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Tumour necrosis factor-alpha synthesis by cerebrospinal-fluid-derived T cell clones from patients with multiple sclerosis

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SUMMARY

T cell clones derived from cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) were analysed for their ability to produce interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin-2 (IL-2) and interleukin-4 (IL-4). The CSF-T clones were compared for their ability to produce cytokines with autologous peripheral T clones and with liver-infiltrating T cell clones from patients with chronic active hepatitis. IL-4 production was also compared with that by peripheral T clones derived from atopic patients. All the CSF-T clones (both $CD4^+$ and $CD8^+$) produced large amounts of IFN-γ and particularly of TNF-α. These cytokines were synthesized in significantly larger amounts by CSF T clones than by reference clones. Moreover, they were capable of secreting IL-2, but not IL-4. We conclude that the CSF–$CD4^+$ T clones could constitute a subset with functional properties similar to those of T helper 1 (Th1)/inflammatory cells of the mouse; and that the large amounts of TNF produced by CSF T cell clones strongly suggest a significant role for this cytokine in MS immunopathogenesis.

Keywords tumour necrosis factor-alpha multiple sclerosis CSF-T cell clones

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease, which affects the white matter of the central nervous system (CNS) (McFarlin & Farland, 1982). Susceptibility to MS is under multigenic control and is linked to HLA-D locus genes on chromosome 6 and possibly to IgG allotype genes (Waksman & Reingold, 1986; McFarlin & Lachmann, 1989). Although numerous hypotheses have been advanced to explain the cause of MS, such as the role of measles, rubella, varicella and HTLV I viruses (Koprowski *et al.*, 1985; Waksman & Reingold, 1986), there is consensus on the fact that a primary defect in immune control mechanisms is involved in MS (Antel, Arnason & Medof, 1978; Hafler, Buchsbaum & Weiner, 1985a; Hafler *et al.*, 1985b; Walsh & Tourtelotte, 1986; Hirsh 1986; Morimoto *et al.*, 1987; Hafler & Weiner, 1989). The similarities between MS and experimental allergic encephalomyelitis (EAE), in which the neuropathological lesions are mediated by T cell reactivity to myelin basic protein (MBP) (Sgroi *et al.*, 1986), strongly suggest that MS could be a disease of the immune system in which the CNS is the target. The autoimmune pathogenesis of MS is also supported by the finding in cerebrospinal fluid (CSF) of an oligoclonal expansion of T cells using a restricted number of T cell receptor (TCR) V gene segments (Hafler *et al.*, 1988; Wucherpfenning *et al.*, 1990), as the specific T cell clones recognizing short MBP peptides in mice or rats with EAE (Acha-Orbea *et al.*, 1988; Urban *et al.*, 1988; Chou *et al.*, 1989).

Moreover, it has been recently suggested that some inflammatory cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), can play a crucial role in tissue injury during MS (Waksman & Reingold, 1986; Panitch *et al.*, 1987; Robbins *et al.*, 1987; Traugott & Lebon, 1988; Frei *et al.*, 1987; Selmaj & Raine, 1988; Benveniste, Sparacio & Bethea, 1989).

Since the cellular source of these cytokines in CSF of MS patients has not yet been clearly elucidated (Hofman *et al.*, 1989), we generated CSF-derived T cell clones from MS patients, in order to investigate their functional properties, with particular regard to the production of IFN-γ and TNF-α in comparison to other cytokines involved in modulation of immune response such as interleukin-2 (IL-2) and interleukin-4 (IL-4). Our data demonstrated that upon triggering by phytohaemagglutinin (PHA), the majority of CSF-derived T cell clones had the potential to produce unusually large amounts of TNF-α, compared with peripheral T clones and with unrelated tissue-infiltrating T lymphocytes, and suggest that this cytokine could play a pivotal role in the pathogenesis of the disease.

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PATIENTS AND METHODS

Patients

The study population consisted of two female patients, aged 30 and 34 years, respectively, with clinically definite MS, according to the criteria of Schumacher *et al.* (1965), and relapsing–progressive disease. The patients were not treated with immunosuppressive therapy for at least 6 months before the beginning of the study. CSF was obtained during an exacerbation phase of the disease.

Isolation of CSF lymphocytes and peripheral blood mononuclear cells (PBMC)

CSF was obtained through lumbar puncture and immediately centrifuged at 300 *g* at 4°C. The cells were washed in RPMI 1640 (Flow Lab, UK) supplemented with 10% heat-inactivated fetal calf serum (Flow Lab), 1% glutamine (Flow Lab), 25 mM HEPES (Gibco), 1% sodium pyruvate (Flow Lab) 100 U/ml pennicillin (Flow Lab) 100 μg/ml streptomycin (Flow Lab) and 2·5 μg/ml fungizone (Flow Lab) (complete medium). The PBMC from the same donors were isolated by Ficoll–Hypaque density gradient centrifugation and washed three times in complete medium (Böyum, 1968).

Generation of CSF-derived and peripheral T cell clones

CSF lymphocytes and PBMC were immediately cloned by limiting dilution at 1 or 0·3 cell/well in the presence of 1 μg/ml PHA, 10^5 alogeneic irradiated PBMC, and 30 U/ml human recombinant IL-2 (rIL-2; La Roche, Basel, Switzerland). After two or three weeks, cell growth was detected using an inverted microscope. The growing cells were expanded in rIL-2-containing medium and allogeneic iPBMC plus PHA every 2–3 weeks.

Surface phenotypic analysis

The surface phenotype of CSF-derived T cell clones was determined by indirect immunofluorescence microscopy, using murine monoclonal antibodies (MoAbs): OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKIa (anti-DR), OKT26a (anti-CD25: IL-2-receptor-bearing lymphocytes) (Ortho, Raritan, NJ), and WT31 MoAb, directed to a common epitope of the alpha/beta heterodimer of the TCR (Becton Dickinson, Mountain View, CA).

Production of cytokines

Cloned T cells (2×10^5) were washed extensively and cultured in complete medium in a total volume of 0·2 ml into flat-bottomed 96-well plates, in the presence or in absence of PHA (1 μg/ml) at 37°C. Supernatants were removed after 48 h for cytokine determination. IL-2, IL-4, and TNF-α production was assessed using ELISA kits (InterTest 2 and interTest 4, Genzyme, Boston, MA; Biokine TNF test kit, T Cell Science, Cambridge, MA), IFN-γ, using a radioimmunoassay test kit (Centocor, Malvern, PA). Culture supernatants showing cytokine levels of 5 s.d. above those obtained using complete medium as control were considered positive.

Cytotoxicity assay

Cytotoxic T lymphocyte (CTL) activity was tested in a 4-h ^{51}Cr release assay. Cloned T cells, used as effector cells, were incubated into round-bottomed microtitre plates (Falcon) with ^{51}Cr-labelled target cells at an effector/target ratio of 10:1. The cell lines used as target in the cytotoxicity assay were the natural killer (NK) sensitive K562 cell line (NK activity), and the NK-resistant murine P815 cell line in the presence or absence of 1 μg/ml PHA (lectin-dependent cell-mediated cytotoxicity: LDCC). The microplates were centrifuged at 100 *g* for 5 min and incubated at 37°C for 4 h. After incubation, the supernatants were analysed for ^{51}Cr release. The specific lysis was calculated as per cent cytotoxicity according to the formula:

$$\frac{\text{Experimental} - \text{Spontaneous release}}{\text{Total} - \text{Spontaneous release}} \times 100.$$

T clones showing specific lysis higher than 20% were considered positive for cytolytic activity.

RESULTS

Generation of CSF-derived T cell clones

After 2–3 weeks from cloning procedure, proliferating cells were apparent in 52 out of 576 wells containing the CSF lymphocytes cloned in the presence of PHA, rIL-2 and allogeneic PBMC. Phenotypic analysis revealed that all the T cell clones expressed the alpha/beta chain of TCR; 40 out of 52 clones were $CD3^+CD4^+CD8^-$, and 12 were $CD3^+CD4^-CD8^+$. All the clones were $CD25^+$.

Production of cytokines

Thirty CSF T clones were assayed for their ability to produce IL-2, IL-4, IFN-γ and TNF-α following PHA stimulation. As shown in Fig. 1, both $CD4^+$ and $CD8^+$ T clones released large amounts of TNF-α and IFN-γ, as well as being capable of secreting IL-2 (Fig. 2). Otherwise, no detectable production of IL-4 was observed (Fig. 2). The CSF-derived T clones were compared for their ability to produce cytokines with 20 autologous random peripheral T clones and with a series of cytotoxic T cell clones derived from lymphocytes infiltrating the liver of two patients with hepatitis B surface antigen (HBsAg) positive chronic active hepatitis (CAH). These last T clones were generated with the same cloning procedure of CSF-derived T clones and were demonstrated to produce large amounts of inflammatory cytokines (Barnaba *et al.*, 1989; Franco *et al.*, 1990). CSF-derived T clones were also compared with peripheral T clones obtained from atopic patients producing large amounts of IL-4 (Benvenuto *et al.*, 1991) for the production of this cytokine. The TNF-α level in supernatants of CSF-derived T cell clones was significantly higher than in supernatants of both liver-derived T clones and peripheral T clones. None of the clones showed any detectable cytokine production without PHA stimulation (data not shown).

Comparable results for cytokine production were observed in both MS patients.

Analysis of cytolytic activity of CSF T clones

The cytolytic potential of T clones was assessed by an LDCC assay and by an NK assay. The results in Fig. 3 show that all the $CD8^+$ T clones and six out of 20 $CD4^+$ T clones tested were able to lyse the NK-resistant P815 target cells when cultured in the presence of PHA. Moreover, all the $CD8^+$ T clones also showed NK activity, while no $CD4^+$ T clone was able to lyse the NK-sensitive K562 cells. In contrast, none of the clones had cytolytic activity against the NK-resistant P815 cells in the absence of PHA, ruling out the possibility that these clones were lymphokine-activated killer (LAK) cells.

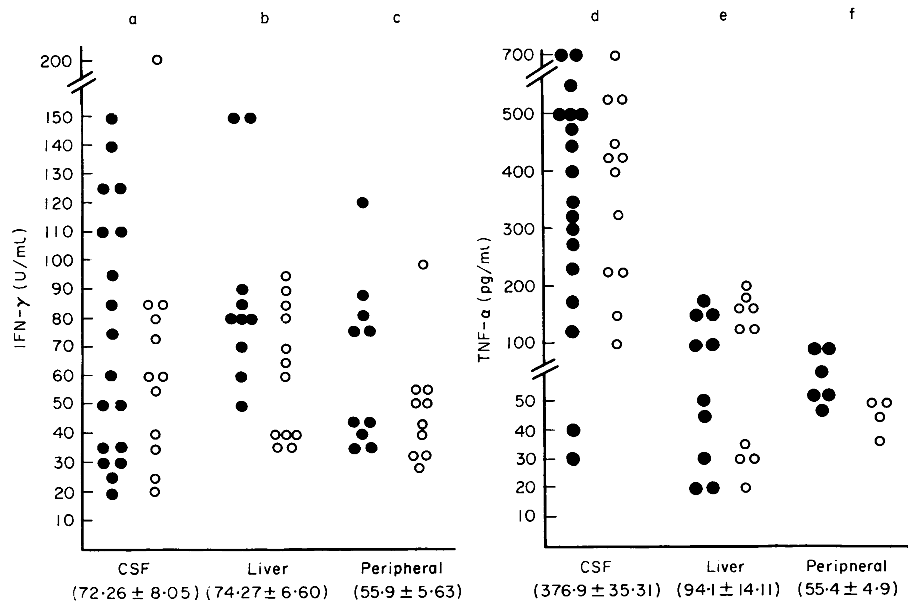

Fig. 1. IFN-γ (a–c) and TNF-α (d–f) production by CSF-derived and peripheral T cell clones from MS patients, and by liver-derived T cell clones from $HBsAg^+$ CAH patients (CD4, ●; CD8, ○). The production of cytokines by CSF-derived T clones, liver-derived T-clones and peripheral T clones were compared and the results were analysed by two-tailed Student's *t*-test for unpaired data. Values in parentheses represent the data of cytokine concentration in supernatants of T clones expressed as mean ± s.e.m.; a *versus* b, NS; a *versus* c, $P<0·05$; d *versus* e, $P<0·0005$; d *versus* f, $P<0·0005$.

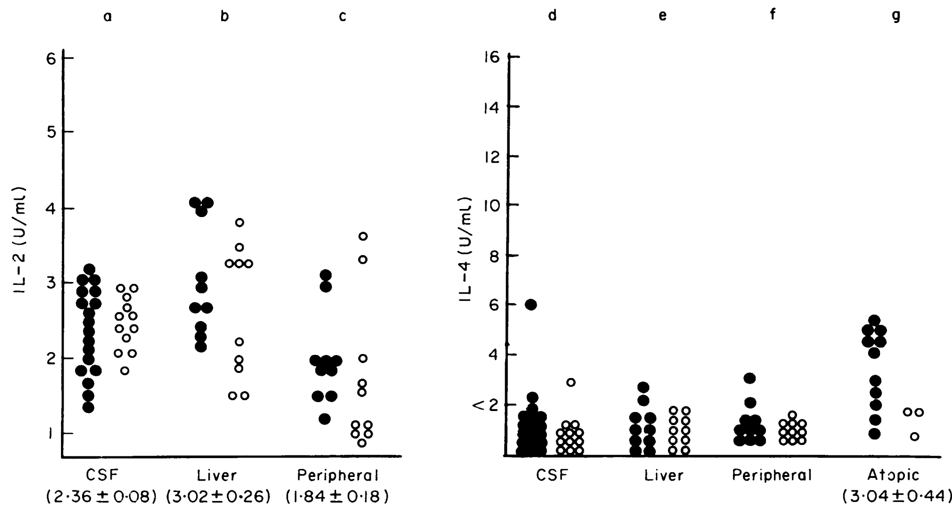

Fig. 2. IL-2 (a–c) and IL-4 (d–g) production by CSF-derived and peripheral T clones from MS patients, by liver-derived T clones from CAH patients, and by peripheral T clones from atopic patients (CD4, ●; CD8, ○). Production of cytokines by the different groups of T clones was compared and the results were analysed by two-tailed Student's *t*-test for unpaired data. Values in parentheses represent the data of cytokine concentration in SN of T clones expressed as mean ± s.e.m.; b *versus* a, $P<0·01$; b *versus* c, $P<0·01$; d *versus* e, NS; d *versus* f, NS; g *versus* d, $P<0·01$.

DISCUSSION

The study of CSF-derived T lymphocytes is hampered by the small number of lymphocytes that can be obtained through lumbar puncture. Using techniques for growing long-term T clones (Fathman & Fitch, 1984), we were able to establish 52 T cell clones (40 $CD4^+$ and 12 $CD8^+$) from CSF-lymphocytes of MS patients and to study their phenotype and function, with particular regard to their ability to produce cytokines.

All the $CD4^+$ T clones tested, as well as the $CD8^+$ T clones, were found to produce large amounts of IFN-γ and of TNF-α after incubation with PHA, while no detectable level of IL-4 was observed in the supernatants of stimulated cultures.

Several lines of evidence have suggested that $CD4^+$ T cells can be categorized in mouse into two subclasses, T helper (Th) 1 and Th2, based on the array of cytokines they secrete (Mosmann *et al.*, 1986). It has been found that Th1 produce IL-2, IFN-γ and lymphotoxin, whereas Th2 produce IL-4 and interleukin-5

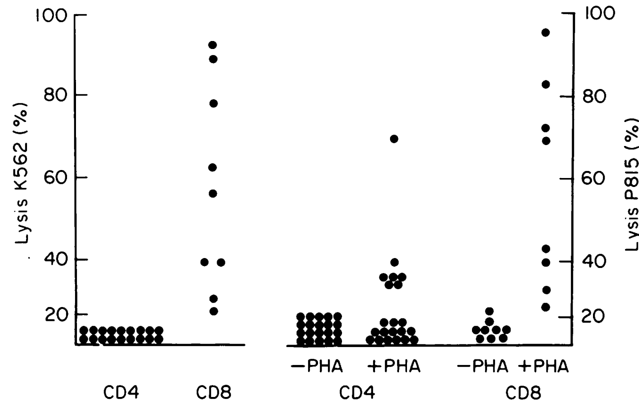

Fig. 3. NK and LDCC cytolytic activity of CSF-derived T cell clones. Results are given as per cent of specific lysis at 10 : 1 effector/target ratio.

(IL-5). Interleukin-3 (IL-3) is apparently produced by cells of both subsets, and granulocyte-macrophage factor (GMF) and TNF are preferentially but not exclusively produced by Th1 cells (Mosmann & Coffmann, 1989). The Th1 cell subset could help polyclonal B cell response, mediate delayed-type hypersensitivity (DTH) and kill appropriate target cells. The Th2 subset should help mainly specific B cell response, failing to mediate killing and DTH (Cherwinski *et al*, 1987). Several reports suggest that Th1 and Th2 cells represent two different subsets of late-memory T cells that have differentiated from a common precursor subset with a much less restricted lymphokine pattern (Firestein *et al.*, 1989; Street *et al.*, 1990). These precursor cells, for which the designation of Th0 cells has been proposed, produce IL-2 and IFN-γ as well as IL-4 (Firestein *et al.*, 1989).

It has been postulated that in humans $CD4^+$ cells could be divided in two subsets, according to their functional properties (Bottomly, 1988), but the identification of a specific marker is still controversial. Large numbers of random and alloreactive human $CD4^+$ T cell clones produce IL-2, IFN-γ and IL-4 simultaneously (Paliard *et al.*, 1988; Maggi *et al.*, 1988), thereby resembling murine Th0 cells. Only some T clones have been described with Th1- or Th2-like lymphokine profiles (Maggi *et al.*, 1988; Umetsu *et al.*, 1988). Little is known, however, about the distribution and functional role of human Th1- and Th2-like T cells.

Our data seem to suggest that $CD4^+$ T cell precursors isolated from the CSF of patients with MS could constitute a subset with functional properties, similar to those of Th1 subset of the mouse, and like Th1 cells could be responsible for inflammatory response rather than for helper activity.

The cytotoxic activity of our cytolytic CSF-derived T clones could be mediated by the production of IFN-γ and TNF-α. TNF has been reported to mediate cytotoxicity by direct lysis of target cells (Beutler & Cerami, 1986) and to act synergystically with IL-1 and IFN-γ to augment monocyte/macrophage cytotoxicity (Philip & Epstein, 1986; Kehrl, Miller & Fauci, 1987). The findings that in MS patients the levels of TNF, IFN-γ and IL-1 were shown to be increased in blood and spinal fluid (Merril *et al.*, 1989), that their enhanced production by peripheral blood cells seems to precede clinical exacerbations of the disease (Beck *et al.*, 1988), and that treatment of MS patients with IFN-γ provoked an exacerbation of the disease (Panitch *et al.*, 1987) suggest that these cytokines may be relevant in MS progression. Recently, the possible role of genes on chromosome 6 other than those of HLA-D locus was suggested to contribute to the pathogenesis of MS, particularly those encoding TNF-α, TNF-β, and complement components (McFarlin & Lachmann, 1989).

In addition, the overproduction of TNF and IFN-γ could modulate the class II MHC antigen expression on astrocytes (Wong *et al.*, 1984) and endow these last cells with the capacity to perform antigen presentation function with respect to autoreactive cytolytic T lymphocytes, as demonstrated in animal EAE models (Fontana, Fiers & Wekerle, 1984; Massa, Dorries & ter Meulen, 1986; Sun & Wekerle, 1986).

More patients should be studied with these techniques to determine is similar patterns of cytokines produced by CSF T clones can be identified, in order to extend these data to the general underlying mechanisms of MS disease; however, the pathogenetic role of TNF in MS is emphasized in our study by the demonstration that CSF T cell clones synthesized this cytokine in significantly larger amounts than unrelated tissue-infiltrating T cell clones generated from liver of chronic active hepatitis patients and showed to produce high levels of inflammatory cytokines (Barnaba *et al.*, 1989; Franco *et al.*, 1990).

Furthermore, recent immunohistochemical studies demonstrated the presence of TNF^+ cells identified as astrocytes, as well as macrophages, at the plaque site of MS patients (Hofman *et al.*, 1989). Although activated astrocytes and microglial cells have been demonstrated to synthesize and secrete TNF (Robbins *et al.*, 1987; Frei *et al.*, 1987), the study of Hofman *et al.* (1989), showing TNF binding to astrocytes, did not identify the cellular source of this cytokine. Our results indicate that the selective accumulation of T lymphocytes producing large amounts of TNF in CSF in the course of MS could account for the presence of TNF^+ astrocytes, and that this factor may be involved in the demyelination and disease progression. This last hypothesis is also supported by the demonstration that TNF participates in oligodendroglial lysis and degeneration of myelin sheat (Selmaj & Raine, 1988).

The TNF involvement in MS pathogenesis could suggest new therapeutic strategies involving immunological manipulations, and studies of EAE and immune reactivity to MBP could provide the basis for treatment with antibodies to TNF.

The antigen to which our CSF T cell clones were directed remain unknown. Our experiments did not show any proliferative response to MBP (data not shown), unlike other studies, in which a low specific response was observed (Fleisher *et al.*, 1984; Hafler *et al.*, 1985c). Taken together, these findings seem to suggest that MBP is not the only target for CSF-derived T cells and that other antigens, like viruses or different white matter components may play a role in MS.

NOTE ADDED IN PROOF

While this manuscript was being processed for publication, Ruddle *et al.* (1990) published a paper entitled 'An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis' (*J. exp. Med.* **172,** 1193).

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