSF according to diagnosis

Disease	No. of cases	No. of cases with	
		Toxic serum*	Toxic CSF*
Groups 1–3			
Multiple sclerosis	38	28	30
Group 5: Other neurological diseases			
Chronic encephalitis	3	3	3
Acute encephalitis	2	2	2
Degenerative myelitis	2	2	2
Tabes dorsalis	1	0	1
Muscular dystrophy	3	0	0
Friedreich's ataxia	1	0	0
Choreoathetosis	1	0	0
Amyotrophic lateral sclerosis	2	0	0
Neurological disease of uncertain diagnosis	5	1	1
Psychosis	8	0	0
Group 6			
Controls	24	4	3

The serum and CSF from cases of encephalitis and degenerative myelitis of unknown aetiology were as toxic for cultured nerve cells as were samples from cases of MS.

* Toxic = cytotoxic index >4+.

whose serum and CSF were highly toxic, these being five cases of acute and chronic 'non-specific encephalitis', two cases of 'myelitis of unknown aetiology' and one patient with tabes dorsalis whose CSF was toxic (Table 4).

Cytotoxic factor in viral diseases

Serum from ninety-three cases of various viral diseases was tested for toxicity for cultured nervous tissue (Table 5). Eleven sera were toxic, these being from eight of twelve cases of coxsackie B2 infection, from two of three cases of infectious mononucleosis, and from the one case of Q fever. The mean cytotoxic index of sera from the twelve patients with coxsackie

TABLE 5. Cytotoxic effect of serum from patients with various viral diseases tested on cultured nervous tissue

Viral diseases	No. of sera tested	Virus specific antibody titre in serum (range)	No. of sera toxic for cultured nervous tissue	Mean cytotoxic index of serum for all cases
Influenza I, II, III	19	8–64	0	0·2
Adenovirus A	20	8–64	0	0·6
Adenovirus B	20	8–28	0	0·4
Poliomyelitis types I, II, III	9	89–512	0	0·1
Coxsackie B2	12	45–2048	8	10·6
Psittacosis	5	16–64	3	5·2
Mumps	3	64–128	1	2·3
Measles	1	—	0	0
Q fever	1	8	1	16·0
Infectious mononucleosis	3	60–1280*	2	6·1

We tested only the second serum sample, i.e. that showing an increase of specific virus antibody titre, for cytotoxicity on cultured nervous tissue.

* Titre of heterophile agglutinin.

B2 infection was 10·6, while the only Q fever serum completely destroyed the tissue cultures giving a maximum cytotoxic index of 16.

Correlation of cytotoxic index and γ-globulin level of the CSF

We compared for each group of patients the mean percentage of γ-globulin in the CSF with the cytotoxic index of the CSF. Groups 1 and 2 had a high level of γ-globulin which correlated with the high cytotoxic index. For group 3 the mean γ-globulin percentage was normal, being 6·6% of the total CSF protein, but the mean cytotoxic index was high at 8·3. Multiple sclerosis was the only disease in the present series in which a high γ-globulin was associated with a high cytotoxic index in the CSF.

DISCUSSION

Kabat, Moore & Landow in 1942 showed that there was an increase in the γ-globulin level in the CSF in multiple sclerosis. This is the most consistent and probably the most significant laboratory finding in multiple sclerosis, and was present in 83% of Kabat's patients (Kabat, Glusman & Knaub, 1948; Kabat *et al.*, 1950; Yahr, Goldensohn & Kabat, 1954). Moreover the total protein of the CSF in multiple sclerosis is not increased despite the rise in γ-globulin.

In the present study all fifteen definite cases of multiple sclerosis in an acute exacerbation (group 1) had a raised γ-globulin level in the CSF and the mean value of 20·1% of total protein was more than three standard deviations greater than the mean for the controls (group 6). The γ-globulin in the CSF in our cases was considerably higher in the acute than in the inactive stage of multiple sclerosis; hence estimation of CSF γ-globulin could be of value in diagnosis and assessment of progress. There are in the literature widely varying figures cited for the percentage of γ-globulin in the CSF in multiple sclerosis, from 47–55% (Bauer, 1956; Bauer & Heitmann, 1958) to 93% (Schapira & Park, 1961). However, we believe that this variation can be explained by the differing biochemical techniques used, by inclusion of mis-diagnosed cases, and by failure to separate patients according to the stage of the disease.

We showed by immunoelectrophoresis that 50% of cases of multiple sclerosis have in the CSF an abnormal fast γ-globulin which extended the γ-globulin line toward the anode, and perhaps this component accounted for the increase in total CSF γ-globulin and for the cytotoxicity of the CSF. Our failure to detect this fast component in the other 50% of cases could have had several explanations: firstly the technique, especially the concentration of the CSF, was not adequate to demonstrate it; secondly this component may have been present in the CSF only for a short period; thirdly it could have become fixed on to cells like cytophilic antibody.

There are many unanswered questions concerning the significance of the increased γ-globulin in the CSF in multiple sclerosis. In regard to its origin, it could be derived from the serum by transfer across the CSF-blood-barrier either free or fixed to lymphoid cells. There is some evidence for local synthesis, since Levine & Wenk (1965) showed that in hyperacute EAE in Lewis rats, mononuclear cells penetrated the CSF-blood-barrier probably before the occurrence of lesions. We doubt whether the increase in CSF γ-globulin could be explained by degeneration of the nervous tissue as suggested by Field & Ridley (1960), since our fast γ-globulin reacted with an anti-human serum protein, and Yokoyama & Roboz-Einstein (1965) found that the various basic encephalitogens, which moved toward the cathode in electrophoresis, were absent from the CSF of patients with multiple sclerosis.

Many autoimmune diseases are characterized by an increased serum γ-globulin (Mackay & Burnet, 1963) which is usually due to the presence of autoantibody, and some would regard this autoantibody as being partly or wholly responsible for the pathogenesis of the disease in question. We are still not certain whether this applies to the increase in CSF γ-globulin in multiple sclerosis, since we cannot say whether the increase precedes or follows exacerbations, and whether it causes damage *in vivo* as it appears to do *in vitro*. In rhesus monkeys with EAE, the CSF γ-globulin increases in parallel with the development of cytotoxicity of the CSF, and we showed that this occurred weeks before the onset of

symptoms of EAE (Lamoureux *et al.*, 1966). Our present studies showed a similar parallel increase in CSF γ-globulin and cytotoxicity of CSF in acute multiple sclerosis, and hence we can infer that the γ-globulin in the CSF is responsible for the cytotoxic action *in vitro*. If this were so, the next step would be to show that a globulin in the CSF in multiple sclerosis is responsible for damage *in vivo*. However, it must be emphasized at this point that the cytotoxic antibody is not wholly specific for multiple sclerosis and in the present study was found in other neurological diseases including acute and chronic encephalitis and myelitis, and in serum of patients with coxsackie B2 virus infection. These other neurological diseases could, of course, resemble multiple sclerosis in pathogenesis in being caused by an abnormal immune response in the central nervous system. It is of interest that Bornstein & Appel (1965) reported a neurocytotoxic effect with nine of fifteen sera from cases of amyotrophic lateral sclerosis, and Field & Hughes (1965) found such an effect with thirteen of twenty-one sera from cases of motor neurone disease.

The presence of a cytotoxic autoantibody in the serum and CSF in multiple sclerosis raises further questions in regard to its origin, and to the antigenic determinants with which it reacts. The latter may be related to the basic encephalitogenic peptide (Carnegie, Lamoureux & McPherson, 1966) which is extracted from myelin and which provokes encephalitis when injected into animals in minute quantities with adjuvant. How immunization to nervous tissue could occur spontaneously in man is unknown. One may speculate that the antigens of myelin, which are presumably 'inaccessible' and therefore immunogenic, might as a result of trauma or viral infection be released into the circulation either free or coated onto a virus particle; if an autoimmune process were thereby set up, it could be self-perpetuating as in the case of Hashimoto's thyroiditis (Mackay & Burnet, 1963). Secondly antibody to the antigens of a virus, e.g. coxsackie B2, could cross-react with antigens of myelin, an analogue being the myocardial autoantibody which is stimulated by streptococcal antigens (Kaplan & Svec, 1964). Thirdly there may be weakness in homeostatic processes allowing production over threshold levels of 'natural' antimyelin autoantibodies like those described by Field, Caspary & Ball (1963) in 20% of normal subjects.

We would conclude by emphasizing that multiple sclerosis is closely related to EAE not only histologically but also immunologically. Thus there are similar histopathological lesions in the two diseases, and plaque-like lesions also follow injections of nervous tissue in man whether for rabies vaccination (Shiraki & Otani, 1959) or for other therapeutic purposes (Jellinger & Seitelberger, 1958). Finally we have shown that the serum and CSF in both diseases contains cytotoxic antibodies for cultured nerve cells, and the degree of cytotoxicity correlates with the increase in γ-globulin in the CSF. It is virtually certain that in EAE the cytotoxic antibody or the cells which produce it are the causative agents of the disease: the same pathogenesis must now be strongly considered for multiple sclerosis.

The practical aspect of the present work relates to the laboratory diagnosis of multiple sclerosis by means of electrophoresis and immunoelectrophoresis of CSF and by use of cultured nervous tissue. Features suggestive of multiple sclerosis on electrophoresis of CSF include a raised CSF γ-globulin, a low β–γ-globulin ratio and a decrease of α_1-globulin, and on immunoelectrophoresis the presence of a fast IgG causing an extension of the IgG line toward the anode, and the presence of IgA. Finally it would be quite practical for hospital laboratories to establish cultures of nerve cells to facilitate the diagnosis of neurological diseases.

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