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G-CSF Therapy of Ongoing Experimental Allergic Encephalomyelitis Via Chemokine- and Cytokine-Based Immune Deviation

Flora Zavala, Sébastien Abad, Sophie Ezine, Véronique Taupin, Annie Masson, and Jean-François Bach*

Converging evidence that G-CSF, the hemopoietic growth factor of the myeloid lineage, also exerts anti-inflammatory and pro-Th2 effects, prompted us to evaluate its direct therapeutic potential in autoimmune diseases. Here we report a novel activity of G-CSF in experimental allergic encephalomyelitis, a murine model for multiple sclerosis, driven by Th1-oriented autoaggressive cells. A short 7-day treatment with G-CSF, initiated at the onset of clinical signs, provided durable protection from experimental autoimmune encephalomyelitis. G-CSF-treated mice displayed limited demyelination, reduced recruitment of T cells to the CNS, and very discrete autoimmune inflammation, as well as barely detectable CNS mRNA levels of cytokines and chemokines. In the periphery, G-CSF treatment triggered an imbalance in the production by macrophages as well as autoreactive splenocytes of macrophage inflammatory protein-1α and monocyte chemoattractant protein-1, the prototypical pro-Th1 and pro-Th2 CC chemokines, respectively. This chemokine imbalance was associated with an immune deviation of the autoaggressive response, with reduced IFN-γ and increased IL-4 and TGF-β1 levels. Moreover, G-CSF limited the production of TNF-α, a cytokine also associated with early CNS infiltration and neurological deficit. These findings support the potential application of G-CSF in the treatment of human autoimmune diseases such as multiple sclerosis, taking advantage of the wide clinical favorable experience with this molecule. The Journal of Immunology, 2002, 168: 2011–2019.

Granulocyte CSF, a key hemopoietic factor of the myeloid lineage, has been extensively used for >10 yr in the treatment of neutropenia as well as for bone marrow reconstitution and stem cell mobilization. Apart from these well-known activities, a series of reports have demonstrated that G-CSF displays immunoregulatory properties: G-CSF is able to expand the monocyte/macrophage subset and to promote an anti-inflammatory pattern conferring protection in murine endotoxemia (1). Moreover, T cell allogeneic and mitogenic reactivities are inhibited in G-CSF-treated individuals (2), corresponding to a reduced IFN-γ production capacity (3). Recipients of G-CSF-mobilized peripheral blood stem cells do not display higher incidence of graft-vs-host disease than bone marrow recipients (4) despite much larger numbers of T cells in unmanipulated stem cell grafts. Murine studies have shown that G-CSF-induced protection from acute graft-vs-host disease is associated with a shift toward Th2 of the production of T cell-derived cytokines, with a reduction of Th1-oriented cytokine production capacity, particularly IFN-γ (5). A similar shift in cytokine production pattern has been recently confirmed in human peripheral blood T cells (6).

Here we address the hypothesis that these immunoregulatory properties of G-CSF might be beneficial in autoimmune diseases. We investigated the therapeutic potential of G-CSF in experimental autoimmune encephalomyelitis (EAE),2 a murine model for the human demyelinating disease multiple sclerosis (MS). EAE (7) is mediated by the activation of autoaggressive Th1 cells, which trigger an inflammatory reaction. Immunoprevention of EAE has been hitherto achieved either by suppressing the production and/or action of the neurological deficit-associated cytokines TNF-α or lymphotoxin-α (8–10) or by eliciting regulatory Th2/Th3 cells that release anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β and impair activation of the Th1 pathogenic cells (11, 12). More recently, therapeutic strategies have been aimed at the neutralization of chemokines, based on reports showing that expression of chemokines, particularly the CC chemokine macrophage inflammatory protein-1α (MIP-1α), and chemokine receptors in the CNS correlated with EAE disease symptoms (13) and was increased in patients with acute MS (14, 15). Treatments of proteolipid protein- or myelin basic protein (MBP)-immunized SJL/J mice with anti-MIP-1α was able to inhibit acute EAE severity (16). Of particular relevance to the role of chemokines in autoimmune settings is the fact that in addition to driving leukocyte attraction and accumulation to tissue sites of inflammation, chemokines play a regulatory role in T cell proliferation, differentiation, and Th1/Th2 balance in cytokine production (17–19). In vivo and in vitro studies have identified MIP-1α and macrophage chemoattractant protein-1 (MCP-1) as prototypical pro-Th1 and pro-Th2 chemokines, respectively (20–22).

We now demonstrate that a short treatment with G-CSF at the onset of clinical signs provides durable protection of SJL/J mice from EAE. G-CSF reduces the T cell infiltration and autoimmune

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2 Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; AID, autoimmune disease; MBP, myelin basic protein; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage-inflammatory protein-1α; MS, multiple sclerosis; RPA, RNase protection assay.

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inflammation within the CNS, an effect based on immunoregulatory events that take place in the periphery and include an imbalance in the chemokine (MIP-1α/MCP-1) production ratio and an immune deviation toward Th2 of the T-cell autoreactive response together with a reduction of systemic and lymphocyte TNF-α production.

Materials and Methods

**EAE induction**

Female SJL/J mice (8–10 wk of age, purchased from Elevage Janvier, Le Genest St. Isle, France) were immunized by s.c. injection at two sites at the tail base on day 0, and boosted on day 7 in the flanks, with 400 μg guinea pig MBP (Sigma, St. Louis, MO) emulsified in CFA containing 50 μg heat-inactivated Mycobacterium tuberculosis H37Ra (Sigma), in a volume of 50 μl/site. The disease developed with an acute phase characterized by loss of weight as well as progressive ascending clinical paralysis, followed by periods of remission with weight recovery, and subsequent relapses or chronic disease. Clinical symptoms were scored as follows: 0, no symptoms; 1, flaccid tail; 2, impairment of righting reflex or abnormal gait; 3, severe hind limb weakness; 4, complete hind limb paralysis; and 5, paraplegia, moribund. The mean clinical score was defined as the mean of all clinical scores in a group including animals with no symptoms at a given time point or as the mean of the mean clinical scores at all time points over the entire disease length, when specified. The mean disease incidence was also calculated as the mean of incidences at all disease time points.

**G-CSF treatment**

Recombinant human G-CSF (Amgen, Thousand Oaks, CA), which is active in mice (5), was injected s.c. at 200 μg/kg/day. Excipient consisted of 5% dextrose in sterile H2O.

**Histology and immunohistochemistry**

Mice were anesthetized with sodium pentobarbital and perfused intracardially through the left ventricle with ice-cold 10% formalin for OCT-embedded tissues. Five-micrometer sections of brain and spinal cord were stained with toluidine blue or Luxol Fast Blue to assess demyelination and included tissues. Five-micrometer sections of brain and spinal cord were stained with H&E to assess leukocyte infiltration. Histologic scores were determined on three mice per group on 20 sections per animal, using the following scale: 0, no mononuclear cell infiltration; 1, one to five perivascular lesions per section with minimal parenchymal infiltration; 2, five to ten perivascular lesions per section with minimal extension of parenchymal infiltration; and 3, >10 perivascular lesions per section with extensive parenchymal infiltration. Immunohistochemical staining was performed with Histomouse immunostaining kits (Zymed, South San Francisco, CA) according to the manufacturer’s instructions, except for the use of secondary Ab, which was a biotin-goat anti-rat IgG (H+L) (Zymed) used at 7.57 μg/ml in PBS buffer containing 1% BSA and 0.3% Triton X-100. The primary rat Abs used were anti-CD3 (clone KT3), anti-Mac-1/CD11b (clone M1/70), anti-Gr-1 (clone RA3-8C5), and anti-F4/80 (all from BD PharMingen, Le Pont de Claix, France).

**TNF-α concentration in serum**

TNF-α was measured in sera diluted 1/2 with commercial ELISA from R&D Systems Europe (Abingdon, U.K.).

**Assessment of T cell autoreactive response**

Splenocytes were harvested on day 40 after MBP immunization. Proliferation was assessed using 2 × 105 viable splenocytes/well in the presence of MBP (50 μg/ml, final concentration) or PBS in RPMI Glutamax medium supplemented with 5% FBS (Life Technologies, Cergy Pontoise, France), 1% penicillin and streptomycin, and 5 × 10−5 M β-ME in 96-well culture plates. T cell proliferative responses were quantified at 96 h after a 14-h pulse with [3H]thymidine. Supernatants for cytokine and chemokine determinations were generated by 72-h culture in the above-described conditions.

**Cytokine and chemokine measurements**

Cytokine and chemokine levels were determined by commercial ELISAs for TNF-α, MIP-1α, and MCP-1 (R&D Systems Europe) and by sandwich ELISA with mAbs prepared in our laboratory from hybridoma or purchased from BD PharMingen for IFN-γ (R46A2 and AN18), IL-4 (11B11 and BV6D), and IL-10 (JES2.A5 and JES5-16E3) as previously described (23).

**Preparation of RNA**

Total RNA was also extracted from PBS-perfused CNS spinal cords using RNeasy (Eurobio, Les Ulis, France) following the manufacturer’s instructions. Splenocytes (4 × 106 cells/well in 24-well plates) were incubated with PBS or MBP (50 μg/ml) for 5 h, and total RNA was extracted with RNeasy. Control RNA consisted of total RNA extracted from the spleen of BALB/c mice 60 min after i.v. injection of 5 μg anti-CD3 (clone 145 2C11) Ab in 150 μl PBS.

**RNase protection assay (RPA)**

mRNA levels for the cytokines, chemokines, and hemopoietic factors were determined using multiprobe RPA III kit from Ambion (Ambion, Austin, TX). [α-32P]UTP-labeled antisense RNA transcripts prepared from mCK5, mCK1b, mCK3b, or mCK4 DNA templates (Riboquant, PharMingen, San Diego, CA) using MAXIscript in vitro transcription kit (Ambion) were hybridized with total RNA (20 μg for spinal cords and 10–15 μg for splenocytes) overnight at 56°C according to the manufacturer’s instructions. Bands were detected by phosphorimaging using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results were calculated as a ratio of the volume of the band of interest to the mean of the volumes of the bands for the housekeeping genes large ribosomal protein L32 and GAPDH.

**Macrophage isolation**

Macrophages were isolated from the spleens of mice after 5 days of treatment with G-CSF or vehicle and were purified by 24-h adherence (98% pure as assessed by FACS analysis using Mac-1+ staining) in 24-well culture plates (1 × 105 cells/well). After incubation with anti-CD40 (5 μg/ml, clone 3/23, Valbiotech, Paris, France) or PBS for 24 h, supernatants were collected, and their contents of cytokines and chemokines were measured by ELISA. At the end of the incubation, cellular protein content was measured by the bicinchoninic acid colorimetric method (Pierce, Rockford, IL) in adherent cells disrupted in PBS/0.01% Tween after three freeze-thaw cycles.

**FACS analysis**

The cell surface phenotype of splenocytes was analyzed by flow cytometry. All cells were incubated in cold PBS supplemented with 2% FCS and 0.02% azide. One million cells per sample were preincubated in 20 μl for 15 min at room temperature under constant shaking with anti-FcγRIII/II (clone 2G12) to reduce nonspecific binding, followed by mAbs (anti-CD4 (clone GK1.5), anti-CD8 (clone 53-67.2), anti-CD3 (clone 145-2C11), anti-αβ TCR (clone H57-597), anti-B220 (clone RA3-6B2), anti-CD19 (clone I3D3), anti-Mac-1/CD11b (clone M1/70), anti-Gr-1 (clone RA3-8C5), and anti-F4/80) (all from BD PharMingen, Le Pont de Claix, France).

**Results**

**G-CSF administration before and at disease onset reduces EAE severity**

EAE was actively induced in the genetically sensitive SJL/J mouse by immunization on days 0 and 7 with MBP emulsified in CFA. We treated mice s.c. with recombinant human G-CSF (200 μg/kg/day). No effect was observed when the treatment was initiated at the induction phase of the disease, i.e., from day −1 before immunization to day 8 postimmunization (not shown). However, when treatment was started on day 10 after the first immunization, approximately 1 wk before the appearance of the first clinical signs, and continued daily until day 30, a significant protection from the disease was observed (Fig. 1A), with a reduction in the maximal mean clinical score as well as in the incidence of disease (p < 0.01), thus demonstrating that G-CSF was able to prevent EAE.

Importantly, the therapeutic properties of G-CSF were also apparent when treating the MBP-immunized mice starting on day 17.
G-CSF-treated mice are protected from demyelination and show limited cellular infiltration in CNS

CNS inflammation and demyelination were assessed in MBP-immunized mice, treated with excipient or G-CSF on day 40 postimmunization. Toluidine blue was used to stain myelin in Fig. 2, A and D. Loss of myelin was extensive in the cerebellum of excipient-treated mice (Fig. 2A), but remained limited in G-CSF-treated mice (Fig. 2D). In excipient-treated mice, H&E staining (Fig. 2B) showed extensive parenchymal infiltration with perivascular cuffs in cerebellum, that were not observed in G-CSF-protected mice, which displayed few cells around the vessels (Fig. 2E). The mean histologic scores (Table I) were significantly reduced by G-CSF treatment. Immunohistochemical staining revealed that infiltrating cells near these perivascular cuffs consisted mainly in T cells (Fig. 2C) and Mac-1+ cells (Fig. 2F), including essentially macrophages and fewer granulocytes (Fig. 2G). Gr1+ granulocytes were detected in the perivascular zone and fewer in the surrounding parenchyma in both excipient-treated (Fig. 2G) and G-CSF-treated (Fig. 2J) mice, but at much lower rate in the latter. Mac-1+ staining (Fig. 2I) correlated with F4/80+ staining (Fig. 2H) and was also observed on activated microglia with dendritic morphology in the surrounding parenchyma of excipient-treated mice, whereas G-CSF-treated mice displayed almost no F4/80 label (Fig. 2K) and very limited Mac-1 staining (Fig. 2L).

RPA analysis of mRNA production of cytokines, chemokines, and hemopoietic growth factors in spinal cords of mice with EAE

Spinal cords of G-CSF-protected mice expressed no or very limited amounts of mRNAs for cytokines, chemokines, and growth factors as analyzed by RPA. G-CSF treatment prevented the induction of both Th1-oriented cytokines (such as IFN-γ and IL-2) as well as of regulatory cytokines, which are expressed in EAE undergoing SJL/J mice treated with excipient, including IL-10 (Fig. 3A), as previously observed by Tran et al. (24), and TGF-β1 (Fig. 3B). Levels of TNF-β, lymphotixin-β, and TNF-α mRNAs were also significantly reduced by G-CSF treatment (Fig. 3B). Similar findings were obtained for chemokines (Fig. 4). RANTES, and MCP-1, predominantly expressed in spinal cords of excipient-treated mice, were barely detectable in G-CSF-treated mice. No particular chemokine among those analyzed was increased by G-CSF treatment.

We further analyzed the production of hemopoietic growth factors. M-CSF is constitutively expressed by human and murine astrocytes, and GM-CSF and G-CSF can be produced by these cells after inflammatory or viral stimulation (25–28). We therefore investigated whether expression and/or regulation of these factors might be implicated in the EAE disease process. RPA results (Fig. 5A) show that G-CSF mRNA, although expressed at levels below quantitation limits in spinal cords of mice developing EAE, was not apparently enhanced in mice treated with G-CSF, thus suggesting that G-CSF treatment did not significantly induce its own
production at this site. Instead, M-CSF mRNA was expressed constitutively at high levels in spinal cords from naive mice and was further increased in mice injected with excipient and presenting with EAE, yet returned to basal levels in mice treated with G-CSF (Fig. 5B). Although expressed at much lower levels than M-CSF, IL-6 and stem cell factor mRNA levels followed the same trend.

Together, these results show that the CNS of G-CSF-treated mice is essentially free from immune activation, and that this protection is related to the restriction in T cell infiltration and autoimmunity inflammation. This suggests that immunoregulation is taking place at the periphery and targets the T cell activation process and/or their entry into the CNS. We therefore investigated the modifications of the autoreactive immune response in spleen and lymph nodes of G-CSF- vs excipient-treated mice.

**Cellular subsets affected by G-CSF**

We first investigated the cellular changes induced by G-CSF in EAE. The hemopoietic properties of G-CSF were confirmed by extramedullary hemopoiesis, with an increase in total spleen cell number (p < 0.05) in G-CSF-treated, MBP-immunized mice (208 ± 28 × 10⁶ cells/spleen; n = 3) relative to excipient-treated animals (117 ± 12 × 10⁶ cells/spleen; n = 3) and to control nonimmunized animals (126 ± 15 × 10⁶ cells/spleen; n = 3).

Moreover, FACS analysis of spleens of animals on day 40 after immunization essentially revealed that macrophages (CD11b<sup>high</sup> Gr-1<sup>−</sup> cells), which represented 2.44 ± 0.2% of total spleen cells in naive SJL mice (n = 3) and 3.28 ± 0.3% in MBP-immunized, excipient-treated mice (n = 3), increased to 3.92 ± 0.2% in G-CSF-treated mice (n = 3), while granulocytes (CD11b<sup>high</sup> Gr-1<sup>+</sup>) changed from 1.5 ± 0.2 to 3.2 ± 0.3 and 6.8 ± 0.3% in the same cell populations, respectively (Fig. 6). No major alterations were observed in the percentages of the other immune cell subsets, including T cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>) and B cells (CD19<sup>+</sup>, B220<sup>+</sup>). G-CSF had no particular effect at this stage of the disease on the respective proportions of the dendritic cell subsets, i.e., myeloid, characterized as CD11c<sup>+</sup>, CD11b<sup>high</sup>, vs lymphoid, CD11c<sup>+</sup> CD11b<sup>low</sup>, at variance with the preferential pro-lymphoid effect reported in man (29). Essentially similar results were obtained in lymph nodes (not shown).

**Imbalance of the chemokine MIP-1α/MCP-1 ratio in macrophages and autoreactive splenocytes**

Since the migration of T cells to the CNS is under the control of chemokines, we measured chemokine production by 1) macrophages isolated by adherence from spleens of excipient- or G-CSF-treated animals and activated with anti-CD40 to mimic the T cell cognate interaction, and 2) autoreactive splenocytes. We observed that the chemokine content measured by specific ELISA in supernatants of macrophages from G-CSF- compared with excipient-treated mice showed 2-fold reduced production of MIP-1α (Fig. 7A), whereas MCP-1 levels were 2-fold increased (Fig. 7B), leading to a final 4-fold reduction in the MIP-1α/MCP-1 balance (Fig. 7C). Similarly, in supernatants of MBP-stimulated splenocytes, the levels of MIP-1α were 2.5-fold reduced, whereas those of MCP-1 were enhanced 2.5-fold in G-CSF-treated mice relative to mice.
injected with excipient. The overall MIP-1α/MCP-1 protein ratio was reduced approximately 5-fold (Fig. 7, D–F), close to the ratio of the corresponding chemokine mRNA levels analyzed by RPA (Fig. 7, G–I).

**Suppression of TNF-α production in anti-CD40-stimulated macrophages and reduced systemic levels of TNF-α in EAE**

TNF-α activates endothelial cells (30, 31) and facilitates T cell migration to the CNS. We observed that production of TNF-α was 2-fold reduced in macrophages isolated from spleens of G-CSF-treated animals and activated with anti-CD40 to mimic the T cell cognate interaction (Fig. 8A), relative to cells isolated from excipient-treated mice. Moreover, a dramatic reduction in TNF-α serum levels was observed, with 11.5 ± 1.26 vs 85.67 ± 14.77 pg/ml TNF-α (p < 0.002) at the acute phase of the disease and 4.2 ± 1.53 vs 41 ± 16.34 pg/ml (p < 0.005) at the chronic phase, in G-CSF-treated mice relative to excipient-treated animals, respectively (Fig. 8B).

**Immune deviation of the T cell autoreactive cytokine response**

The autoreactive T cell response to the recall Ag MBP was substantially affected by G-CSF treatment. Although proliferation to the autoantigen did not differ between the two groups of mice (not shown), analysis of the cytokine contents on day 4 of incubation in supernatants of MBP-stimulated splenocytes from G-CSF-treated mice, relative to mice treated with excipient, revealed significant inhibition of the production of Th1 cytokines, such as IFN-γ and TNF-α, whereas IL-4 was increased (Fig. 9A). IL-10 levels were unaffected. By contrast, RPA analysis revealed an increase in TGF-β1 mRNA levels in splenocytes of G-CSF- vs excipient-treated mice (Fig. 9B).

**FIGURE 3.** RPA analysis of cytokine mRNA expression in spinal cords. Spinal cord mRNAs were extracted as indicated in Materials and Methods from mice on day 40 postimmunization with MBP that had been treated at the onset of clinical signs with excipient or G-CSF. Samples from two mice per group were compared with mRNA extracted from a naive SJL/J mice, using RPA multiprobe mCK1b (A) and mCK3b (B). The same exposure time was used for the genes of interest and for the housekeeping genes, but in A contrast enhancement, performed after the PhosphorImager scan identically for all lanes in each frame, was kept minimal for L32 and GAPDH. Quantitative analysis by ImageQuant software of the levels of the mRNAs are shown below (C corresponding to RPA A, D corresponding to RPA B). They were calculated as the mRNA ratio of the volume of the bands of the genes of interest to the mean of the volumes of the bands for the housekeeping genes L32 and GAPDH, all measured at the same exposure level. TGF-β2 and -β3, and MIF mRNA levels did not vary between lanes and are omitted from Fig. 3D.

**FIGURE 4.** RPA analysis of chemokine mRNA expression in spinal cords. mRNA was extracted from spinal cords of MBP-immunized mice that were treated with excipient (two mice) or G-CSF (two mice) and compared with that from naive SJL/J mice. The individual EAE score is indicated at the top of each lane. RPA was performed with the multiprobe mCK5. The positions of the different bands were attributed after comparison with mRNA extracted from spleens of BALB/c mice, 60 min after i.v. injection of 5 μg anti-CD3 Ab (145-2C11 clone) in 150 μl PBS (left lane). Quantitative analysis of the bands above detection limits is shown below, calculated as the mRNA ratio of the volume of the bands of the chemokines to the mean volume of the bands for the housekeeping genes L32 and GAPDH. IP-10, IFN-γ-inducible protein 10. TCA-3, mouse T cell activation gene 3.
Discussion

In the present study we demonstrate that a short 7-day treatment with G-CSF not only prevents the development of EAE when administered 1 wk before the appearance of clinical signs, but also protects from the disease when initiated later, at the onset of clinical signs. The protective effect was stable, being observed at the acute phase as well as the chronic phase of the disease, i.e., >1 mo after cessation of treatment. These therapeutic properties of G-CSF in SJL/J mice compare favorably with those of several other cytokines active at the effector phase of the disease, among them IFN-γ, which inhibits Th1 cytokine production, but for which discontinuation of treatment after 10 days caused rapid onset of EAE with enhanced clinical signs and prolonged duration of the disease (32). Interestingly, the therapeutic window for G-CSF treatment appears similar to that reported for soluble TNF receptor-stabilized molecules or IL-1 receptor antagonist (8, 9, 33).

Peripheral administration of G-CSF was able to significantly reduce the EAE-associated CNS pathology. Whether this results from a local CNS effect of G-CSF, which may potentially cross the blood-brain barrier which is damaged at the onset of clinical signs when G-CSF s.c. administration is initiated, cannot be excluded. Analysis of mRNA in spinal cords does not support an endogenous role of brain-derived G-CSF, but a regulatory effect of G-CSF therapy on brain-derived M-CSF was observed. At variance with M-CSF, which has been found at increased levels in the cerebrospinal fluid of patients with a variety of neurological diseases, including brain tumors, bacterial meningitis, and AIDS-dementia complex (34), but not in MS patients, G-CSF was found only in patients with bacterial meningitis. Constitutive expression of M-CSF has been previously reported in normal mouse brain tissue; the transcripts are detected from embryonic day 13 through adulthood (35). In vivo expression of G-CSF has been reported in neoplastic astrocytes (36–38), but was lost at higher degrees of dedifferentiation. Inflammatory and viral stimuli enhance M-CSF expression and promote G-CSF production by human and murine astrocytes (25, 26). We report here that the constitutive, high level expression of M-CSF in spinal cords increases in mice with EAE, but returns to basal levels in mice given G-CSF, whereas G-CSF itself was not significantly induced by either EAE disease or G-CSF treatment.

All mediators of immune response that we analyzed in brains of mice given G-CSF appeared to be inhibited, including proinflammatory cytokines such as IFN-γ, IL-2, TNF-α and -β, and lymphotoxin-β, as well as regulatory cytokines such as IL-10 and TGF-β. Chemokine expression was also altered, with no enhancement of any particular member of this family.
G-CSF treatment exerted a marked influence on cellular infiltration to the CNS. Experiments conducted in rats (39) demonstrating that increased absolute neutrophil count in rats given G-CSF did not increase posttraumatic brain neutrophil accumulation, suggested that the ability of G-CSF-stimulated neutrophils to migrate into injured tissue may be impaired. Likewise, the expansion of the neutrophil and macrophage subsets that we observed in spleens of our G-CSF-treated mice, which illustrated the hematopoietic properties of G-CSF, did not occur in CNS. Instead, T cell infiltration and the inflammatory reaction that normally follows were remarkably limited. These processes are under control of several sets of molecules, including the chemokines and TNF-α whose production in the periphery was shown to be altered and/or reduced by G-CSF treatment.

The role of chemokines in EAE pathogenesis differs according to the strains of mice and Ag used to induce disease. Whereas MOG-immunized C57BL/6 mice show early CNS expression of MCP-1, and lack of MCP-1 expression results in protection from the disease (an effect that may reflect the absence of recruitment of CCR2-bearing, inflammatory macrophages), in the MBP/CFA and incubated (2 × 10^6/well) for 72 h with MBP (50 μg/ml) or PBS, respectively, we were similarly analyzed by ELISA for their contents of MIP-1α and MCP-1 (D and E), and the ratios of MIP-1α vs MCP-1 production were calculated (F). Data represent the mean ± sem of triplicate results (*, p < 0.05; **, p < 0.02). Splenocytes (4 × 10^6 cells/well of 24-well plates) from excipient- or G-CSF-treated mice on day 40 after immunization with MBP were stimulated for 4 h with PBS or MBP (50 μg/ml), and their mRNA was extracted and analyzed for the content in chemokine mRNAs by RPA and PhosphorImager detection (G). The positions of the different bands were attributed after comparison with mRNA extracted from spleens of BALB/c mice, 60 min after i.v. injection of 5 μg anti-CD3 Ab (145-2C11 clone) in 150 μl PBS. H and I, mRNA ratio of the volume of the bands of MIP-1α and MCP-1 to the mean of the volumes of the bands for the housekeeping genes L32 and GAPDH.

show that the protective effect of G-CSF correlated with a reduced MIP-1α/MCP-1 production ratio in the autoactive response at both mRNA and protein levels.

This imbalance in MIP-1α/MCP-1 production ratio in macrophages and autoreactive splenocytes may first affect T cell migration to the CNS, particularly via reduction of MIP-1α production, in keeping with the inhibitory effect on the primary acute phase of EAE of anti-MIP-1α Ab treatment observed by Karpus et al. (16). Moreover, both MIP-1α reduction and MCP-1 increase may participate in immune deviation of the T cell autoreactive response in the periphery. A large body of data have demonstrated the existence of cross-regulation between cytokines and chemokines (17).

MIP-1α was shown to be induced predominantly in a Th1 setting and to, in turn, activate IFN-γ production, whereas MCP-1 is preferentially produced in Th2 settings and promotes IL-4 production (20, 21). MCP-1-deficient mice are unable to mount Th2 responses and are resistant to infection by Leishmania major (40). G-CSF treatment of MBP-immunized SJL/J mice, by reducing the production of MIP-1α and enhancing that of MCP-1 at both protein and mRNA levels, creates a pro-Th2 imbalance. Interestingly, the reduction of the MIP-1α/cIL-1β ratio matched the reduction of the IFN-γ/IL-4 ratio measured in the same splenocyte supernatants.

The production of TGF-β1 was also increased by G-CSF treatment. While the role of this potent immunoregulatory factor in promoting immune deviation is well documented in autoimmune diseases, it should be noted that it is also able to reduce MIP-1α...
Whether immune deviation of the autoreactive response is effectively associated with either reduced encephalitogenic potential of the lymphocytes in G-CSF-treated mice and/or regulatory properties of these cells deserves further investigation using adoptive transfer and cotransfer experiments.

In addition to the imbalance in chemokine production and the immune deviation toward Th2, the protection against EAE by G-CSF treatment was associated with a reduced production of TNF-α, a cytokine involved in the disease pathogenesis, particularly at the early inflammatory phase (10). This property may be related to the potent promotion of chemokine synthesis by this cytokine, often exerted in synergy with IFN-γ, on both endothelial cells (30, 31) as well as astrocytes (42). Whether this reduction of TNF-α levels is a primitive anti-inflammatory effect of G-CSF, as reported in experimental models of endotoxemia (1), or is secondary to the anti-Th1 and pro-Th2 properties also displayed by this molecule (3, 5, 6) remains to be investigated. Particularly relevant to the latter hypothesis is the fact that IL-4 is known to inhibit the macrophage production of IL-1 and TNF-α (43). The dual capacity of G-CSF to reduce the production of TNF-α by both macrophages and autoreactive splenocytes confers high regulatory potential to this molecule in the context of inflammatory reactions associated with cell-mediated autoimmune diseases.

In conclusion, we demonstrate that a short treatment with G-CSF, which limits the potential risk of immunization against the molecule, exerts remarkable and long-lived protective effects on the clinical course of EAE, even at a late stage of the disease process, when clinical signs are first detected. The progression of the disease is inhibited in the CNS of G-CSF-treated animals, which display only limited demyelination and almost no inflammation. Whether remyelination takes place under the influence of G-CSF treatment remains to be investigated. No signs of immune activation, including the expression of regulatory cytokines or chemokines, are observed in the brain tissue of G-CSF-protected mice, suggesting that this protection from autoimmune disease is secondary to reduced T cell infiltration in the CNS. We have shown that G-CSF protection is strikingly associated with important alterations of the peripheral immune system, some of which...
were previously shown to be induced by this factor in allogeneic and inflammatory settings (1, 5).

G-CSF has been used in humans for >10 yr with a remarkable tolerance. It has been shown to decrease infectious episodes and acute inflammatory response in patients with a variety of brain pathologies, including brain tumors, acute traumatic brain injury, and cerebral hemorrhage (44). Together with recent data from this laboratory (23), in which treatment with G-CSF was able to protect mice from the development of another AID, spontaneous systemic lupus, the present results, which demonstrate that G-CSF targets mechanisms critical for the pathogenesis of EAE, constitute a rationale for the clinical evaluation of G-CSF in human AID, such as MS.

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References


