Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis

D J Mahad, S J L Howell, M N Woodroffe

Objective: To define the chemokine profile in the CSF of patients with multiple sclerosis (MS) and compare it with three control groups; patients with benign headache (headache), non-inflammatory neurological diseases (NIND), and other inflammatory neurological diseases (IND). In addition, the correlations of CSF chemokine concentrations with chemokine receptor expression on CSF CD4+ T cells and with clinical disease activity were assessed.

Methods: Forty three patients with MS, 24 with IND, 44 with NIND, and 12 with benign headache undergoing diagnostic or therapeutic lumbar puncture were included. Supernatant fluid from CSF was assessed for four β (CXCL2, CCL3, CCL4, CCL5) and two α (CXCL9, CXCL10) chemokines by enzyme linked immunosorbent assay (ELISA). Chemokine receptors CCR3, CCR5, and CXCR3 on CD4+ T cells from eight patients with MS were analysed using directly conjugated fluorescent labelled monoclonal antibodies and flow cytometry.

Results: CXCL10, formerly interferon-γ inducible protein-10 (IP-10), was significantly increased and CCL2, formerly monocyte chemoattractant protein-1 (MCP-1), was significantly reduced in the CSF of patients with MS and IND compared with those with benign headache and NIND. Concentrations of CXCL10 were significantly greater in patients with relapsing-remitting compared with secondary progressive MS and correlated significantly with CXCR3 expression on CSF CD4+ T cells from patients with MS. Concentrations of CXCL10 decreased and CCL2 concentrations increased as time from the last relapse increased in patients with MS.

Conclusion: Increased CXCL10 and decreased CCL2 concentrations in the CSF are associated with relapses in MS. Although serial values from individual patients were not available, this study suggests that CXCL10 and CCL2 may return towards baseline concentrations after a relapse. Correlation of CXCL10 with CD4+ T cell expression of CXCR3 was consistent with its chemoattractant role for activated lymphocytes. Thus CXCL10 neutralising agents and CXCR3 receptor antagonists may be therapeutic targets in MS.

Chemokines are a subgroup of cytokines with selective chemoattractant properties. They are key mediators of inflammation and have major effects on migration of cells to sites of inflammation as well as activation of recruited and resident CNS cells. Chemokines are divided into four families depending on the number of amino acids between the two N-terminal cysteine residues, α, β, γ, and δ. Most α-chemokines attract neutrophils, whereas CXCL10, formerly interferon-γ inducible protein-10 (IP-10), and CXCL9, formerly monokine induced by γ-interferon (Mig), attract T lymphocytes. Hence CXCL10 and CXCL9 as well as the β chemokines, which attract T lymphocytes and monocytes, are of interest in multiple sclerosis (MS).1–4 Chemokines bind to seven transmembrane domain G protein coupled receptors, which are classified as CXCR (1–6), CCR (1–10), CXCR, and CR. Ligation of chemokines to chemokine receptors leads to integrin activation, changes in cytoskeleton, and chemotactic cell migration. T lymphocytes play a critical part in the pathogenesis of MS and chemokines are likely to be involved in the steps which lead to recruitment of leucocytes across the blood-brain barrier. Chemokines and their receptors are expressed by infiltrating and resident cells in MS lesions.6–9 Expression of CCL2 in astrocytes has been demonstrated in active plaques7; CXCL10 is expressed by astrocytes and perivascular lymphocytes.8 Recent studies have reported the expression of chemokines in the CSF of patients with MS.10

In one study, CCL3, formerly macrophage inflammatory protein 1 α (MIP-1α), was the predominant chemokine in MS relapses whereas in a second study CXCL9, CXCL10, and CCL5 (formerly regulated upon activation of normal T cell expressed and secreted, RANTES) were significantly increased in patients with MS within 2 weeks of a relapse.11 A recent study analysed serum and CSF chemokine concentrations in patients with MS and confirmed the above finding for CXCL10 and CCL2.12

The aim of this study was to quantify the chemokine expression in the CSF of patients with MS and compare it with three control groups and correlate the concentrations in relapsing-remitting and secondary progressive MS with clinical disease (relapse) activity. CSF white cell count, and CSF CD4+ T cell expression of chemokine receptors CCR3, CCR5, and CXCR3. The control groups included patients with benign headache (headache), non-inflammatory neurological diseases (NIND), and other inflammatory neurological diseases (IND).

PATIENTS AND CLINICAL DETAILS

The study was approved by the local research ethics committee. One hundred and twenty three patients with neurological symptoms were included: 43 patients with Poser criteria definite MS (88% of whom had oligoclonal bands pattern 2 or 3), mean age 38, and sex ratio (women:men) 1.9 (35 patients with relapsing-remitting and eight with secondary progressive disease).13 Clinical details including the time since...
the last relapse were recorded at the time of the lumbar puncture. A relapse was defined according to Schumacher et al. Twelve patients with benign headaches, mean age 41 and sex ratio 2:0, 44 patients with NIND, mean age 35 and sex ratio 0.55, and 24 patients with IND, mean age 45 and sex ratio 3:8. Patients with benign headaches had sudden onset of headache without evidence of subarachnoid haemorrhage and after investigations had no diagnosis other than non-specific headache. Patients in the NIND group had idiopathic intracranial hypertension (n=10), suspected normal pressure hydrocephalus (n=7), motor neuron disease (n=4), axonal neuropathy (n=8), complex migraine (n=6), age related ischaemic changes on MRI (n=4), epilepsy with normal CT head scans (n=2), cerebellar atrophy (n=2) and multisystem atrophy (n=1). Patients in the IND group had partial cord syndromes/transverse myelitis (n=7), acute disseminated encephalomyelitis (n=3), cerebral lupus/vasculitis (n=3), cerebrovascular infarct (n=2), viral meningitis/encephalitis (n=5), antiphospholipid syndrome (n=3), and globlastoma multiforme (n=1). None of the patients had received immunomodulatory therapy for 3 months before the lumbar puncture.

MATERIALS AND METHODS

CSF

A sample of CSF (10 ml) was removed and immediately centrifuged at 250 g for 10 minutes. The supernatant was decanted and stored within 30 minutes of the lumbar puncture at −70°C.

Samples of CSF with greater than 12×10⁶ cells/ml were stained and fixed within 4 hours for flow cytometry. A white cell count in CSF was obtained through routine microscopy. Protein and diffusion ratios for CSF were also determined.

Enzyme linked immunosorbent assay (ELISA)

Chemokines

Frozen CSF samples were thawed and the chemokine concentrations quantified using paired monoclonal anti-CXCL10 and anti-CXCL9 antibodies (R and D Systems, Minneapolis, USA) on 96 well plates. A capture antibody concentration of 4 µg/ml for CXCL10 and CXCL9 with a detection antibody concentration of 100 ng/ml for CXCL10 and 200 ng/ml for CXCL9 were used after optimisation using recombinant CXCL9 and 10 (R and D Systems, UK). The detection limits of the assays were 9.0 pg/ml. Intra-assay variability was <10%, with an interassay variability of 12%. All samples were analysed undiluted in duplicate according to the manufacturer’s instructions and reanalysed when variability between duplicates was greater than 20%.

β chemokines

Quantikine kits for CCL2, 3, 4, and 5 were used according to the manufacturer’s guidelines (R and D Systems, UK). The detection limits of the assays were 5 pg/ml for CCL2, 10 pg/ml for CCL3, 4 pg/ml for CCL4, and 8 pg/ml for CCL5. All samples were analysed undiluted in duplicate.

Flow cytometry

Seven patients with relapsing-remitting MS and one patient with secondary progressive disease (with superimposed relapse) had an adequate number of cells (>12 white cells/µl) for T cell phenotyping using flow cytometry. Blood was obtained and lymphocytes separated using a Histopaque (Sigma) gradient. Peripheral blood and CSF lymphocytes were labelled with directly conjugated monoclonal antibodies following the manufacturer’s instructions using anti-CD4 cytokrome (Pharmingen), anti-CCR3 phycoerythrin, anti-CCR5 phycoerythrin, and anti-CXCR3 FITC (R and D Systems) as well as isotype specific antibody controls. Antibodies were used at 10 µl/10⁶ cells in 50 µl FACS buffer (PBS+2% heat inactivated fetal calf serum). Cells were fixed in 2% paraformaldehyde and stored in the dark before analysis using a Becton and Dickinson FACSC flow cytometer.

STATISTICS

Data were analysed using SPSS version 10 and non-parametric tests (Kruskal-Wallis and Mann-Whitney). Spearman’s correlation coefficient was calculated using EXCEL. Multiple linear regression analysis was used to establish a correlation between CXCL10 and CCL2 when corrected for time since last relapse. p Values <0.05 were considered to be statistically significant.

RESULTS

Chemokines in CSF

Concentration of CXCL10 was significantly increased in patients with MS as well as in inflammatory and non-inflammatory neurological diseases when compared with benign headache controls (p<0.001, fig 1 A). Although CXCL10 in MS and IND did not significantly differ (p=0.528), CXCL10 in MS and IND was significantly increased compared with NIND (p=0.002). Patients with secondary progressive disease had significantly lower CXCL10 concentrations (mean 88 pg/ml, n=8) compared with relapsing-remitting patients without progression (mean 255 pg/ml, n=20, p=0.004, fig 1 B). In the benign headache group, concentrations of CXCL10 were detectable above the sensitivity limit in only three samples, all from older patients. Spiking the samples with recombinant chemokines (500 and 1000 pg/ml) resulted in the detection of the expected concentrations (87% recovery). Concentrations of CCL2 were significantly reduced in MS compared with headache, NIND, and IND controls (p=0.003, p=0.005, p=0.024 respectively, fig 1 C). The range of CCL2 concentrations within the IND group was dependent on disease type and was wide (148–2084 pg/ml). Patients with partial cord syndromes and transverse myelitis had lower values compared with patients with viral meningitis, CNS vasculitis, and active cerebral lupus. Concentrations of CXCL9 in the CSF of patients with MS compared with those with NIND were just significantly increased (p=0.045, fig 1 D). Two patients who relapsed within a few days after the lumbar puncture (one within 24 hours and the other within 7 days) had low CCL2 (160, 210 pg/ml) and low CXCL10 concentrations (97, 99 pg/ml) compared with the mean values for the group.

Concentrations of three other β chemokines CCL3, CCL4, and CCL5 did not show a significant difference between groups. Concentrations of CCL3 were all below the detection limit (range 4.5–6 pg/ml). Concentrations of CCL5 ranged from 10–15 pg/ml and 11% of samples were below the detection limit. All CCL4 concentrations were detectable above the minimum limit of the assay in all groups. The headache control group had the highest mean concentration (n=5, 303 pg/ml), which was not significantly greater than the other groups (MS, n=23, 162, NIND, n=17, 156, IND, n=10, 128).

Chemokine correlation with disease activity

Concentrations of CXCL10 correlated significantly and negatively with time from relapse (r=-0.51, p<0.02, fig 2 A). The CSF white cell count (n=37, r=0.64, p<0.001 fig 2 C) also correlated significantly with CXCL10 concentrations. One patient with secondary progressive disease who had relapsed 180 days before the lumbar puncture had increased CXCL10 (609 pg/ml). All other patients with secondary progressive disease did not have superimposed clinical relapses. Concentrations of CCL2 also correlated significantly and positively with time from relapse (n=25, r=0.51, p<0.02, fig 2 B). The CSF IgG concentrations, the diffusion ratios, and the CSF protein concentrations did not correlate significantly with chemokine concentrations (data not shown).
**CSF T cell chemokine receptor expression**

Eight CSF samples from patients with MS with an adequate number of cells (seven relapsing-remitting and one secondary progressive) were analysed using flow cytometry for CD4, CCR5, CCR3, and CXCR3. The receptor for CXCL10, CXCR3, was present on 86% of the CD4+ cells in the CSF of patients with MS; CCR5 was present on 7% of the CD4+ T cells, and CCR3 was present on 1.20% of CD4+ T cells in the CSF of patients with MS. Most CCR5 (86%) and CCR3 (84%) staining was expressed within the CXCR3+ subset of CD4+ T cells. The percentage of CXCR3 expressing CD4+ T cells in the CSF correlated significantly with CXCL10 concentrations (n=7, p<0.05, r=0.76, fig 2 D).

**DISCUSSION**

Interaction between chemokines and their receptors is an important stage in the process of leucocyte recruitment to sites of inflammation. Chemokines and their receptors have been described in various neurological disorders. These include inflammatory myopathies, uveitis, AIDS dementia, viral and tuberculous meningitis, HTLV-1 associated myelopathy, and glioblastoma multiforme.15–20

There is increasing evidence for an association of changes in CSF chemokine concentrations in patients with multiple sclerosis as well as with other inflammatory diseases affecting the CNS. Our findings on CXCL10 and CCL2 are in agreement with previous reports suggesting an association of increased CXCL10 and reduced CCL2 with clinical relapses in MS.11–12 In addition, we showed in patients with MS, a significant inverse correlation of concentrations of CXCL10 and a significant positive correlation of CCL2 in the CSF with time from last clinical relapse. Also there was a significant difference in CXCL10 concentrations between patients with relapsing-remitting and those with secondary progressive MS and a low concentration of CXCL10 and CCL2 in the CSF immediately (up to 7 days) before relapse in two patients with MS. Similar changes were found for CXCL10 concentrations in the CSF of patients with other inflammatory neurological diseases.

CXCL10 has a chemotactic role in T cells via their receptor CXCR3. Astrocytes, glial cells, endothelial cells, macrophages, and T lymphocytes can express CXCL10.14–15 CXCL9 and 10 are expressed by macrophages within plaques and by astrocytes in surrounding parenchyma in acute demyelinating lesions in MS brain tissue.7 The finding of CXCR3+ T cells within plaques and perivascular cuffs as well as the increased number of CXCR3+ lymphocytes in the CSF of patients with MS compared with their peripheral blood is consistent with a chemotactic role for CXCL10.16 The significant correlation between CXCL10 and the percentage of CXCR3 expressing

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**Figure 1** Chemokine concentrations in CSF. Numbers on the x axis indicate the number of patient samples analysed in each group. RR, relapsing-remitting MS; S progressive, secondary progressive MS.
lymphocytes found in the present study provides further evidence for the chemotactic role of CXCL10. Disease pathogenesis may be influenced in other ways by CXCL10 as it induces the production of nitric oxide and reactive oxygen species by macrophages and induces differentiation and activation of lymphocytes, which may result in demyelination in the CNS. Animal models suggest a pathogenic role for CXCL10 in experimental autoimmune encephalomyelitis; CXCL10 is upregulated at disease onset and during relapse in this disease. The significant correlation of CXCL10 with time from last relapse in patients with MS shown in this study implies that CXCL10 tends to decrease with time from a clinical relapse. The correlation coefficient between CXCL10 and time from last clinical relapse may have been somewhat reduced by the asymptomatic MS disease activity, as MRI studies show that this may occur up to 20-fold more often than symptomatic disease activity. Concentrations of CXCL10 were significantly lower in patients with secondary progressive disease than with relapsing-remitting disease. This is consistent with relatively less gadolinium enhancing lesions in patients with secondary progressive disease than those with relapsing-remitting disease. In agreement with this study, Franciotta et al found significantly low CXCL10 in stable relapsing-remitting MS compared with patients within 2 months of a clinical relapse. These findings suggest a role for CXCL10 in the inflammatory and demyelinating processes involved in MS relapses. Two patients with relapsing-remitting MS in this study had a clinical relapse immediately after the lumbar puncture (within 24 hours and 7 days). The finding of low CXCL10 in the two patients who relapsed within a few days after the lumbar puncture suggests that the increases in CXCL10 in relation to clinical relapse occurs just before or immediately after the onset of relapse related symptoms.

The finding of lower CCL2 in CSF during MS relapses, although in agreement with two previous studies, contradicts the proinflammatory role suggested for CCL2 in studies of experimental autoimmune encephalomyelitis and findings on postmortem MS brain tissue, which show astrocytes and microglia within MS lesions strongly reactive for CCL2. CCL2 transgenic mice overexpressing CCL2 showed increased numbers of monocytes and macrophages in the CNS and anti-CCL2 antibodies suppressed experimental autoimmune encephalomyelitis relapses. A possible explanation for this discrepancy is that sequestration of CCL2 within MS lesions or impaired secretion of CCL2 by astrocytes and microglia may lead to localised high CCL2 concentrations within MS lesions and consequently reduced concentrations in the CSF. This is by contrast with physiologically high CSF concentrations and no CNS tissue expression of CCL2 in normal people. These changes in MS may lead to a dual role for CCL2 in the pathogenesis of MS. High concentrations of CCL2 within MS lesions would lead to chemoattraction of CCR2 inflammatory cells to the lesion and reduced CSF CCL2 concentrations may bias the Th1 to Th2 ratio of CSF lymphocytes towards Th2. Both patients with a clinical relapse immediately after lumbar puncture (within 24 hours and within 7 days) showed low concentrations of CCL2. Thus the reduced CCL2 concentration in CSF may be present at least 7 days, before the onset of clinical relapse and precede the increase of CXCL10.

Although the concentrations of CCL4 were not significantly different in the CSF in the four groups, it was present at detectable concentrations in all groups, suggesting a physiological role for this chemokine. Our findings for CCL3 and CCL5 are not in agreement with the two previous studies. Increased CCL3 concentrations in CSF in acute relapsing-remitting MS, reported by Miyagishi et al, were also below the minimum detection limit of the ELISA (R and D Systems MIP-1α, Quantikine). Hence, the findings of our study and that of Sorensen et al cast doubt on the relevance of the increased CCL3 CSF concentrations reported by Miyagishi et al. The fact that CSF concentrations of β chemokines were undetectable does not exclude a pathogenic role for β chemokines in MS as CCL3, CCL4, and CCL5 have been demonstrated within MS lesions.

This study demonstrates that CXCL10 and CCL2 are likely to play a part in the pathogenesis of MS; thus these ligands and their receptors may be targets for immunomodulatory therapy. In addition, the study suggests that the reduction of CSF CCL2 occurs before the onset of clinical relapse and the increase in CXCL10 occurs after the onset of relapse. Indirect
data from this study suggest that increased CXCL10 and reduced CCL2 concentrations tend to revert towards baseline concentrations with time from a clinical relapse. CXCL10 is more likely to be significant in the pathogenesis of relapsing-remitting disease than in secondary progressive MS without superimposed relapses.

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