Neuroprotection and Immunomodulation With Mesenchymal Stem Cells in Chronic Experimental Autoimmune Encephalomyelitis

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Objective: To investigate the therapeutic potential of mesenchymal stromal cells (MSCs) in the chronic model of experimental autoimmune encephalomyelitis (EAE).

Design: Mesenchymal stromal cells were obtained from the bone marrow of naïve C57BL and green fluorescent protein–transgenic mice and cultured with Eagle minimum essential medium/alpha medium after removal of adhering cells. Following 2 to 3 passages, MSCs were injected intraventricularly or intravenously into mice in which chronic EAE had been induced with myelin oligodendrocyte glycoprotein 35-55 peptide.

Results: In 8 separate experiments, the intravenously and intraventricularly injected green fluorescent protein–positive MSCs were attracted to the areas of central nervous system inflammation and expressed galactocerebroside, O4, glial fibrillary acidic protein, and β23 type III. The clinical course of chronic EAE was ameliorated in MSC-treated animals (0% mortality; mean [SE] maximal EAE score, 1.76 [1.01] and 1.8 [0.46] in the intraventricular and intravenous groups, respectively, vs 13% and 21% mortality and 2.80 [0.79] and 3.42 [0.54] mean maximal score in the controls). A strong reduction in central nervous system inflammation, accompanied by significant protection of the axons (86%-95% intact axons vs 43% in the controls) was observed in the animals injected with MSCs (especially following intraventricular administration). Mesenchymal stromal cells injected intravenously were detected in the lymph nodes and exhibited systemic immunomodulatory effects, down-regulating proliferation of lymphocytes in response to myelin antigens and mitogens. Mesenchymal stromal cells cultured with fibroblast growth factor and brain-derived neurotrophic factor in vitro acquired neuronal-lineage cell morphology and expressed β23, nestin glial fibrillary acidic protein, and O4.

Conclusions: Our results indicate that stem cells derived from bone marrow may provide a feasible and practical way for neuroprotection, immunomodulation, and possibly remyelination and neuroregeneration in diseases such as multiple sclerosis.

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The current treatments for multiple sclerosis are only partially effective, probably owing to defective remyelinating and regenerating mechanisms. This results in chronic, cumulative disability and irreversible axonal and neuronal damage.1,2 Additionally, it appears that the immunotherapies now in use for multiple sclerosis do not exert significant in situ immunomodulating effects in the central nervous system (CNS). It has been suggested that 2 pathogenetic processes (inflammatory and degenerative) run in parallel in this disease.

Several studies have provided evidence of neurodegeneration in multiple sclerosis.3,4 Transplantation of stem cells may enhance neuroprotective mechanisms and induce neuroregeneration. Embryonic stem cells have been studied in vitro and in animal models of neurodegeneration and CNS trauma.3,4 Despite some reservations, neuronal stem cells (injected as neuronal spheres) have proved to be successful in downregulating experimental autoimmune encephalomyelitis (EAE) by exerting peripheral and in situ immunomodulating effects. They also have the ability to provide a potential source for remyelination and neuroregeneration.3

Another potential source of stem cells is bone marrow. It hosts 2 kinds of stem cells: hematopoietic and nonhematopoietic, mesenchymal stromal cells (MSCs) being the latter type. Mesenchymal stromal cells can be isolated from other bone marrow cells by adhering to tissue culture plastic and have a spindle-shaped, fibroblastlike morphology.3 Mesenchymal
stromal cells are multipotent, self-renewing cells that can differentiate into osteoblasts, chondrocytes, and adipocytes. Several studies have shown in models of neural injury in vivo that MSCs can differentiate into neural-like and glial-like cells, mainly in vitro, and have potential for neuroregeneration. Mesenchymal stromal cells were also found to possess significant immunomodulating properties. In 2 published studies, intravenous injection of MSCs was shown to successfully suppress EAE through induction of peripheral immunosuppression. Based on these data, it seems that MSCs are ideal candidates for application in human neurological diseases because they can be obtained from adult bone marrow and injected autologously without the need for immunosuppression.

In this study, we tested the therapeutic potential of MSCs derived from bone marrow, administered intravenously or intrathecally, in the chronic progressive model of EAE, which, owing to its chronic course and extensive axonal pathology, is similar to multiple sclerosis. The immunomodulatory effects of MSC treatment in vitro and in vivo were also evaluated along with their potential for neuroprotection.

### METHODS

#### MICE

The C57BL6 mice and green fluorescent protein (GFP)-transgenic C57BL6 mice (provided by T.B.-H.) were used under the approval of the Hadassah-Hebrew University Medical School’s Ethics Committee and the experiments were conducted in accordance with the US Public Health Policy on Human Care and Use of Laboratory Animals. All animals were kept in pathogen-free animal facilities.

#### CHRONIC-EAE INDUCTION

Chronic EAE was induced in 6- to 7-week-old female C57BL6 mice by immunization with an emulsion containing 300 µg of purified myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide in phosphate-buffered saline (PBS) and an equal volume of complete Freund adjuvant containing 3 mg of H37Ra (Difco Laboratories, Detroit, Michigan). A 0.2-mL volume of the inoculum was injected subcutaneously on the day of induction (day 0) and at day 7. In addition, 300 ng of Bordetella pertussis toxin in 0.2 mL of PBS were injected intraperitoneally on days 0 and 2. Animals with EAE were scored daily for neurological symptoms according to the EAE clinical severity scale: 0 = asymptomatic; 1 = partial loss of tail tonicity; 2 = tail paralysis; 3 = hind limb weakness; 4 = hind limb paralysis; 5 = 4-limb paralysis; 6 = death.

#### BONE MARROW MSC ISOLATION

Femurs and tibias were removed from 6- to 9-week-old C57BL and GFP-transgenic C57BL female mice and bone marrow cells were collected by flushing the bones with Eagle minimum essential medium/alpha medium supplemented with 10% fetal calf serum, 1% vitamins, 1% glutamine, and 1% nonessential amino acids. The cells were centrifuged, resuspended in fresh culture medium, and counted. Subsequently, the isolated bone marrow cells were seeded in 75-cm² flasks and grown in an incubator at 37°C and 7% carbon dioxide for 48 hours. The plates were then washed with PBS to remove all nonadhering cells; the attached cells were cultured for 14 days with an exchange of medium twice weekly and then harvested and reseeded at a ratio of 1:3.

#### CHARACTERIZATION OF ISOLATED MSCs BY FLOW CYTOMETRY

Mesenchymal stromal cells (passages 2-3) were harvested by trypsinization, divided into polystyrene fluorescence-activated cell sorter (FACS) tubes, and labeled with antimouse CD45 fluorescein isothiocyanate (1:200), antimouse CD29-phycocerythrin (PE) (1:100), and antimouse CD44-PE (1:100) antibodies for 45 minutes in the dark. Fluorescence data were collected from 10 000 cells and measurements and analysis were performed using a FACScan (BD Biosciences, Franklin Lakes, New Jersey).

#### MSC TRANSPLANTATION IN VIVO

In 8 separate experiments, 104 C57BL6 mice were immunized with MOG to induce chronic EAE. Of these, 55 served as controls and were administered saline injections intravenously or intrathecally. Of the remainder, 22 were injected intrathecally with MSCs on day 10 after EAE induction and 27 received MSCs intravenously. Ten days after EAE induction, mice were anesthetized with intraperitoneal injection of pentobarbital (0.6 mg/10 g) and were fixed in a stereotactic device for the intrathecinal transplantation. Quantities of 10⁶ MSCs/10 µL of saline were used for this route. For the intravenous route, 10⁵ MSCs/100 µL of saline were used. Control animals for each group were treated under the same conditions with an equivalent amount of saline only.

#### IN VITRO MSC DIFFERENTIATION ASSAY

Bone marrow cells obtained from C57BL mice were cultured with Eagle minimum essential medium/alpha medium after depletion of nonadhering hematopoietic progenitors to develop a pure MSC population. To induce MSCs to differentiate into various cell phenotypes, 200 000 cells were plated in culture dishes and allowed to reach confluence. Osteogenic differentiation medium, consisting of 10% Eagle minimum essential medium/alpha medium and fetal bovine serum, supplemented with 50-mg/mL ascorbic acid, 10mM β-glycerophosphate, and 10⁵M dexamethasone, was exchanged twice a week for 3 weeks. For adipocytic differentiation, the medium was supplemented with 1-methyl-3-isobutylxanthine, 10⁵M dexamethasone, 3mM insulin, and 5mM indomethacin. For neural-glial differentiation, MSCs from passages 3 to 4, were seeded on fibronectin-coated 24-well plates and cultured in Eagle minimum essential medium/alpha medium enriched with 7% bovine serum, 1% glutamine, 1% vitamins, 1% penicillin-streptomycin, and 1% ampicillin, then a solution of 40mM alizarin red (pH 4.0) was added and left for 15 minutes at room temperature. Stained cells were then washed with PBS to remove all nonadhering cells; the attached cells were cultured for 14 days with an exchange of medium twice weekly and then harvested and reseeded at a ratio of 1:3.

#### DETECTION OF OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION

To detect osteoblastic differentiation, alizarin red dye, which stains the mineralized matrix excreted by bone-differentiated tissues, was used. The cells were fixed with cold methanol for 5 minutes on ice; a solution of 40mM alizarin red (pH 4.0) was added and left for 15 minutes at room temperature. Stained cells were then washed with PBS to remove all nonadhering cells; the attached cells were cultured for 14 days with an exchange of medium twice weekly and then harvested and reseeded at a ratio of 1:3.

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were washed with double-distilled water and air dried. To detect adipogenic differentiation, oil red O (10 mg/mL) was added and left for 20 minutes at room temperature. The cells were washed 3 times with PBS and fixed with paraformaldehyde, 4%, for 20 minutes.

**IMMUNOSTAINING ASSAY FOR IN VITRO NEURAL DIFFERENTIATION**

The medium was aspirated and the cells were washed gently with 0.05% Tween 20 diluted in PBS and (Figure 1) then fixed with fresh paraformaldehyde, 4%, for 20 minutes at room temperature. To stain the intracellular components, the cells were permeabilized with Triton X-100, 0.9%, for 7 minutes. For blocking nonspecific binding, the cells were rinsed with 5% bovine serum albumin in PBS for 60 minutes at room temperature on a slowly rotated plate. Then, the cells were washed 3 times with 0.05% Tween 20 diluted in PBS, and incubated with the following primary antibodies: antineuron nestin, antineuron β-tubulin type III, antineuron glial fibrillary acidic protein (GFAP), and antineuron O4 diluted to the required concentrations with FACS buffer containing 1% bovine serum albumin. After washing, the cells were incubated with goat antimouse fluorescein isothiocyanate–conjugated and goat antirabbit tetramethylrhodamine isothiocyanate–conjugated secondary antibodies diluted in bovine serum albumin buffer, 1%, on a slowly rotated plate for 45 minutes in the dark at room temperature. The cells were mounted on slides with mounting solution and examined under fluorescence and light microscopy.

**HISTOPATHOLOGIC EXAMINATION**

Mice (5 per group; mean EAE severity score at day of euthanasia, 2.7) were anesthetized with a lethal dose of pentobarbital and the brains and spinal cords were perfused with ice-cold PBS followed by paraformaldehyde, 4%, on days 42 to 80 (median, 61 days) after chronic-EAE induction. The inflammatory process and axonal pathology of chronic EAE were quantified using axial frozen sections at predetermined levels. Sections were stained with a modified Bielschowsky technique for simultaneous evaluation of the axonal pathology and inflammation. Brain, corpus callosum, cerebellar, and spinal cord sections were evaluated under 20 × magnification of optical fields. An examiner blinded to treatment and clinical severity counted the total number of perivascular mononuclear infiltrates in hematoxylin-eosin sections and estimated the severity of axonal injury and axonal loss (modified Bielschowsky stain). To grade inflammation, the number of perivascular infiltrations and the number of cells per perivascular cuff were counted. To grade the axonal injury, we established a scale of severity as follows: 0=normal tissue; 1=a few scattered injured axons; 2=focused (up to 25% of an ×10 magnified field area) mild to moderate axonal injury; 3=moderate mild to severe axonal injury; and 4=scattered severe axonal injury. For axonal loss we used a similar scale: 0=normal axonal density; 1=focused mild to moderate axonal loss; 2=moderate mild to moderate axonal loss; 3=focused severe axonal loss; and 4=scattered severe axonal loss.

**IMMUNOFLOUORESCENT STAINING OF DIFFERENTIATED CELLS IN VIVO**

For immunostaining, brains and spinal cords were prepared as described previously. The following antibodies were used for co-localization: Alexa 488–conjugated goat antimouse IgM (1:100), goat antirabbit IgG (1:100), or goat antimouse IgG (1:100) was added as a secondary antibody. Immunofluorescence staining was performed on 6- to 8-µm frozen axonal brain sections obtained 2.85 mm below the bregma. Frozen sections were warmed to room temperature and washed 3 times with PBS. The sections were then incubated for 45 minutes at room temperature with 5% bovine serum albumin in PBS to block nonspecific binding and incubated with the primary antibodies overnight at 4°C and with the secondary antibody for 45 minutes in the dark. The slides were mounted with 4′,6-diamidino-2-phenylindole.

**IN VITRO PROLIFERATION OF LYMPHOCYTES**

Draining lymph nodes were excised from C57BL mice with EAE on postimmunization day 10 and cultured as single-cell suspensions. Lymph node cell (LNC) proliferation was assayed in vitro by ³H-thymidine incorporation. All cultures were carried out in triplicate in 96-well, flat-bottom, microtiter plates. The assay was carried out by seeding 4 × 10⁵ cells/well in 0.2 mL of RPMI medium (Sigma, Rehovot, Israel) supplemented with 2.3% fetal calf serum, 1mM L-glutamine, and antibiotics. Basal ³H-thymidine incorporation was determined in response to concanavalin A (1 µg/mL) or purified MOG 33–55 (10 µg/mL). To examine the effect of MSCs on LNC proliferation, different numbers of MSCs were added and cocultured with the LNCs. The cultures were incubated for 48 hours in a humidified atmosphere of 5% carbon dioxide at 37°C and then pulsed for 16 hours with ³H-thymidine (1 µC/well). Cells were harvested on fiberglass filters using a multiharvester and the radioactivity was counted.

**STATISTICAL ANALYSIS**

Data from 8 experiments were pooled to provide the mean (SD) for each experimental group. For histopathology, we initially tested the normality of the data using the Shapiro-Wilk and Kolmogorov-Smirnov tests. If data were found to violate the normality assumption and a logarithmic transformation could not be applied, we used the nonparametric equivalent tests for group comparison. The values are expressed as the
RESULTS

ISOLATION AND CHARACTERIZATION OF MSCs

After 14 days of culture of the bone marrow–derived cells, spindle-shaped fibroblastlike cells began to appear in the plates. Cells from passages 2 to 3 were tested by FACS analysis for the expression of the markers CD45 (hematopoietic lineage marker), CD29, and CD44 (adhesion molecules, largely expressed on MSCs). More than 90% of the cells presented a CD45−/CD44+/CD29+ phenotype (data not shown), consistent with nonhematopoietic MSCs.19,20 To prove their mesodermal nature, we cultured MSCs in adipogenic and osteogenic media. Adipogenesis was evident morphologically owing to the transformation of MSCs into rounded cells containing lipid vesicles, visualized by oil red O. When induced to differentiate into osteoblasts, the cells formed mineralized structures, as manifested by alizarin red staining of calcium deposits (data not shown).

DIFFERENTIATION OF MSCs IN VITRO INTO CELLS EXPRESSING NEURAL AND GLIAL MARKERS

To test their neurodifferentiation potential, purified MSCs (passages 2-3) were cultured with a mixture of growth factors, including fibroblast growth factor 2, brain-derived neurotrophic factor, and fibroblast growth factor 8. Under these conditions, MSCs showed morphological changes (development of thin and long processes) resembling neuronal-like and glial-like cells. In each differentiation set, there was positive immunostaining for the neural-lineage cell markers nestin and -tubulin type III (neural markers), GFAP (astrocytic marker), and O4 (oligodendrocytic marker) (Figure 1).

CLINICAL IMPROVEMENT AND AXONAL PROTECTION FOLLOWING MSC TRANSPLANTATION

The clinical course of chronic EAE was ameliorated in animals treated with MSCs following both intraventricular and intravenous administration (Figure 2). As
presented in Table 1, mice treated intraventricularly with MSCs had a mortality rate of 0% and a mean (SE) maximal clinical score of 1.76 (1.01) compared with 13% mortality (P < .001, Mann-Whitney U test) and a mean (SE) maximal clinical score of 2.8 (0.79) in the saline-injected control group (P < .001, t test). In animals treated with MSCs intravenously, the mortality rate was again 0% vs 21% in the controls (P < .001, Mann-Whitney U test); mean (SE) maximal clinical scores were 1.8 (0.46) vs 3.42 (0.54), respectively (P < .001, t test) (Table 1). All deaths were related to EAE disease severity. In addition to the saline-treated control group, we used a mesenchymal cellular control, in which mouse fibroblasts were injected. Injection of the fibroblasts did not induce any beneficial effect on the clinical course of chronic EAE (data not shown).

Histopathologic evaluation of brains and spinal cords of MSC-treated mice at day 42 after chronic-EAE induction revealed a reduction in the total number of infiltrates in both the intravenously and the intraventricularly MSC-treated animals (P < .001, Mann-Whitney U test) (Figure 3A). The size and cellularity of the lymphocytic infiltrates were significantly reduced only in the intraventricularly MSC-treated group (P < .001, Mann-Whitney U test) (Figure 3B). The latter may indicate more efficient local immunomodulation when MSCs are injected intraventricularly.

A neuroprotective effect was observed following treatment with MSCs and was more profound in the group treated intraventricularly (Table 2). In animals with chronic EAE that were treated with saline or left untreated, more than half of the axons were significantly damaged; whereas in mice treated with intravenous or intraventricular MSCs, the great majority of the axons (85% and 95.8%, respectively) were found intact at 42 to 80 days (median, 61 days) following treatment. This neuroprotective effect was also evident when axonal loss was graded (Table 2).

Table 2. Axonal Damage and Loss in Mice With Experimental Allergic Encephalitis (EAE) Treated With Mesenchymal Stromal Cells (MSCs)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Normal</th>
<th>Few Scattered</th>
<th>Focused Mild to Moderate</th>
<th>Scattered Mild to Moderate or Focused Severe</th>
<th>Scattered Severe</th>
<th>Axonal Loss Score, Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40 (5)</td>
<td>26.7 (5)</td>
<td>10.0 (5)</td>
<td>20.0 (5)</td>
<td>3.3 (5)</td>
<td>1.75 (0.3)</td>
</tr>
<tr>
<td>Mice with EAE</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Intraventricular PBS</td>
<td>56.4 (5)</td>
<td>23.1 (5)</td>
<td>5.1 (5)</td>
<td>12.8 (5)</td>
<td>2.6 (5)</td>
<td></td>
</tr>
<tr>
<td>Intravenous PBS</td>
<td>46.0 (5)</td>
<td>30.0 (5)</td>
<td>10.0 (5)</td>
<td>10.0 (5)</td>
<td>4.0 (5)</td>
<td></td>
</tr>
<tr>
<td>Intraventricular MSCs</td>
<td>85.8 (5)^b</td>
<td>4.2 (5)^b</td>
<td>0</td>
<td>0</td>
<td>0.3 (0.15)</td>
<td></td>
</tr>
<tr>
<td>Intravenous MSCs</td>
<td>85.0 (5)^c</td>
<td>10.0 (5)^c</td>
<td>5.0 (5)</td>
<td>0</td>
<td>1.3 (0.4)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: PBS, phosphate-buffered saline.

^a^ Axonal loss: 0 = normal axonal density; 1 = focused mild to moderate axonal loss; 2 = scattered mild to moderate axonal loss; 3 = focused severe axonal loss; and 4 = scattered severe axonal loss.

^b^ For statistical comparisons, the χ^2^ test for between-group comparison and the nonparametric Kruskal-Wallis test were applied, P < .001.

^c^ For statistical comparisons, the χ^2^ test for between-group comparison and the nonparametric Kruskal-Wallis test were applied, P < .05.
Mice were injected with MSCs on day 10 after chronic-EAE induction. The intravenously injected GFP-positive MSCs were detected in the inflamed areas of the CNS (as visualized by fluorescence microscopy) (Figure 4A and B), showed morphological and immunohistological features of neuronal lineage cells, and expressed neuronal (positive staining for β-tubulin type III) (Figure 4D), astrocytic (positive staining for GFAP) (Figure 4F), and oligodendrocytic (positive staining for galactocerebroside) (Figure 4H) markers. Mesenchymal stromal cells transplanted intravenously were also detected in the white matter (in close proximity to the EAE lesions) and acquired neural and glial morphology and specific cell marker expression: GFP-positive transplanted cells stained positively for β-tubulin type III, GFAP, and O4 (Figure 4C, E, and G, respectively).

Most of the incorporated GFP-positive MSCs were those expressing astrocytic markers (GFAP positive, data not shown), their pattern of biodistribution in the white matter of the CNS being in direct correlation with the degree of inflammation (Figure 5). In areas where there was no significant inflammation (Figure 5A), the transplanted cells did not penetrate into the white matter and only surrounded the ventricular lining (Figure 5B). In contrast, in areas where significant inflammation was observed (Figure 5C), transplanted cells were found in the inflammatory lesion (Figure 5D).

IMMUNOMODULATORY EFFECTS OF MSCS IN VITRO AND IN VIVO

Coculture of MSCs with myelin-sensitized lymphocytes (obtained from mice administered MOG 35-55 for EAE induction) induced a dose-dependent suppression of the proliferation (Figure 6A). When equivalent quantities of fibroblasts were used as cellular controls, no suppression of lymphocyte proliferations was observed (data not shown). Proliferation assays revealed that LNCs isolated from mice with EAE that were pretreated with MSCs did not proliferate in the presence of the mitogen concanavalin A, in contrast with the LNCs isolated from saline-treated mice with chronic EAE (Figure 6B).

MIGRATION OF INTRAVENOUS MSCS TO LYMPH NODES

To further elucidate the in vivo immunomodulatory mechanisms of MSCs, we examined whether intravenously injected MSCs migrate to the peripheral lymph nodes. Immunohistology revealed that at early stages (starting on day 3 after MSC injection), GFP-positive cells (derived from the bone marrow of GFP-transgenic donors) could be detected in the lymph nodes.
nodes (Figure 7A), indicating that they may exert a peripheral immunomodulatory effect. This could account for the observed decrease in infiltrates in the CNSs of mice given MSCs intravenously. Green fluorescent protein–positive cells were also detected in the lymph nodes at later stages of the disease (day 40), as shown in Figure 7B.

**COMMENT**

Our results show the neuroprotective effect of MSCs in the mouse model of chronic EAE. Mesenchymal stromal cells injected intravenously or intraventricularly suppressed the clinicopathologic manifestations of chronic EAE and induced a protective effect on the axons (prevention of axonal damage), which was more pronounced with intraventricular administration. Our data, showing a significant reduction in the number of infiltrating cells in the brains of MSC-treated animals with chronic EAE, reconfirm the recently described immunomodulatory effects of MSCs. In our experiments, MSCs downregulated (both in vitro and in vivo) the proliferative reactivity of lymphocytes to MOG 35-55 and to the concanavalin A mitogen. In 2 previously published studies, intravenous injection of MSCs was shown to successfully suppress EAE through induction of peripheral immunomodulation.\(^{17,18}\) In contrast to those findings, in our experimental setting we demonstrated the advantages of direct injection of MSCs into the ventricles of the brain, where they induced a more pronounced reduction in infiltrating lesions, indicating an additional and possibly more important local in situ immunomodulatory effect. The peripheral immunomodulatory effects of MSCs are equally important and the migratory ability of these cells in the lymph nodes (when injected intravenously) argue in favor of this assumption.

We also show that MSCs can, under special conditions, in vitro (presence of growth factors) and in vivo (existence of neuroinflammation due to chronic EAE), express neuronal lineage markers and that they can effectively enter the inflamed white matter areas of the CNS. Moreover, the extent of MSC biodistribution was in direct correlation to the degree of inflammation in the CNS. This putative transdifferentiation ability of MSCs in our chronic EAE model represents a novel finding not evident in the previous publications, in which the authors argue in favor of a sole immunomodulatory mechanism of action of MSCs.\(^{17}\)

In multiple sclerosis, one of the main reasons for the relatively limited efficacy of immunomodulating treatments is the significant destruction and loss of axons, which leads to irreversible CNS atrophy and progressive disability, which is evident even following the administration of extreme cytotoxic regimes.\(^{22}\) Additional reasons for the observed lack of efficacy of immunomodulation in multiple sclerosis may include the limited ability of the immunomodulatory drugs to reach the affected areas of multiple sclerosis lesions deep in the white matter.

Clinical experience with various neuroprotective strategies in neurodegenerative processes, as in the case of cerebrovascular events and CNS trauma, have not been successful so far.\(^{23}\) The use of stem cells has, therefore, become the focus of research in the past decade as a possible solution for induction of neuroregeneration and neuroprotection.

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**Figure 6.** A, Significant dose-dependent suppression of lymph node cell (LNC) proliferation in mice with chronic experimental allergic encephalitis treated with mesenchymal stromal cells (MSCs). B, Suppression of the proliferation of lymphocytes isolated from mice pretreated with MSCs. ConA indicates concanavalin A; MOG, myelin oligodendrocyte glycoprotein; *P < .001.

**Figure 7.** Fluorescence microscopy of frozen lymph node sections from mice with chronic experimental allergic encephalitis (EAE) treated with mesenchymal stromal cells (MSCs). There are a significant number of green fluorescent protein (GFP)–positive cells as early as 3 days after MSC injection (A) but also later on day 40 after chronic-EAE induction (B). Arrow indicates GFP-positive cell; *, lymph node parenchymal cells.
The use of bone marrow–derived MSCs in our study provides several advantages over conventional neuronal, embryonic, and hematopoietic stem cells used in other studies. MSCs can be obtained from adult bone marrow, are readily cultivated and expand in large numbers, can be injected autologously without the need for immunosuppressive means to prevent rejection, and are less prone to genetic abnormalities during multiple in vitro passages, carrying a lower risk for induction of malignancies compared with other types of stem cells. As suggested by earlier studies, including those from our group, the mechanisms of action of stem cells in general (whether neuronal, embryonic, or adult stem cells) are multiple and include a neuroprotective effect mediated by the newly injected cells and/or the activation of local resident CNS stem cells and progenitors through growth factors locally produced by the injected stem cells, a neuroregenerating mechanism, possibly through the growth factors provided by the injected stem cells or through transdifferentiation of the injected stem cells, and a systemic or local immunomodulatory effect of the stem cells.

Indeed, systemic immunoregulation by stem cells has been demonstrated by several groups. That the biodistribution of the injected MSCs correlates well with the degree of inflammation in the white matter indicates that the focal immunomodulatory and neuroprotective effects can be induced precisely in the proximity of the active lesions. Local immunomodulation may be especially important because the immunomodulators in use probably have a limited ability to act directly at the site of lesions in multiple sclerosis. Moreover, it seems that the axonal damage in multiple sclerosis is related to local, “departmentalized” inflammation and therefore the only successful way to induce local immunomodulation is by injecting cells that can migrate in and around the lesion areas. According to our data, this can be achieved more efficiently by intraventricular administration of MSCs.

Our data confirm all 3 mechanisms of action of stem cells in MSCs. We believe that whatever the dominant mechanism, there is a clear neuroprotective effect, which was evident and prominent throughout the 2-month observation in our experiments. The chronicity of the clinical paralytic signs in our chronic-EAE model and the prominent axonal pathology render this model more reminiscent of human multiple sclerosis. Our data, therefore, seem to be more relevant for future applications of such treatment modalities in patients with neuroimmunologic diseases.

The expression of surface markers specific for neurons, oligodendrocyte progenitors, and astrocytes in significant proportions, both in vitro cultures of MSCs (in the presence of a mixture of growth factors) and in vivo (in the proximity of CNS lesions in the white matter) may support the transdifferentiation of MSCs. However, great uncertainty exists in the literature regarding this possibility in contrast to fusion mechanisms, which may equally explain the colocalization of those neuronal lineage surface markers on GFP-positive donor MSCs (Figure 4). In addition, we found a significant portion of positively stained recipient CNS cells in chronic-EAE lesions (for neuronal lineage progenitor markers), indicating parallel activation of the resident stem cell repertoire (Figure 4).

In summary, the practical advantages of using bone marrow–derived MSCs, together with the clinicopathologic efficacy we now report in the model of chronic EAE, may provide the scientific basis for the use of MSCs as a future treatment for induction of neuroprotection (and effective in situ immunomodulation) in diseases such as multiple sclerosis. Based on our data, the intrathecal approach might be more advantageous because it induces a local immunomodulatory effect and stronger neuroprotection.

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