Improvement in disability after alemtuzumab treatment of multiple sclerosis is associated with neuroprotective autoimmunity


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Treatment of early relapsing–remitting multiple sclerosis with the lymphocyte-depleting humanized monoclonal antibody alemtuzumab (Campath [registered trade mark]) significantly reduced the risk of relapse and accumulation of disability compared with interferon β-1a in a phase 2 trial [Coles et al., (Alemtuzumab vs. interferon β-1a in early multiple sclerosis. N Engl J Med 2008; 359: 1786–801)]. Patients treated with alemtuzumab experienced an improvement in disability at 6 months that was sustained for at least 3 years. In contrast, those treated with interferon β-1a steadily accumulated disability. Here, by post hoc subgroup analyses of the CAMMS223 trial, we show that among participants with no clinical disease activity immediately before treatment, or any clinical or radiological disease activity on-trial, disability improved after alemtuzumab but not following interferon β-1a. This suggests that disability improvement after alemtuzumab is not solely attributable to its anti-inflammatory effect. So we hypothesized that lymphocytes, reconstituting after alemtuzumab, permit or promote brain repair. Here we show that after alemtuzumab, and only when specifically stimulated with myelin basic protein, peripheral blood mononuclear cell cultures produced increased concentrations of brain-derived neurotrophic factor, platelet-derived growth factor and ciliary neurotrophic factor. Analysis by reverse transcriptase polymerase chain reaction of cell separations showed that the increased production of ciliary neurotrophic factor and brain-derived neurotrophic factor after alemtuzumab is attributable to increased production by T cells. Media from these post-alemtuzumab peripheral blood mononuclear cell cultures promoted survival of rat neurones and increased axonal length in vitro, effects that were partially reversed by neutralizing antibodies against brain-derived nerve growth factor and ciliary neurotrophic factor. This conditioned media also enhanced oligodendrocyte precursor cell survival, maturation and myelination. Taken together, the clinical analyses and laboratory findings support the interpretation that improvement in disability after alemtuzumab may result, in part, from neuroprotection associated with increased lymphocytic delivery of neurotrophins to the central nervous system.
Introduction

Multiple sclerosis is an immune-mediated disease in which foci of inflammation within the central nervous system cause reversible conduction block, demyelination and acute axonal transection. Early in the relapsing–remitting phase relapse-related symptoms often resolve fully through plasticity, removal of physiological conduction block and by partial or complete remyelination. Later, patients accumulate apparently fixed deficits after relapses, commonly attributed to non-inflammatory mechanisms: failure of adaptation, axonal transection and persistent demyelination (Compston and Coles, 2008). Therefore, suppression of inflammation should not reverse fixed deficits, but would at most prevent further accumulation of disability. However, we have previously observed an improvement in disability following treatment of early relapsing–remitting multiple sclerosis with the lymphocyte-depleting monoclonal antibody alemtuzumab [Campath (registered trade mark)] (Coles et al., 2006, 2008). Here, we explore the mechanism for this effect.

Alemtuzumab is a humanized monoclonal antibody that targets CD52, found on lymphocytes and monocytes. Already licensed as a treatment for chronic lymphocytic leukaemia, annual pulses of alemtuzumab are being tested as a potential treatment of relapsing–remitting multiple sclerosis with the lymphocyte-depleting monoclonal antibody alemtuzumab [Campath (registered trade mark)] (Coles et al., 2006, 2008). Here, we explore the mechanism for this effect.

Disability improvement after alemtuzumab was first observed in an uncontrolled open-label study of 22 patients with active relapsing–remitting multiple sclerosis, with mean disease duration of 2.7 years and mean score on the Expanded Disability Status Scale (EDSS; Kurtzke, 1983) of 4.8 (Coles et al., 2006). In the year before alemtuzumab, their mean annualized relapse rate was 2.9 and disability had worsened on average by (+) 2.2 EDSS points. Alemtuzumab reduced their relapse rate to 0.2 (P=0.001) and, at 6 months, mean disability had improved by (−) 1.2 points compared with baseline. At subsequent time points, up to 36 months after alemtuzumab, smaller but still significant incremental reductions in disability were seen. This improvement in disability after alemtuzumab was confirmed in a phase 2 trial in which it was compared with interferon β-1a in patients with early relapsing–remitting multiple sclerosis (Coles et al., 2008). The risks both of relapse and sustained accumulation of disability over 36 months were reduced by over 70% compared with interferon β-1a. In addition, the mean disability of patients treated with alemtuzumab improved by (−) 0.39 EDSS points over 36 months follow-up, whereas those receiving interferon β-1a continued to acquire disability by (+) 0.38 points (P=0.0001), both from a mean baseline score of 2.0. MRI T1 brain volume in the alemtuzumab group increased from month 12 to 36 (avoiding the initial pseudo-atrophy of anti-inflammatory treatment) but decreased in the interferon β-1a group (P=0.02), suggesting tissue restoration after alemtuzumab.

Inflammation promotes healing in several contexts. In multiple sclerosis and experimental models of demyelination, remyelination is most prominent within areas of inflammation (Morell et al., 1998; Wolswijk, 2002; Zhao et al., 2006) and is impaired in mice lacking either T cells (Bieber et al., 2003) or the pro-inflammatory cytokines interleukin (IL)-1β (Mason et al., 2001) and tumour necrosis factor alpha. In rodent models of optic nerve crush, contusive spinal cord injury and retinal ganglion cell damage induced by glutamate toxicity, the presence and activation of peripheral brain-reactive T lymphocytes reduces secondary neuronal loss (Moalem et al., 1999; Hauben et al., 2000a, b; Barouch and Schwartz, 2002; Kipnis et al., 2002b, 2004). Production of neurotrophic factors by these T cells may contribute to repair (Kerschensteiner et al., 1999; Moalem et al., 2000). This phenomenon has been termed ‘neuroprotective autoimmunity’.

We have considered three possible explanations for the improvement in disability following alemtuzumab. Firstly, the disability at baseline results from ongoing inflammation, perhaps due to soluble factors such as nitric oxide causing reversible conduction block (Moreau et al., 1996; Redford et al., 1997; Smith et al., 2001), and subsequent improvement in disability follows restored propagation of the action potential when alemtuzumab suppresses this inflammation. Secondly, alemtuzumab may permit endogenous repair mechanisms by suppressing cerebral inflammation more completely than interferon β-1a. Thirdly, alemtuzumab could actively promote tissue restoration by modifying the immune response to brain self-antigens and inducing neuroprotective autoimmunity. We have investigated these hypotheses by post hoc analyses of the phase 2 CAMMS223 trial and by in vitro studies of neurotrophins produced by peripheral blood mononuclear cells (PBMCs) from patients before and after alemtuzumab treatment.

Materials and methods

Patient cohorts

Retrospective analysis of disability data from CAMMS223 included all 334 patients entered into this phase 2, randomized, blinded trial (Coles et al., 2006, 2008). The risks both of relapse and sustained accumulation of disability over 36 months were reduced by over 70% compared with interferon β-1a. In addition, the mean disability of patients treated with alemtuzumab improved by (−) 0.39 EDSS points over 36 months follow-up, whereas those receiving interferon β-1a continued to acquire disability by (+) 0.38 points (P=0.0001), both from a mean baseline score of 2.0. MRI T1 brain volume in the alemtuzumab group increased from month 12 to 36 (avoiding the initial pseudo-atrophy of anti-inflammatory treatment) but decreased in the interferon β-1a group (P=0.02), suggesting tissue restoration after alemtuzumab.
et al., 2008). Briefly, all patients were treatment naïve, had early relapsing–remitting multiple sclerosis with baseline disability scores of 3.0 or less on the EDSS and a disease duration of ≤3 years, with at least two relapses in the previous 2 years, at least one relapse in the previous 12 months, and at least one gadolinium enhancing lesion on any one of four consecutive monthly screening MRI scans. Patients were randomized to receive either subcutaneous interferon β-1a (at a dose of 44 μg) three times per week or two to three annual intravenous cycles of alemtuzumab (at a dose of 12 mg or 24 mg per day, for 5 days for the initial cycle and 3 days for subsequent cycles) over 36 months. There were no differences between the two doses of alemtuzumab in any outcome measure.

To study the effect of alemtuzumab on the production of neurotrophins by PBMCs we sampled a longitudinal cohort of 15 patients (aged 27–51 years, four males) receiving alemtuzumab at one trial centre (Cambridge, UK); 12 were participants in the CAMMS223 trial (LREC 02/315), and 3 were subjects in an investigator-led trial (CAMMS224; LREC 03/078) involving almost identical inclusion criteria to CAMMS223 and with the same treatment schedule (but confined to the 24 mg/day dose). Venous blood samples were taken prior to alemtuzumab and at three months intervals for 12 months thereafter. Controls were 15 healthy volunteers (aged 26–57 years, 4 males), and 10 patients treated with interferon β-1a (aged 21–49 years, 2 males).

A cross-sectional cohort of patients were used to explore the cellular source neurotrophins; this consisted of 5 healthy controls (1 male), 5 people with untreated multiple sclerosis (2 males) and 8 patients 9–12 months after alemtuzumab treatment (2 males). A further cross-sectional cohort was used to study the effect of conditioned media on rat neuronal and oligodendrocyte cultures; this consisted of 8 healthy controls (4 males), 8 untreated patients with multiple sclerosis (3 males) and 10 patients 9–12 months after alemtuzumab treatment (3 males). All patients were participants either in CAMMS223 or CAMMS224 and were seen at Cambridge, UK. The term ‘untreated patient’ is used in the text to distinguish cross-sectional groups from our cohort of 15 patients followed longitudinally who are designated ‘pre- and post-alemtuzumab’. All patients and controls consented to venepuncture for research purposes (LREC 02/263) and all were free from exposure to other disease modifying agents, including corticosteroids, for at least one month prior to blood sampling.

Isolating and culturing PBMCs

PBMCs, isolated from heparinized blood by centrifugation on a Ficoll density gradient (Pharmacia Biotech UK), were cultured at a concentration of 10⁶/ml in Roswell Park Memorial Institute media (RPMI-1640: 21875-034; Gibco) containing 1% penicillin, 1% streptomycin and 10% foetal calf serum (FCS: S5394; Sigma-Aldrich), unstimulated or with: (i) 50 μg/ml myelin basic protein (MBP) (RDI TRKV8M79; Research Diagnostics Inc.); (ii) 1 μg/ml tetanus toxin (T3644; Sigma-Aldrich), (iii) 10 μg/ml myelin oligodendrocyte glycoprotein (a35–55) (IP-03-371P; Raybiotec), (iv) 100 μg/ml collagen type II fragment a245–270 (H-1604; Bachem), (v) 10 μg/ml myelin basic peptide a87–99 (350264; Abbiotec), (vi) 10 μg/ml keyhole limpet haemocyanin (H7017; Sigma-Aldrich), (vii) 10 μg/ml recombinant human insulin (90177C; Sigma-Aldrich) or (viii) polyclonally stimulated with 1 μg/ml anti-CD3 (550368; BD Pharmingen) and 1 μg/ml anti-CD28 (kind gift from M. Frewin, Department of Pathology, University of Oxford, UK) monoclonal antibodies. Antigen concentrations were chosen following dose–response experiments (data not shown). Culture supernatants were harvested after 72 h (chosen following time-course experiments) and were stored at –80°C until used.

MBP used in these experiments was tested for endotoxin by Cambrex (http://www.cambrex.com) using the limulus amoebocyte lysate kinetic chromogenic assay. Very low levels of endotoxin were detected (equivalent to 0.044 ng/ml lipopolysaccharide). Stimulation of PBMCs with lipopolysaccharide (Sigma L-7895) at this and higher (up to 1000 ng/ml) concentrations neither induced neurotrophic factor secretion nor mimicked any of the results obtained with MBP (data not shown).

Quantifying neurotrophins and cytokines in culture supernatants

Cell culture supernatants were interrogated for neurotrophins/ cytokines by enzyme-linked immunosorbent assay (ELISA) and/or Bio-Plex. Commercially available ELISA kits were used to detect: brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), glial derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF-BB), and fibroblast growth factor (FGF-basic) (Duoset R&D Systems, DY248, DY291, DY256, DY267, DY268, DY212, DY257, DY220 and DY233, respectively). Culture supernatants were also assayed for cytokines using the Bio-Plex system for the simultaneous quantification of IL-2, IL-17, IFN-gamma, IL-10, IL-4, PDGF and FGF, used according to the manufacturer’s instructions. Briefly, premixed standards were reconstituted with 500 μl of RPMI/10% FCS, and then serially diluted in the same culture medium to generate a nine-point standard curve. The assay was performed in a 96-well filtration plate (171-025001; Bio-Rad). An amount of 50 μl of premixed beads, coated with target capture antibodies, were transferred to each well and washed twice with the vacuum manifold. Pre-mixed standards or samples (50 μl) were added to each well. The plate was shaken for 30 s then incubated at room temperature for 60 min with low-speed shaking. After further wash steps, pre-mixed detection antibodies (25 μl/well) were added, followed after 30 min incubation by streptavidin-PE (50 μl/well) for 10 min at room temperature. After washing three times, the beads were resuspended in Bio-Plex assay buffer and read on the Luminex system. Data were analysed using Bio-Plex Manager software with SPL curve fitting.

In vitro simulation of lymphocyte depletion

PBMCs from two healthy controls were negatively separated into monocytes and T and B lymphocytes, using 20 μl of magnetic beads (Miltenyi Biotec: T cells 130-091-156, B cells 130-091-151, and monocytes 130-091-153) per 1 x 10⁷ cells loaded into a MACS® LS Column (cell purity consistently 94–98%, data not shown). Following separation, cells were resuspended in growth medium at proportions mimicking those from peripheral blood of patients sampled before and at 3, 6, 9 and 12 months after alemtuzumab (ratio of B cells:T cells:monocytes, 10:70:20 pre-treatment; 25:25:50 at 3 months; 30:35:35 at 6 months; 25:45:30 at 9 months and 25:50:25 at 12 months) and cultured with MBP (as described earlier). After 72 h, supernatants were harvested and stored at –80°C until used.
Cell separation and neurotrophin expression analysis

PBMCs, immediately ex vivo or after culture with MBP or polyclonal stimulation, were positively separated using 20 μl of magnetic beads (Miltenyi Biotec; CD19 Microbeads, CD3 Microbeads, CD14 Microbeads) per 1 x 10⁷ cells loaded into a MACS® LS Column. Magnetically retained cells were eluted, washed and stored in RNA Later™ at ~7°C (cell purity consistently 95–98%, data not shown). BDNF, CNTF, PDGF and FGF expression was determined by semi-quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). Mitochondrial RNA was extracted from cells stored in RNA Later™ using the RNeasy Mini Kit (QIAGen) and reverse transcribed to cDNA using the Pro-STAR First Strand RT–PCR Kit (Stratagene). PCR primers and probes were designed using Primer Express (PE Biosystems, Foster City, CA, USA) and purchased from Oswe1 DNA service. Mitochondrial RNA sequence information was obtained from Genbank. Quantitative real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System (Perkin Elmer) using PCR Mastermix containing ROX (Eurogentec; RT-QP2X-03). Primer and probe sequences were: BDNF For 5'-GCT GAC ACT TCT GAA CAC ATG A-3', Rev 5'-CTG GAC GTG TAC AAG TCT GGG T-3', JOE-labelled probe 5'-CTG TTG GAT GAG GAC CAG AAA GTT CGG C-3', CNTF For 5'-GCC AGT AGG AAC CAC TCA T-3', Rev 5'-GGA AGG TAC GAT AAG CCT GAA GGT-3', BDNF AGG TAC GAT AAG CCT GAA GGT-3', JOE-labelled probe 5'-CTG ACC GAG GCA GAG CGA CCT CAA-3'; PDGF For 5'-AGA GGC CAG GGC AGC AA-3', Rev 5'-CAT ACA ATG TCC TGG TGC AGA ACA C-3', FAM-labelled probe 5'-CAA CCC AGC CAG TGC TGC CAT G-3' and for FGF For 5'-TGC CCA TAG GGT AAA CAT GAT TAG-3', Rev 5'-AGC GAT CCT CCT GGT G-3', JOE-labelled probe 5'-CAG TGG CTC ATG CCT ATA TCT CCT GCA-3'.

A bioassay of the effect of soluble factors from patients’ peripheral immune cells on rat neuronal and oligodendrocyte growth

Media was ‘conditioned’ by culturing PBMCs for 72 h with MBP, after which cells were harvested, washed three times in phosphate buffered saline (PBS) and suspended for 24 h in minimal—Dulbecco’s Modified Eagle’s Medium (DMEM—GIBCO 41966) plus insulin-free Feto containing: bovine serum albumin (100 μg/ml), transferrin (100 μg/ml), progesterone (0.06 μg/ml), putrescine (16 μg/ml), selenite (0.04 μg/ml), thyroxine (0.04 μg/ml), and triiodothyronine (0.04 μg/ml). Following centrifugation at 250 x g for 5 min and passage through a 20 μm filter, the resulting supernatant was stored at ~20° C until used.

Neuronal cultures were prepared from the cortices of Day 16 rat embryos (E16). All procedures were performed in compliance with national and institutional guidelines (UK Animals Scientific Procedures Act 1986 and the University of Cambridge Animal Care Committees). Briefly, the pregnant Sprague Dawley female (time-mated) was sacrificed and the embryos removed. The embryonic forebrains were isolated, meninges stripped and cortices dissected from the underlying subcortical structures. After enzymatic dissociation, cells were plated onto poly-α-lysine-coated eight-well plastic chamber slides at a density of 100,000 cells in 300 μl of 2% B27 supplemented DMEM. After 5 days at 37° C in a humidified atmosphere of 5% CO₂, the growth medium was changed, and the cells resuspended in either: (i) fresh 2% B27 supplemented DMEM; (ii) fresh ‘minimal’; or (iii) PBMC derived conditioning medium diluted 1:1 with fresh medium, for a further 3 days. For blocking experiments, neutralizing antibodies to BDNF (2 μg/ml—Sigma B5050), CNTF (0.2 μg/ml—Peprotech 500-P140), FGF (2 μg/ml RnD systems AB-233-NA) and PDGF (20 μg/ml RnD systems AN-20-NA) were added at the time of resuspension in PBMC derived conditioning medium. Following fixation with 4% paraformaldehyde, cells were permeabilized and non-specific antibody binding blocked by pre-treatment with 5% normal goat serum (NGS) in 0.2% triton/PBS for 1 h. Primary antibodies were: 1:200 β-tubulin (Sigma UK T8660), and 1:1000 neurofilament antibody SM1-312 (Covance AB5320). Secondary antibodies coupled to fluorochromes Alexa Fluor® 555® and Alexa Fluor® 488® (Invitrogen) were used to visualize primary antibody staining. Hoechst 33258 (1:4000) was used (10 min at room temperature) for nuclear identification and morphological assessment of cell survival. Neuronal survival was determined by counting live β-tubulin positive cells, taking five random fields per culture and at least three cultures per experiment. Axonal length was determined by measuring SMI-312 positive processes using ImageJ software, version 1.37 (Abramoff et al., 2004), available online at http://rsb.info.nih.gov/ij/. The 10 longest axons per field were measured and the mean calculated. In all cases control cultures, grown throughout the experimental period in DMEM/2% B27, were also analysed and observations for experimental conditions divided by this value in order to standardize results between experiments.

Oligodendrocyte precursor cell cultures were derived from mixed glial cell cultures, prepared following the protocol of McCarthy and de Vellis (McCarthy and de Vellis, 1980). Briefly, forebrains of newborn rat pups were removed, the meninges stripped and cortices dissected from the underlying subcortical structures before mechanical and enzymatic dissociation. The resulting cell suspension was plated onto poly-α-lysine coated 75 cm² tissue culture flasks. Culture medium (DMEM plus 10% FCS) was changed at 24 h then three times weekly until the cells reached confluence after 10–12 days. At this stage, a loosely adherent superficial layer of cells, representing oligodendrocyte precursors and microglia lying on a confluent astrocyte layer, was isolated by two-stage differential adhesion. Microglia were removed by vigorous shaking of the cultures at 240 rpm, followed by a change to fresh medium. Top-dwelling oligodendrocyte precursors were dislodged by overnight shaking of the cultures at 160 rpm and the supernatant placed into uncoated Petri dishes for 20 min to allow adherence of any residual microglia. The loosely adherent oligodendrocyte precursors were dislodged by gentle manual shaking. The final supernatant from these shaken cultures contained 85–90% oligodendrocyte precursors. Cells were resuspended in oligodendrocyte precursor expansion media: 1% N2 (GIBCO 17502-048), 2% B27 (GIBCO 17504-044) DMEM-Penicillin–Streptomycin–Amphotericin B (PSF GIBCO 15240-062), 10 μg/ml PDGF-alpha (PDGF-A RnD systems 221-AA) and 10 μg/ml FGF minus heparin (RnD 223-FB), to enrich further the oligodendrocyte cultures. After 48 h the cells were washed and resuspended in either: (i) fresh 2% B27 supplemented DMEM; (ii) ‘minimal’; or (iii) PBMC derived conditioning medium diluted 1:1 with fresh medium, for a further 5 days. Blocking antibodies to BDNF (2 μg/ml—Sigma B5050), CNTF (0.2 μg/ml—Peprotech 500-P140), FGF (2 μg/ml RnD systems AB-233-NA) and PDGF (20 μg/ml RnD systems AN-20-NA) were added to oligodendrocyte cultures at the time of resuspension in PBMC derived conditioning medium. ‘Live’ oligodendrocyte precursors were stained with primary antibodies against the cell surface markers galactocerebroside (GalC—1:5: omitted when MBP staining was performed) and O4 (1:5: both derived from hybridoma lines; European Collection of Cell Cultures, Salisbury, UK). Secondary antibodies,
coupled to fluorochromes Alexa Fluor® 555red and Alexa Fluor® 488green, were used to visualize primary antibody staining. After further wash steps, fixed cells were blocked and permeabilized with 0.2% triton, 5% NGS in PBS. Primary antibodies against NG2 (Chemicon—1:10000) and MBP (MBP aa82-87—Sero tec 1:100) were added in 0.2% triton in PBS and incubated overnight at 4°C. After washing, secondary antibodies coupled to Alexa Fluor® 680 (1:1000) and Alexa Fluor® 488green (1:1000) were used to visualize primary staining. Hoechst 33258 (1:4000) was used (10 min at room temperature) for nuclear identification and morphological assessment of cell survival. Live oligodendrocyte precursors were identified and counted, taking five random fields per culture and at least three cultures per experiment. Maturation was assessed by counting the number of cells positive for the stage-specific markers NG2, O4 and GalC. Maturation data are expressed as a percentage of total oligodendrocyte precursors. In addition, the average number of MBP positive cells per field was determined.

Statistical analysis

Analysis of EDSS data was performed using a mixed model for repeated measures with study visit, EDSS at baseline, country, treatment group and study visit by treatment group interaction as covariates. Models were fitted using data from specific patient subsets where appropriate, restricted to patients who were treated. Missing data for the T2 lesion load assessment were handled using the last-observation-carried-forward imputation approach. For biological studies, parametric (Student’s t-test) or non-parametric (Wilcoxon/Mann–Whitney) tests were performed. The reported P-values are two-sided and not adjusted for multiple testing.

Results

Improvement in disability after alemtuzumab is not solely attributable to suppression of inflammation

As previously reported, the mean disability score of patients treated with alemtuzumab improved significantly from baseline over 36 months in the CAMMS223 trial; this was not seen in those patients who received interferon β-1a. Most of this disability improvement occurred in the first 6 months after treatment, and was maintained thereafter. Here, by post hoc subgroup analyses of the CAMMS223 trial, we examine the dependence of this disability improvement on alemtuzumab’s ability to suppress inflammation at baseline and during the trial.

First, we considered the possibility that baseline disability might be in part due to ongoing inflammation, perhaps attributable to soluble factors such as nitric oxide causing reversible conduction block; after alemtuzumab, the suppression of inflammation would then release this block, causing improvement in disability. To test this hypothesis, we examined the disability change in those patients in CAMMS223 who did or did not experience clinical disease activity at baseline and during the trial.

We next tested the hypothesis that alemtuzumab improves disability indirectly, through passively permitting endogenous repair by suppressing cerebral inflammation more effectively than interferon β-1a. We stratified patients into those who did or did not display disease activity during the trial (Fig. 1C–F). Whether disease activity was defined by a clinical relapse or an increase in T2 MRI lesion load, the differential effect on disability was maintained. For instance, in the subgroup without on-trial relapse, disability significantly improved from baseline after alemtuzumab by (−) 0.52 (P < 0.0001; Fig. 1C) and non-significantly deteriorated by (+) 0.13 after interferon β-1a, leading to an overall difference of 0.65 points between the treatment arms (P < 0.0005). Likewise, for that subgroup with no increase in T2 lesion load, disability improved by (−) 0.48 points (P < 0.0001) after alemtuzumab and deteriorated non-significantly by (+) 0.09 points after interferon β-1a, giving a mean difference in disability by 36 months of 0.57 points (P < 0.001; Fig. 1E). In contrast, there was no significant disability change for those alemtuzumab patients who experienced a relapse on-trial (Fig. 1D) or had an increase in the MRI T2 lesion load (Fig. 1F), although in both cases the mean disability of the interferon β-1a group significantly worsened on-study.

We concluded that the improvement in disability seen after alemtuzumab cannot be entirely attributed to its anti-inflammatory effects. So we investigated whether alemtuzumab actively promoted neuroprotection and myelin repair through alterations in lymphocytic neurotrophin secretion.

Peripheral mononuclear cell production of BDNF, CNTF, PDGF and FGF increases after alemtuzumab

Fifteen patients treated with alemtuzumab were studied in detail. To confirm that they were representative of patients receiving alemtuzumab, we analysed lymphocyte reconstitution (Fig. 2). Lymphocytes and monocytes were undetectable in peripheral blood within hours after alemtuzumab but then recovered at varying rates. B cells recovered rapidly and superseded pre-treatment levels at 12 months (Fig. 2B); T cells reconstituted more slowly, reaching only 30 and 46% of baseline at 12 months for CD4+ and CD8+ T cells, respectively (Figs 2C and D). Monocytes reached 85% of baseline at 3 months and remained at this level (Fig. 2E). These changes accord with our previous reports (Cox et al., 2005; Thompson et al., 2010). Disability in this cohort also changed as expected: mean EDSS improved over 36 months from 2.6 to 1.5 points (data not shown). Of the
Figure 1  Post hoc subgroup analysis, by mixed model for repeated measures, of the mean change in disability of those patients treated during the CAMMS223 trial with interferon β-1a or alemtuzumab. Both dose levels of alemtuzumab are illustrated but only pooled alemtuzumab data are presented in the text. Mean EDSS change for cohorts stratified by the absence (A) or presence (B) of relapses in the 3 months before treatment; by the absence (C) or presence (D) of relapses in the 36 months after treatment; and by the improvement (E) or worsening (F) of MRI T2 lesion load in the 36 months after treatment. Unbracketed stars indicate the significance of the comparison between mean disability of either the pooled alemtuzumab group or the interferon β-1a group compared with baseline, whereas bracketed stars indicate the significance of the comparison between the mean disabilities of the pooled alemtuzumab and interferon β-1a groups (*P<0.05, **P<0.01, ***P<0.001).
15 patients studied, 5-year data were available in 13 (the other two being lost to follow-up). In nine cases, EDSS at month 60 was unchanged from month 36 (including three with EDSS of 0). Three patients showed continued improvement between months 36 and 60, with one reducing to EDSS 0. One patient had a significant relapse at month 60, which increased her disability to EDSS 7.5, from which she continues to make a slow recovery, now at EDSS 7.0.

Twelve months after alemtuzumab, PBMCs derived from this cohort secreted increased BDNF, CNTF, PDGF and FGF but reduced IGF-1, compared with baseline (Fig. 3). BDNF, CNTF and PDGF production followed a similar pattern, with maximal increases in secretion (3- to 6-fold) after alemtuzumab in response to MBP, and less marked increases in unstimulated or polyclonally stimulated cultures (Figs 3A–C). FGF secretion was increased after alemtuzumab, but equally for all culture conditions (Fig. 3D). In contrast, IGF-1 secretion decreased 3-fold after alemtuzumab (Fig. 3E), with no additional response to MBP or polyclonal stimulation. NGF, NT-3, NT-4/5 and GDNF could not be detected in patient or healthy control derived supernatants.

To determine the cellular source of these neurotrophins, PBMCs from five healthy controls, five untreated patients, and eight patients at 9–12 months after alemtuzumab were separated by beads into CD19+ B cells, CD3+ T cells and CD14+ monocytes either immediately ex vivo, or following MBP or polyclonal stimulation, for gene expression analysis by real-time RT–PCR. Under all conditions, CNTF was expressed by only T cells, whereas BDNF was expressed by all cell types. After alemtuzumab, the only significant change was an increase in T-cell expression of BDNF; there was also a trend towards increased T-cell CNTF mRNA (Fig. 3B and Supplementary Data). PDGF and FGF mRNA expression were observed in all cell types with no clear change after alemtuzumab (data not shown).

**Increased BDNF, CNTF and PDGF secretion after alemtuzumab is specific to stimulation with MBP**

To investigate the antigen specificity of increased neurotrophin secretion after alemtuzumab, we stimulated PBMCs collected
Figure 3  Neurotrophin secretion from PBMC cultures from 15 patients before and 12 months after alemtuzumab treatment. Cultures were either unstimulated (unstim) or stimulated with the myelin antigen, MBP or polyclonally stimulated with anti-CD3/anti-CD28 antibodies (CD3/28). Supernatants were harvested after 72 h and assayed for: (A) brain-derived neurotrophic factor, (B) CNTF, (C) platelet-derived neurotrophic factor, (D) FGF, and (E) insulin-like growth factor-1. Error bars represent 95% confidence intervals. (*P<0.05, **P<0.01, ***P<0.001).
from three patients 12 months after alemtuzumab with exogenous antigens (tetanus toxoid and keyhole limpet haemocyanin), myelin antigens (MBP, myelin basic peptide aa87–99 and myelin oligodendrocyte glycoprotein aa35–55) and non-brain self-antigens (collagen and insulin). BDNF, CNTF and PDGF secretion increased specifically in response to MBP (Figs 4A–C), whereas, as expected from the above, IGF-1 and FGF secretion after alemtuzumab was not antigen specific (Figs 4D and E).

**Figure 4** Peripheral blood mononuclear cell neurotrophin secretion from three patients 12 months after alemtuzumab in response to a wide variety of antigens. PBMCs were cultured either unstimulated (unstim) or stimulated with; MBP, tetanus toxoid (TT), myelin oligodendrocyte glycoprotein (MOG), collagen type II fragment aa245–270 (Collagen), myelin basic peptide aa87–99 (MBP-P), keyhole limpet haemocyanin (KLH) or recombinant human insulin (Insulin). Supernatants were harvested after 72 h and assayed for: (A) brain-derived neurotrophic factor, (B) CNTF, (C) platelet-derived neurotrophic factor, (D) insulin-like growth factor-1 and (E) FGF (*P<0.05).
Increased neurotrophin secretion is seen after alemtuzumab treatment but not following interferon β-1a or mimicked by lymphopenia in vitro

We compared the production of neurotrophic factors by PBMCs in response to MBP after alemtuzumab with 10 patients who had been treated with interferon β-1a, for between 9 and 12 months, and 15 healthy controls. Interferon β-1a treatment had no effect on neurotrophin production by PBMCs except to reduce IGF-1 secretion (Fig. 5E). After alemtuzumab, PBMC secretion of BDNF, CNTF and PDGF in response to MBP rose at 6 months and further at 12 months as expected (Figs 5A–C). At three months after alemtuzumab our patients were too lymphopenic to consistently perform these assays; however in available data from four patients at 3 months, BDNF supernatant concentration was elevated at 354 pg/ml, and there was increased CNTF (7.6 pg/ml) and reduced IGF-1 (535 pg/ml). Unexpectedly, we found that pre-treatment multiple sclerosis patients produced significantly lower levels of FGF (Fig. 5D) and PDGF (Fig. 5C) compared to healthy controls. Following alemtuzumab, PBMC secretion of IL-17 (in response to stimulation with MBP) and IL-2 (constitutive and in response to MBP) was reduced at least 2-fold (Figs 6A and E) whereas IL-10 and IL-4 secretion was unchanged (Figs 6C and D). These changes were not seen with interferon β-1a treatment. These findings, and the fall in IGF-1 secretion, suggest that the changes in BDNF, CNTF and PDGF after alemtuzumab occur in the context of complex immunomodulation and

Figure 5 Peripheral blood mononuclear cell neurotrophin secretion, induced by MBP stimulation. PBMCs from 15 healthy controls (HC), 15 patients before (pre) and at three time points (6, 9 and 12 months) after alemtuzumab and from 10 patients treated with interferon β-1a (re bif), were cultured with MBP. Supernatants were harvested after 72 h and assayed for: (A) brain-derived neurotrophic factor, (B) CNTF, (C) platelet-derived neurotrophic factor, (D) insulin-like growth factor-1 and (E) FGF. Error bars represent 95% confidence intervals (**P < 0.01, ***P < 0.001).
are not due to non-specific up-regulation of protein secretion after alemtuzumab.

After alemtuzumab, T cells reconstitute more slowly than B cells or monocytes. To determine if this alone is responsible for increased neurotrophic factor secretion, PBMCs from two healthy controls were separated into T cells, B cells and monocytes and then resuspended, with and without MBP stimulation, at proportions mimicking those found in the peripheral blood of patients before and at 3, 6, 9 and 12 months post-alemtuzumab. Neurotrophin secretion was unchanged (data not shown).

**MBP-stimulated peripheral mononuclear cell cultures from patients after alemtuzumab secrete factors that support neuronal survival, axonal growth and oligodendrocyte survival and maturation**

To explore the functional consequences of increased PBMC neurotrophin secretion after alemtuzumab, neurones derived from

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**Figure 6** Peripheral blood mononuclear cell cytokine secretion. PBMCs from 15 healthy controls (HC), 15 patients before (pre) and at three time points (6, 9 and 12 months) after alemtuzumab, and from 10 patients treated with interferon β-1a (rebin), were cultured either unstimulated or with MBP. Supernatants were harvested after 72 h and assayed for: (A) IL-17, (B) interferon (IFN)-γ, (C) IL-10, (D) IL-4 and (E) IL-2. Error bars represent 95% confidence intervals (*P<0.05).
Day 16 rat embryos were cultured in media conditioned by MBP-stimulated PBMC cultures from healthy controls, untreated multiple sclerosis patients and patients after alemtuzumab. Neuronal survival in conditioned media was equivalent from PBMC cultures derived from healthy controls and untreated patients. In contrast, neuronal survival and axonal length were increased at least 3-fold, compared with controls, in PBMC-conditioned media derived from patients after alemtuzumab (Figs 7A–C). These effects were largely reversed by neutralizing BDNF or, to a lesser degree, after CNTF neutralization (and unchanged by the blocking antibodies alone, confirming they are not neurotoxic; Figs 7D–F). By chance, two patients (one untreated and one 12 months after alemtuzumab) were experiencing a relapse of multiple sclerosis when sampled. PBMC-conditioned media from these patients significantly reduced neuronal survival and axonal length compared with untreated conditioned medium (Figs 7B and C), suggesting the presence of neurotoxic factors during an acute relapse of multiple sclerosis.

PBMC-conditioned media from patients after alemtuzumab enhanced the survival, and promoted the maturation, of oligodendrocyte precursor cells derived from Day 0 rat neonates compared with controls (Figs 8A and C). These media also boosted MBP production (Figs 8D and F). However, unlike the effects on neurones and axons, the pro-oligodendrocyte effects of these media were not reversed by neutralizing antibodies against BDNF, CNTF, FGF or PDGF (data not shown).

Discussion

We have previously shown, both in an open-label study (Coles et al., 2006) and randomized rater-blinded controlled trial (Coles et al., 2008), that disability in patients with early relapsing–remitting multiple sclerosis improves following treatment with alemtuzumab. This clinical effect, associated with increased brain volume, is sustained for at least 3 years and is in marked contrast to the continuing accumulation of disability and progressive brain atrophy seen when similar patients are treated with interferon β-1a. Here we provide indirect evidence that this improvement in disability after alemtuzumab is not solely due to its anti-inflammatory effect. Instead, we propose that immune cells regenerating after alemtuzumab secrete neurotrophins and oligotrophins. However, when alemtuzumab is administered in the progressive phase of the disease, it suppresses inflammation but patients continue to accumulate disability (Coles et al., 2006); suggesting that there is a ‘window of opportunity for repair’, before the neurodegenerative phase of the disease is irretrievably established.

The strongest evidence that alemtuzumab positively promotes repair comes from post hoc analyses of the phase 2 CAMMS223 trial data. Specifically, we examined those alemtuzumab and interferon β-1a treated participants who had no clinical evidence for disease activity in the 3 months before treatment, or clinical and radiological disease activity evidence for 36 months thereafter. Patients in both subgroups, selected for maximal disease inactivity, share equal suppression of cerebral inflammatory activity; each therefore presumably has the same opportunity for endogenous repair, yet disability improvement was seen only after alemtuzumab and not treatment with interferon β-1a.

On this basis, we argue that alemtuzumab induces ‘neuroprotective autoimmunity’. Although one opinion holds that central nervous system autoimmunity is purely detrimental (Jones et al., 2002, 2004) a growing body of evidence suggests that autoimmunity may be beneficial, and perhaps even necessary for central nervous system repair (Moalem et al., 1999; Hauben et al., 2000a, b; Hohlfeld et al., 2000; Kipnis et al., 2002a, b). For instance, activated MBP-specific T cells protect retinal ganglion cells from secondary damage after traumatic optic nerve injury, unlike activated T cells specific for non-central nervous system antigens (Moalem et al., 1999). Rats subjected to low-dose total body irradiation recover better from optic nerve crush or contusive spinal cord injury than non-irradiated controls, but this effect is not seen in immune deficient animals, suggesting that lymphocyte reconstitution after irradiation is required for neuroprotection (Kipnis et al., 2004). Passive transfer of naturally occurring regulatory T cells also abolishes this effect, supporting the idea that immune activation rather than immune suppression is responsible for neuroprotection.

A likely contributor to neuroprotective autoimmunity is the release of neurotrophic factors by immune cells. In addition to their well-recognized role in development, neurotrophins act on mature neurones and cells of the oligodendroglial lineage, providing trophic support and preventing cell death following various pathological insults. In post-mortem material from patients with multiple sclerosis, BDNF is present in T cells and macrophages infiltrating lesions, and its receptor, TrkB, is expressed within lesions (Stadelmann et al., 2002), demonstrating that the machinery for neuroprotective autoimmunity is already in place. However, under natural conditions neurotrophic factor secretion by immune cells appears unable to prevent the degeneration of oligodendrocytes, axons and neurones, leading to irreversible disability.

We have previously shown that the immune repertoire is radically altered following alemtuzumab, with increased CD4+CD25hiFoxP3+ regulatory T cells, increased CD45RO+ve memory T cells (Cox et al., 2005), reduced CD27+ memory B cells (Thompson et al., 2010), reduced cytokine, Tbet and GATA3 expression (Cox et al., 2005) and enhanced lymphocyte apoptosis and proliferation (Jones et al., 2009). Here we have investigated neurotrophin secretion. We show that immune cells regenerating after alemtuzumab produce significantly greater BDNF, CNTF PDGF and FGF. And, in keeping with the original suggestion that neuroprotective autoimmunity requires reactivity to central nervous system antigens (Moalem et al., 1999), we show that BDNF, CNTF and PDGF secretion occurs specifically in response to the myelin antigen, MBP. These changes are not seen after interferon β-1a treatment and are not due to lymphopenia alone.

Increased peripheral immune cell BDNF secretion has been described following glatiramer acetate treatment of multiple sclerosis, but at a lower level (Azoulay et al., 2005; Blanco et al., 2006). Increases in peripheral lymphocyte CNTF and PDGF secretion have not been previously described. We have shown that BDNF and CNTF, derived from T cells regenerating after alemtuzumab, are capable of promoting the survival of neurones and the
Figure 7  The effect of PBMC-conditioned media on rat neuronal growth. (A, B) Example digital images (×20) of rat neurones exposed to PBMC-conditioned media from untreated patients (UnTx), patients post-alemtuzumab (post) and from patients during a clinical relapse (relapse). Panel 1 shows double staining with β-tubulin (red) and Hoechst nuclear stain (blue); panel 2 shows staining with SMI312 (green). (B and C) box and whisker plots showing neuronal survival and axonal length; min = minimal medium alone. (C) Example digital images (×20) of neurons exposed to post-alemtuzumab conditioned-media, with and without blocking antibodies to BDNF and CNTF. (D and E) box and whisker plots showing neuronal survival and axonal length in minimal media alone (min), and post-alemtuzumab conditioned media, with and without blocking antibodies to BDNF and CNTF. (n = 8 healthy controls, 8 untreated patients, 10 patients post-alemtuzumab, 2 patients during relapse; *P < 0.05, **P < 0.01, ***P < 0.001).
growth of axons in culture. Factors secreted by peripheral immune cells after alemtuzumab, which remain to be characterized, also induce proliferation and maturation of oligodendrocyte precursors in culture. This allows for the possibility that lymphocytes from patients after alemtuzumab may migrate to areas of demyelination, where oligodendrocyte precursors accumulate, to promote their differentiation and proliferation (Scolding et al., 1998; Wolswijk, 2002).

We speculate that increased lymphocytic secretion of neurotrophins after alemtuzumab arises as a homeostatic response to lymphocyte depletion. That would account for the non-antigen specific changes in FGF and insulin-like growth factor-1 secretion.
after alemtuzumab. For example, insulin-like growth factor-1 has effects on T cell maturation, proliferation and effector functions, and is a strong inhibitor of T cell apoptosis (Johnson et al., 1992; Tu et al., 1999, 2000; Walsh and O’Connor, 2000; Walsh et al., 2002). We cannot explain the specificity of BDNF and CNTF response to MBP; it remains to be determined whether this is confined to patients with multiple sclerosis using alemtuzumab or is a generic finding in any lymphopenic patient. We also report the novel finding that PDGF and FGF secretion by lymphocytes from untreated patients with multiple sclerosis is reduced; these are pivotal factors in the survival, proliferation and migration of oligodendrocyte progenitor cells (Noble et al., 1988; Raff et al., 1988; Grinspan et al., 1990; McKinnon et al., 1990, 1991; Wolswijk and Noble, 1992; Oudega et al., 1997; Simpson and Armstrong, 1999).

Our study has several limitations. We have not shown that T cells secreting neurotrophic factors migrate into the central nervous system in these patients. However, there is good evidence from post mortem and animal studies that peripheral immune cells do enter the central nervous system and produce neurotrophic factors in situ (Stadelmann et al., 2002; Aharoni et al., 2003, 2005). We have not shown directly that the improvement in disability, correlating with altered growth factor production and effects on rat neurones and oligodendrocyte precursors in vitro, results from improved tissue repair. Until recently, there has been no satisfactory animal model of alemtuzumab treatment to explore these issues; now, the development of a transgenic mouse expressing human CD52 (hCD52) allows appropriate investigations to be designed and carried out (Hu et al., 2009).

With those caveats, the evidence that human immune cells regenerating after alemtuzumab treatment produce bioactive neurotrophic factors capable of promoting neuronal survival, axon growth and the survival and maturation of oligodendrocytes in vitro may, at least in part, explain the sustained improvement in disability seen in patients with early active relapsing–remitting multiple sclerosis treated with alemtuzumab.

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Conflict of interest

J.J. reports receiving consulting fees and lecture fees from Bayer Schering Pharma and lecture fees from Genzyme. E.J.F. reports receiving consulting fees and grant support from Genzyme and consulting fees and membership in a speakers’ bureau for Bayer. A.C. reports receiving consulting fees, lecture fees and grant support from Genzyme and lecture fees from Bayer Schering Pharma on behalf of himself and the University of Cambridge. A.J.C. reports receiving consulting fees, lecture fees and grant support from Genzyme.

Supplementary material

Supplementary material is available at Brain online.

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