T lymphocyte-derived demyelinating activity in multiple sclerosis patients in relapse

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SUMMARY Supernatants of cultured T lymphocytes of multiple sclerosis patients were tested for a demyelinating activity in rat cerebellum explant cultures. Supernatants of unstimulated T lymphocytes in seven out of 10 multiple sclerosis patients in relapse produced demyelination when checked by phase contrast microscopy. Supernatants of unstimulated T lymphocytes from healthy subjects did not produce demyelination, but when T cells were stimulated by phytohaemagglutinin (PHA), 50% of tested supernatants produced demyelination, which was, however, never as advanced as in multiple sclerosis supernatant treated cerebellum cultures. The demyelinating activity proved to be heat labile. Gel filtration study revealed two fractions of the demyelinating activity 12.5-29.0 kD and 43.0-66.0 kD. The results suggest that lymphokines can be directly involved in the pathogenesis of demyelination in multiple sclerosis.

The primary pathological features of multiple sclerosis are demyelinating plaques randomly scattered throughout the white matter of the central nervous system (CNS).¹ The aetiology of this disease is almost entirely unknown, but it has been long suspected that the immune system is involved in the pathomechanism leading to myelin loss.² The active demyelinating plaques are prominently infiltrated with mononuclear cells, which though they include B cells and plasmocytes,³ consist predominantly of T cells.⁴⁻⁷ The precise role of these cells and their significance in the immune reactions leading to the formation of demyelinating plaques is not fully understood. A previous report⁸ showed that the supernatants of cultured peripheral blood mononuclear cells of multiple sclerosis patients during an acute exacerbation consistently caused demyelination in vitro in nerve tissue organotypic culture.

We demonstrate here that the supernatants of cultured T lymphocytes of multiple sclerosis patients during an acute exacerbation produced, in the majority of cases, demyelination in vitro in nerve tissue organotypic culture. The gel filtration study showed that lymphokines are the most likely substances responsible for this demyelinating activity.

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Material and methods

Subjects

The lymphocyte donors were 10 multiple sclerosis patients seen in the Outpatient Department and wards of the Department of Neurology, Charing Cross Hospital, London UK. All patients had the remitting and relapsing form of definite multiple sclerosis⁹ and were studied in acute exacerbation no later than 2 weeks after the appearance of new neurological signs. There were eight females and two males in this group. Age ranging from 20 to 51 years (mean 32). Duration of the disease from 3 to 15 years. The clinical manifestations included pyramidal, cerebellar, brain stem and spinal cord signs. The patients were classified on the Kurtzke disability status scale from 3 to 7 grades. No patient had received steroids or immunosuppressive drugs for at least 3 months before sampling. Age- and sex-matched healthy subjects were used as controls in each case; blood samples were taken and processed at the same time as those of the patients with multiple sclerosis.

Lymphocyte culture

Peripheral blood was collected in heparinised tubes and mononuclear cells were isolated using Ficoll-paque (Pharmacia Fine Chemicals) gradient centrifugation (density 1.077) for 30 min at 400 g.¹⁰ The adherent cells were removed by incubation of the cell suspension on plastic surfaces (Petri dishes) for 1 h at 37°C.¹¹ The nonadherent cells were then decanted and washed off twice with RPMI 1640 medium (GIBCO). Cell viability was checked with trypan blue. T lymphocytes were isolated by rosette technique.¹² The rosette-forming cells (E-positive) were separated from the non-rosette forming cells (E-negative) by density gra-

dient centrifugation (density 1.077), 12 min at 1000 g. E-positive cells were cultured at a concentration of 5×10^5 /min in RPMI 1640 medium plus 10% fetal calf serum (Sera-Lab) in flat bottom wells (NUNC, GIBCO) from 2 to 6 days. Phytohaemagglutinin (Wellcome Labs) at a concentration of 1 µg/ml was added to duplicate or triplicate cultures; simultaneously E-positive cells were cultured without PHA. E-negative cells were cultured at the same concentration and in the same conditions as the E-positive cells but only for 2 days and without PHA. The supernatants from both E-positive and E-negative cells were harvested and dialysed against distilled water and then against PBS. Supernatants were stored below -20°C before use.

Gel filtration

The separation of the various major components of the T lymphocyte supernatants was undertaken using gel filtration chromatography. A sample (4–6 ml) of T lymphocyte supernatant was layered on to 90 cm $\times$ 2.5 cm Sephadex G-100 (Pharmacia Fine Chemicals) column. The flow rate 65 ml $\times$ h⁻¹ was controlled by the LKB 2115 Multiperex pump and fractions were collected using an LKB 7000 Ultrarac fraction collector. The G-100 column was calibrated using a series of protein of known molecular weight (all obtained from Sigma Chemical Co): cytochrome C (12.5 kD), carbonic anhydrase (29.0 kD), ovalbumin (43.0 kD), bovine serum albumin (66.0 kD) and blue dextran (200.0 kD).

Rat cerebellum culture

Neonatal rats (Wistar) on the day of birth were used for cerebellum culture. Each cerebellum was cut into eight parasagittal sections. These fragments were transferred on to collagen coated glass cover slips and maintained in nutrient medium consisting of 50% human heat inactivated serum, 40% Earle's lactalbumin solution (Flow Labs) with 2% FCS, chicken embryo extract (Flow Labs), glucose at final concentration 600 mg%, penicillin (100 U/ml) and strep-

tomycin (100 µg/ml) in six well plates (Flow Labs). The supernatants of E-positive and E-negative cells were added to aliquots of usual feeding medium in a concentration of 50%, 25%, 10% and 5% after 16–18 days in vitro (16–18 DIV) of cerebellum culture. Substantial myelination occurs in such cultures on 10–12 days, thus allowing us to investigate the effect of T cell supernatants on already myelinated cultures. The chromatographic fractions were added in a concentration of 50%, each sample being added to at least two cultures. As controls, sister cultures were fed with usual nutrient medium plus 50% RPMI 1640 medium. By the end of 72 h observation the level of K⁺ in culture medium was measured as a test for cell viability. For the same purpose some cultures were stained with trypan blue.

After 6, 12, 24, 48 and 72 h the living tested cerebellum cultures were examined for the presence of demyelination by reverse phase contrast microscope (Reichert). The readings were carried out independently by two observers using a coding system without knowing which T cell supernatant had been added to the culture.

The presence of myelin having been confirmed in selected untreated cultures by electron microscopy (Philips), these cultures were fixed in 2% phosphate buffered glutaraldehyde and were processed in the standard way for electron microscopy.¹³

Results

Eighty per cent of E-positive cells reacted with monoclonal antibodies specific for pan-T cell marker Leu-1 (Bio-Yeda Immunochemicals), whilst 85% of E-negative cells reacted with anti-human immunoglobulin serum (Coulter Electronic Ltd) specifically for B lymphocytes. Cell viability tested by trypan blue before and after cell separation was over

Fig 1 Outgrowths of axons lower down the explant. This area shows a field of several myelinated axons. Phase contrast; ($\times 1020$.)

Fig 2 Electron micrograph of an intact myelin sheath (16 DIV) showing characteristic osmiophilic laminated membranes. (Uranyl acetate, lead citrate. $\times 48\,000$.)

95% and, after the termination of cell culture, over 85%.

The cerebellum cultures growing in standard conditions displayed features characteristic for such a system.¹³ Myelinated fibres were present within the neuroglia sheet surrounding the explant in a large proportion of the cultures after 10–12 DIV (fig 1). Myelin showed typical electron microscopic appearance (fig 2).

A summary of the effect of the supernatants tested is given in the table. Supernatants of unstimulated T lymphocytes of seven multiple sclerosis patients showed demyelinating activity, this reaction beginning by the end of 24 h of exposure to the supernatants. At about that time myelin sheaths appeared less bright and less distinct against the usual background. This process continued and apparently intact myelin gradually lost its characteristic birefringence under phase contrast microscope. By the end of 72 h, myelin faded away and was indistinguishable from the background (fig 3). Not infrequently, necrotic cells were seen but not on a large scale when checked

by trypan blue. The K^+ level in the incubation medium was also raised only slightly (less than 2%) in tested cultures at the end of 72 h observation when compared with control cultures. The demyelinating activity was observed to the same degree in supernatants of T lymphocytes cultured for 2 and for 6 days.

The supernatants added to the rat cerebellum cultures in a concentration of 25% of total nutrient medium continued to cause myelin damage in similar degree as in concentration of 50%, but in a concentration of 10% no myelin destruction was observed. Supernatants of T lymphocytes of multiple sclerosis patients stimulated by phytohaemagglutinin showed similar but enhanced demyelinating activity.

Supernatants of unstimulated T lymphocytes of three multiple sclerosis patients did not show demyelinating activity as well as the control subjects. There were no clear differences in clinical status between these three multiple sclerosis patients and the seven patients who showed T cell derived demyelinating activity. Not infrequently, demyelinating changes were observed in cerebellum cultures exposed to supernatants of phytohaemagglutinin stimulated T lymphocytes of control subjects, but never were these as advanced as in the multiple sclerosis patients.

Supernatants of unstimulated B lymphocytes of multiple sclerosis patients cultured for two days did not exhibit demyelinating activity in the rat cerebellum culture. The appearance of myelin in phase contrast microscopy did not differ from the appearance of myelin in control cultures. Similarly, no myelin damage was detected in nerve tissue culture treated with supernatants of B lymphocytes from control subjects.

Chromatographic fractions of T lymphocyte-derived proteins were combined in five groups as follows: I: 66.0 to about 100.0 kD, II: 43.0 to 66.0 kD, III: 29.0 to 43.0 kD, IV: 12.5 to 29.0 kD and V: up to 12.5 kD. The demyelinating activity was detected in

Table Demyelinating activity of T lymphocyte supernatants. Score applied: – no changes, + minimal changes (less than 20% of axons affected), ++ moderate changes (20–50% of axons affected), +++ maximal changes (more than 50% of axons affected)

No	Multiple sclerosis patients		Control subjects	
	Spontaneous activity	PHA induced activity	Spontaneous activity	PHA induced activity
1	+++	+++	–	–
2	++	++	–	+
3	+++	+++	–	+
4	+++	+++	–	–
5	++	+++	–	+
6	–	++	–	–
7	–	+	–	+
8	+++	+++	–	–
9	+++	+++	–	+
10	–	+	–	–

PHA = phytohaemagglutinin.

Fig 3 The culture from fig 1 treated with supernatant of multiple sclerosis patient T lymphocytes for 72 h. This area shows an extensive field of myelin destruction. Most myelin has been reduced to small refractile debris; (phase contrast; $\times 1020$.)

fraction II and IV (fig 4). The time of action and morphological features observed in the rat cerebellum cultures under phase contrast microscopy were similar to those described above for the crude T lymphocyte supernatants, but fraction II appeared to be more active in causing myelin damage. Necrotic cells were also more commonly observed in nerve tissue cultures treated with fraction II than with fraction IV.

Discussion

Whilst there are several mechanisms leading to demyelination in pathological conditions of the CNS, immunological mechanisms are of particular importance in primary demyelinating diseases, the commonest of which is multiple sclerosis. Immunologically mediated demyelination in experimental models is seen in the presence of antimyelin antibodies,¹⁴ as a result of cell-mediated cytotoxicity¹⁵ or as a consequence of antibody-dependent cytotoxicity,¹⁶ but there is no convincing evidence that any of these mechanisms is responsible for demyelination in multiple sclerosis.¹⁷

The results of this study may suggest another mechanism leading to immune mediated demyelination dependent on the soluble factors derived from T lymphocytes. Lymphokines are known to be intercellular regulatory factors, so that the possibility of interaction between lymphokines from multiple sclerosis T lymphocytes and the cells in nerve tissue culture have to be considered. From the theoretical point of view, two such interactions could have occurred in our experimental model. T lymphocytes in culture pro-

duce macrophage activation factor(s)¹⁸ which can drive and stimulate macrophages to phagocytosis as well as hydrolytic enzyme release. Phospholipases¹⁹ and proteases²⁰ secreted by activated macrophages have been shown to mediate breakdown of isolated myelin and myelin basic protein, and morphological studies have implicated macrophages in the demye-

Fig 4 Lymphokine myelinotoxic activity in fractions obtained from Sephadex G-100 chromatography of T lymphocyte supernatant of multiple sclerosis patients (4 subjects were studied). Optic density at 280 nm. Score: - no changes, ++ moderate changes (20-50% axons affected), +++ maximal changes (more than 50% axons affected).

lination of multiple sclerosis.²¹ The molecular weight of macrophage activation factor seems to be heterogeneous²² and may overlap with the molecular weight of the chromatographic fractions causing demyelination in this study. Another cell potentially stimulated by T lymphocyte derived lymphokine is the astrocyte, which possesses hydrolytic enzymes capable of degrading myelin.²³ Histochemical staining for various hydrolytic enzymes showed astrocytes as the main hydrolase-rich cells, apart from macrophages, in both plaque and periplaque tissue.²³ Only recently has a relationship between astrocytes and immune cells been suggested by in vitro studies demonstrating lymphokine-enhanced-proliferation of murine and rat astrocytes.^{24, 25} Two distinct lymphokines are possible candidates for this interaction, glial cell stimulating factor²⁴ and glial growth-promoting factor.²⁵ The molecular weight of glial cell stimulating factor overlaps with the molecular weight of the chromatographic fraction IV causing demyelination in this study. At this stage it is difficult to assess the role of astrocytes or macrophages in the lymphokine mediated demyelination seen in this study. However, another explanation for the observed demyelination in rat cerebellum culture might be a direct lymphokine myelinotoxic effect. Some lymphokines possess enzymatic activity which could produce myelin damage. The timing of demyelination after nerve tissue treatment with T cell supernatants (24–72 h) and the molecular weight of the demyelinating fractions suggests some similarities with lymphotoxin.²⁶ Possible mechanisms related to lymphotoxin activity are an increased content of lysosomal enzymes²⁷ or increased permeability to calcium ions,²⁸ each of which could easily be involved in the pathomechanism of myelin damage. The production of lymphotoxin by lymph node cells of rats with experimental allergic encephalomyelitis may support this suggestion.²⁹

In summary, we described a demyelinating activity of T lymphocyte supernatant of multiple sclerosis patients in relapse. Gel filtration study revealed two fractions of demyelinating activity, 12.5–29.0 kD and 43.0–66.0 kD. Whether this is a new lymphokine with demyelinating activity or a new property of an already known lymphokine is a subject for further study.

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