

Granulocyte Macrophage Colony Stimulating Factor Produced in Lesioned Peripheral Nerves Induces the Up-Regulation of Cell Surface Expression of MAC-2 by Macrophages and Schwann Cells

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Abstract. Peripheral nerve injury is followed by Wallerian degeneration which is characterized by cellular and molecular events that turn the degenerating nerve into a tissue that supports nerve regeneration. One of these is the removal, by phagocytosis, of myelin that contains molecules which inhibit regeneration. We have recently documented that the scavenger macrophage and Schwann cells express the galactose-specific lectin MAC-2 which is significant to myelin phagocytosis. In the present study we provide evidence for a mechanism leading to the augmented expression of cell surface MAC-2. Nerve lesion causes nonneuronal cells, primarily fibroblasts, to produce the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF). In turn, GM-CSF induces Schwann cells and macrophages to up-regulate surface expression of MAC-2. The proposed mechanism is based on the following novel observations. GM-CSF mRNA was detected by PCR in vitro and in vivo degenerating nerves, but not in intact

nerves. The GM-CSF molecule was detected by ELISA in medium conditioned by in vitro and in vivo degenerating peripheral nerves as of the 4th h after injury. GM-CSF activity was demonstrated by two independent bioassays, and repressed by activity blocking antibodies. Significant levels of GM-CSF were produced by nerve derived fibroblasts, but neither by Schwann cells nor by nerve derived macrophages. Mouse rGM-CSF enhanced MAC-2 production in nerve explants, and up-regulated cell surface expression of MAC-2 by Schwann cells and macrophages. Interleukin-1 β up-regulated GM-CSF production thus suggesting that injury induced GM-CSF production may be mediated by interleukin-1 β . Our findings highlight the fact that fibroblasts, by producing GM-CSF and thereby affecting macrophage and Schwann function, play a significant role in the cascade of molecular events and cellular interactions of Wallerian degeneration.

PERIPHERAL nerve injury is followed by Wallerian degeneration of the nerve segment situated distal to the lesion site (26). Molecular and cellular events that take place during the course of Wallerian degeneration turn the degenerating nerve into an environment that supports the regeneration of peripheral and central adult neurons (4, 7). Cellular events that characterize Wallerian degeneration are the breakdown of axons, the proliferation of Schwann cells, the recruitment of macrophages from the circulation, and the removal of myelin by phagocytosis by macrophages and Schwann cells. It has been recently demonstrated that myelin-associated glycoprotein (MAG)¹, a component of central and peripheral myelin, is

a potent inhibitor of axonal growth (20, 22). A rapid and efficient mechanism for the removal of myelin is required, therefore, for successful regeneration.

We have recently documented (28) that Schwann cells and blood born macrophages express the galactose specific lectin MAC-2 in their cytoplasm and on their surfaces during the course of Wallerian degeneration of mice peripheral nerves. We have further provided evidence suggesting that cell surface MAC-2 mediates myelin phagocytosis. Furthermore, MAC-2 is considered a "macrophage activation marker" due to the fact that its expression is up-regulated in activated phagocytic macrophages (10). Therefore, MAC-2 is significant to myelin removal and, in turn, to nerve regeneration for either one or two reasons. One, MAC-2 may be directly involved in mediating myelin phagocytosis. Second, MAC-2 marks activated macrophages regardless of the type(s) of phagocytic mechanism(s) they use.

The vast majority of Schwann cells and activated macrophages that take part in Wallerian degeneration express MAC-2 on their surfaces (28). In contrast, Schwann cells that reside in intact nerves do not express MAC-2 at all

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1. *Abbreviations used in this paper:* CM, conditioned medium; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-1, interleukin-1; MAG, myelin-associated glycoprotein; M-CSF, macrophage colony-stimulating factor; MP, macrophage precursor.

(28). Macrophages are recruited into injured nerves from the pool of blood monocytes of which only 60–70% express cell surface MAC-2 at very low levels (23, 24). The percentage of macrophages that display cell surface MAC-2 and levels of expression of MAC-2 by individual cells are subject to modulation by changing environmental conditions (12, 23, 24). Furthermore, granulocyte macrophage colony stimulating factor (GM-CSF) activates macrophages and concomitantly up-regulates MAC-2 expression (10). It is most probable, therefore, that macrophages that become activated while being recruited into degenerating nerves are also induced to up-regulate surface expression of MAC-2. In the present study, we sought after a mechanism which, in injured nerves, up-regulates cell surface expression of MAC-2 in macrophages and Schwann cells.

We have studied GM-CSF expression in intact and injured peripheral nerves of C57/BL/6NHS mice that display the normal rapid progression of Wallerian degeneration. We have previously documented some of the events and reviewed others (28). The nonneuronal cells residing in intact nerves are primarily Schwann, fibroblasts, and endothelial. After nerve transection, segments situated distal to lesion sites undergo Wallerian degeneration. Macrophages start being recruited into the degenerating nerve during the third day of Wallerian degeneration, where they become numerous during the fourth day. Axonal degeneration, myelin destruction, and myelin removal by phagocytosis are completed 5–7 d after axotomy. The rapid progression of Wallerian degeneration is correlated with a concurrent rapid rise of MAC-2 expression by Schwann cells and macrophages.

In the present study we demonstrate that peripheral nerve injury induces the production of GM-CSF, primarily by fibroblasts, within 4–5 h. GM-CSF, in turn, induces Schwann cells and macrophages to up-regulate cell surface expression of MAC-2. The up-regulation of GM-CSF production is mediated by interleukin-1 (IL-1). Our findings stress the significant role that fibroblasts play in the cascade of molecular events and cellular interactions of Wallerian degeneration.

Materials and Methods

Animals and Surgical Procedures

The experimental protocol was approved by the authority for research and development of the Hebrew University of Jerusalem. C57/BL/6NHS mice (Harlan Sprague Dawley Inc., Jerusalem, Israel), 6–8-month old were used. Surgical procedures were performed under anesthesia. Sciatic nerves were transected after leaving the pelvis. Nerve explants were obtained by placing intact nerve segments in culture kept in a humidified incubator, saturated by 5% CO₂ at 37°C. Freeze damaged nerves were obtained by three cycles of freeze/thaw of nerve segments that were placed back into the donor animals.

Culture Medium

Medium used to culture bone marrow derived cells was the Modified Eagle Minimum Essential Medium alpha, supplemented by 10% FCS, and 1% of the following standard solutions (Beit-Haemek, Beit-Haemek, Israel): nonessential amino acids, vitamins, sodium pyruvate, and penicillin/streptomycin, and 10 µg/ml transferrin (Sigma Chemical Co., St. Louis, MO). Medium used to culture nerve explants, Schwann cells, and fibroblasts was the DME supplemented by 10% FCS and 1% penicillin/streptomycin solution (Beit-Haemek).

Cell Dissociation and Culture

Nonneuronal single cell type cultures were obtained by enzymatic dissociation and differential adhesion plating from either intact, or in vitro or in vivo degenerating, or freeze-damaged nerves, as previously described (28, 30). The different cell types were distinguished from each other by their distinct morphology and immunocytochemistry (F4/80 for macrophages, S-100 for Schwann, and MAC-2 for both) (28). Single cell cultures used were at least 95% pure.

Bone Marrow-derived Macrophage Precursor Cells

(1, 36). Bone marrow cells from 1–2-month-old BALB/c mice were incubated in 10% medium conditioned by the L929 cell line that produces the macrophage colony stimulating factor (M-CSF). Surviving nonadherent cells, the macrophage precursor cells, were collected and further used for testing GM-CSF activity.

Conditioned Medium

Intact, in vitro degenerating, and in vivo degenerating nerves were cut and washed for 1 h in DME, and then incubated in DME supplemented by 10% FCS (0.25–0.5 ml/nerve) in a humidified incubator saturated by 5% CO₂ at 37°C. Each sample of CM was produced by two to four nerves. Macrophage, Schwann and fibroblast cell cultures were also used to condition medium. Wet weights of tissues and DNA content of cell cultures were determined. Then, the proportion of volumes conditioned by either 1 mg of wet weight tissue, or by the equivalent of 1 ng of DNA of cells were calculated. DNA levels were determined by fluorescence emission of the Hoechst 33258 stain at a wave length of 458 nm (18).

GM-CSF mRNA Detection by In Vitro Amplification of GM-CSF cDNA

Nerve tissues (50 mg) were homogenized in 1 ml lysis/binding buffer (Tris-HCl, pH 8.0, 0.5 M LiCl, 10 mM EDTA, 1% SDS, 1 mM dithiothreitol), centrifuged (30 s at 13,000 rpm), and supernates transferred to tubes containing 1 mg oligo dT linked magnetic beads (DynaL AS, Oslo, Norway). Annealing washing and elution of poly A RNA linked beads was performed by using the Dynal MPC-E magnet according the manufacturers instructions.

cDNA synthesis of poly A RNA was performed using the “first strand cDNA synthesis kit” (Amersham Buckinghamshire, UK) with oligo dT as primer. The GM-CSF cDNA was amplified using GM-CSF sense (AGC-ATGTAGAGGCCAATCAAAGAAGC) and anti-sense (TGCATTCAA-AGGGGATATCAGTCAGA) oligonucleotide primers synthesized (General Biotechnology, Rehovot, Israel) following published sequences (GenBank/EMBL/DDBJ). For in vitro cDNA amplification, 12.5 pmole primers, 64 µM dNTP's and 1.25 U Taq polymerase (Boehringer-Mannheim, Mannheim, Germany) were added to the incubation buffer, and the entire mixture overlaid by mineral oil and heated to 96°C for 4 min in a MJ Research Programmable Thermal Controller. The in vitro amplification was carried out for 40 cycles at the following temperature profile: 96°C for 4 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (primer extension). Negative controls consisted of the identical poly A RNA preparations that were subjected to the same procedures as above but with the omission of reverse transcriptase. Poly A RNA preparations purified from the GM-CSF producing cell line BMS2 were used as positive controls. The amplification products were visualized by ethidium bromide after separation on a 3% agarose gel. DNA molecular weight markers (pBR328/Bgl I and pBR328/Hinf I) were used. The reaction product was sequenced by an Applied Biosystems sequencer to verify that it was indeed the GM-CSF cDNA.

Identification and Quantitation of GM-CSF by ELISA

To identify and quantify GM-CSF in CM we used the two site sandwich ELISA (Pharmingen, San Diego, CA) according to manufacturers instructions. Concentrations of GM-CSF were determined from standard curves that were constructed for each experiment using recombinant GM-CSF (rGM-CSF) at a concentration range from 0–1,600 pg/ml.

Detection of GM-CSF Activity by the Granulocyte-Macrophage (GM) Colony Formation Assay

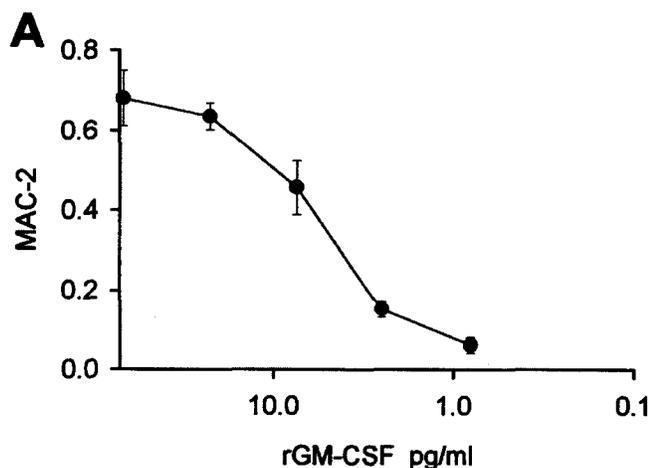
GM-CSF activity can be detected (21) based on its unique ability to form mixed granulocyte-macrophage colonies from single precursor cells. Bone marrow cells (3×10^5 /ml) in soft agar (0.25%) were overlaid on nerve explants undergoing in vitro degeneration or on monolayers of fibroblasts or Schwann cells. After 7–10 d, cells were fixed (PAF), agar dried, colonies stained by hematoxylin, and cell types identified by light microscopy.

Macrophage Precursor Cell Differentiation Assay

We have developed an assay system to detect GM-CSF bioactivity. The assay is based on two facts. One, in the absence of any other cytokine, bone marrow derived macrophage precursor (MP) cells depend on GM-CSF for their survival and differentiation into mature monocytes/macrophages. Second, the ability of a mAb raised against rGM-CSF to inhibit GM-CSF bioactivity. rGM-CSF rescued and induced the differentiation of MP cells into mature macrophages in a dose-dependent manner (Fig. 1). Hence, surviving cells expressed molecules that characterize mature macrophages: MAC-1, MAC-2, the Fc receptor, and the F4/80 antigen (2, 13, 24, 25) (see Fig. 1 for MAC-2 and the F4/80 antigen). The activity of rGM-CSF, judged by the number of surviving cells and levels of MAC-2 expression (see results below and Fig. 3), was inhibited 50% to 60% by a mAb raised against rGM-CSF. We have chosen to follow GM-CSF activity by monitoring levels of cell surface associated MAC-2 by ELISA for two reasons. One, the relationship between GM-CSF and surface expression of MAC-2 is the focus of our interest. Second, one can easily and reliably determine MAC-2 on adherent cells by ELISA (see below).

Bone marrow derived MP cells were seeded in 96 well dishes at a density of 80×10^3 /well. To determine a dose response curve for rGM-CSF, various dilutions of rGM-CSF (Genzyme, specific activity of 10^6 U/mg) were added to test wells in which the final incubation volume reached 0.2 ml. Each dilution was repeated five times. Surviving, adherent cells were studied by ELISA or immunofluorescence light microscopy after 4 d.

To test whether nonneuronal cells that reside in intact or lesioned nerves conditioned medium with GM-CSF activity, various dilutions of CM were added to test wells. For each sample of CM, the concentrations used were the proportions of volume conditioned by 180, 60, 20, 6.6, 2.2, and 0.7 μ g of wet weight tissue, in a final volume of 0.2 ml. These are referred to in the text as dilutions 1 through 7. Concentrations used to test CM produced by either fibroblasts, Schwann cells or macrophages were the proportions of volume conditioned by the equivalents of 180, 60, 20, 6.6, 2.2, 0.7, and 0.23 ng DNA of cells, in a final volume of 0.2 ml. These are referred to in the text as dilutions 1 through 7. The design of experiments was such that each sample of CM was tested in several dilutions to obtain a dose response curve. Each dilution step was tested in quadruplicates. In parallel, a dose-response curve of rGM-CSF was determined.



given in a logarithmic scale. (B) MP cells were incubated for 4 d in the presence of 60 pg/ml of rGM-CSF, and then stained immunohistochemically by a mAb raised against the F4/80 antigen. Surviving adherent cells are mature macrophages since they display the F4/80 antigen which is specific to this cell type. Bar, 50 μ m.

Levels of GM-CSF activity in CM were calculated from the ratio of MAC-2 expression induced by dilution 3 of CM over that induced by 7.4 pg/ml of rGM-CSF.

To determine whether the ability of CM to rescue and lead to the differentiation of MP cells into mature monocytes/macrophages is due to GM-CSF activity, we tested the ability of a mAb raised against rGM-CSF (Genzyme, Boston, MA) to inhibit CM activity. CM (0.1 ml of dilution 3) and rGM-CSF (0.1 ml of 7.4 pg/ml) were incubated for 1 h at 37°C with 2 μ g of the mAb. The mixture was then added to the wells containing MP cells (in 0.1 ml of medium). Percent inhibition was calculated from the ratio of MAC-2 expression induced by the tested sample in the presence of the mAb over that induced by the same sample in the absence of the mAb.

ELISA Determination of Cell Surface-associated MAC-2 Levels

We followed the protocol described in reference 1 with some modifications. Adherent cells were washed three times at 37°C with Ca/Mg/PBS, fixed at 37°C for 30 min with 4% paraformaldehyde (in Ca/Mg/PBS), washed 3 \times in PBS, blocked over night at 4°C with 3% BSA in PBS, washed 3 \times in PBS, incubated up to 2 h at room temperature with a 1:10 dilution (in 1% BSA in PBS) of the mAb M3/38, washed 4 \times in PBS, incubated for 1 h in a 1:5,000 dilution (in 1% BSA in 50 mM Tris, 100 mM NaCl, pH 8) of alkaline phosphatase conjugated goat anti-rat IgG (Jackson ImmunoResearch, Avondale, PA), washed 4 \times in PBS, incubated for 2 h at room temperature with substrate solution (1 mg/ml *p*-nitrophenyl phosphate sodium in 10% diethanolamine, pH 9.8; Sigma Chemical Co., St. Louis, MO). The reaction product was read in a Dynatec ELISA reader at 405 nm wavelength.

ELISA Determination of MAC-2 Levels in Tissues

Nerves were homogenized for 2 min in 50 mM sodium carbonate buffer, pH 10.0, in a volume of 0.3 ml/nerve, and further incubated for 1 h at 37°C. 0.1 ml/nerve of 0.5 M sodium carbonate, pH 9.6, was added to samples that were then vortexed and centrifuged for 10 min at 15,000 g. Protein contents of supernates were determined (protein assay reagent, BioRad Laboratories, Cambridge, MA), protein concentration adjusted to 12.5 μ g/ml with sodium carbonate buffer, pH 9.6, and 50 μ l of serial dilutions were used to coat 96-well plates (Maxisorb Immuno Plates 96F; Nunc, Roskilde, Denmark) over night at 4°C. Wells were washed 2 \times with TBS, blocked for 2 h at 37°C with 2% gelatin (Sigma Chemical Co.) in TBS, washed 2 \times with 0.1% Tween 20 in TBS (TBST), incubated over night at 4°C with mAb M3/38 (diluted 1:50 in 1% BSA in PBS), washed 2 \times with TBST, incubated for 1 h in a 1:5,000 dilution (in 1% BSA in 50 mM Tris, 100 mM NaCl, pH 8.0) of alkaline phosphatase conjugated goat anti-rat IgG (Jackson ImmunoResearch), washed 4 \times in PBS, incubated for as long as required at room temperature with substrate solution (1 mg/ml

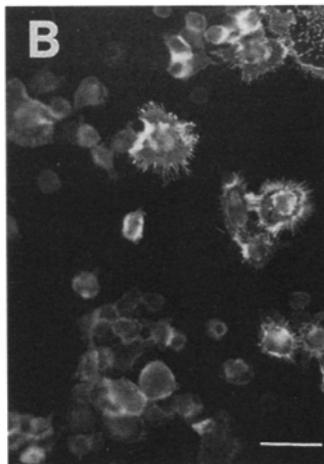


Figure 1. rGM-CSF induces MP cells to differentiate into macrophages that express MAC-2 and the F4/80 antigen on their surface. (A) rGM-CSF induces MP cells to differentiate into macrophages that express MAC-2 on their surface in a dose dependent manner. Levels of cell surface MAC-2 were determined by ELISA on adherent surviving cells, and are presented in optical density units. Each data point is the average of five repetitions, error bars ± 1 SEM. rGM-CSF concentrations are

p-nitrophenyl phosphate sodium in 10% diethanolamine, pH 9.8; Sigma Chemical Co.). The reaction product was read in a Dynatec ELISA reader at 405 nm wavelength.

Immunofluorescence Microscopy

We have used immunofluorescence microscopy to visualize the cell surface antigens MAC-1, MAC-2, Fc, and F4/80, and intracellular MAC-2 and S-100 as previously described (28).

Antibodies Used

Immunocytochemistry. mAbs raised against MAC-1, F4/80 antigen, and Fc receptor (Serotec, Oxford, England), supernates of the hybridoma cell line M3/38 that produces anti MAC-2 mAb (American Type Culture Collection, Rockville, MD), anti S-100 and FITC-conjugated rabbit anti-rat IgG (Bio-Makor, Rehovot, Israel).

ELISA determination of cell surface MAC-2 and tissue content of MAC-2: the anti-MAC-2 mAb, as above.

Two site sandwich ELISA determination of GM-CSF content: capture and detecting antibodies (Pharmingen, USA), alkaline phosphatase conjugated IgG (Jackson, USA).

GM-CSF activity blocking: anti GM-CSF mAb (Genzyme, Cambridge, MA).

Results

GM-CSF Production in Lesioned Peripheral Nerves

Degenerating peripheral nerves produced detectable levels of GM-CSF as of the fourth hour after nerve injury and through the entire 2 wk tested. Intact nerves and nerve segments situated distal to sites of nerve transection were incubated in medium for 5 h. Nonneuronal cells that reside in these tissues conditioned medium (CM) by molecules they synthesize and secrete. CM were tested by ELISA to determine whether and to what extent is GM-CSF produced by the nonneuronal cells that reside in the Wallerian degenerating nerves (Fig. 2). GM-CSF was detected in CM produced by intact nerves during their first 5 h of incubation, immediately after removal from animals (the first data point in Fig. 2). We further tested production during each and every one of these first 5 h using three dis-

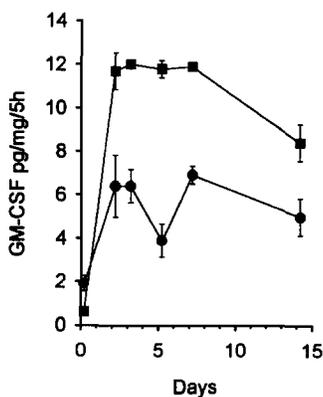


Figure 2. The time course of GM-CSF production by degenerating nerve segments. Nerve segments situated distal to sites of nerve transection were removed from two different batches of mice at various times after surgery, and allowed to condition medium for 5 h. CM were assayed, by two site sandwich ELISA, for GM-CSF content. Then, GM-CSF production was calculated (pg/mg wet weight of nerve tissue/5 h). Each data point is placed on

the time axis in accordance to the post surgery day + 5 h. The first data point for each batch of mice reflects GM-CSF production by the intact nerve during the first 5 h of in vitro degeneration taking place at the time of incubation. All other data points reflect GM-CSF production in Wallerian degenerating nerves. The two batches of mice differ in the magnitude of their response. Data points are the average of three distinct experiments. Error bars ± 1 SEM.

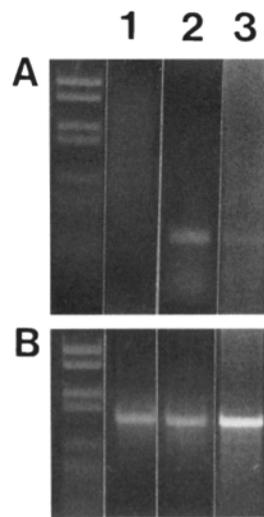


Figure 3. GM-CSF mRNA is expressed in in vitro and in vivo degenerating nerves. PolyA RNA was isolated from intact, 16 h in vitro degenerating nerves, two and six d in vivo degenerating nerves, and from BMS2 cells. Poly A RNA samples were used to synthesize cDNA which was then amplified by PCR. Reaction products were separated by agarose gel electrophoresis. (A) The expected 315-bp PCR reaction product indicating the presence of GM-CSF mRNA was not detected in intact nerves (1), it was detected in 24 h in vitro degenerating nerves (2), and 2 d in vivo degenerating nerves (3). The GM-CSF mRNA was also detected in GM-CSF producing BMS2 cells (not shown). Negative controls were PCR reactions that included polyA RNA samples that had the GM-CSF mRNA but not cDNA in them, and samples containing the GM-CSF sense and antisense primers but neither the GM-CSF mRNA nor cDNA (not shown). (B) The PCR reaction product indicating the presence of β -actin was detected in all preparations.

tinct samples of CM. GM-CSF was first detected (sensitivity of assay 8 pg/ml) in two samples during the fourth, and in all samples during the fifth consecutive hour of incubation. GM-CSF production continued for the entire 14 d of Wallerian degeneration tested. Levels of production differed between animals of different purchases from vendor.

GM-CSF mRNA was detected in in vitro and in vivo degenerating but not intact nerves. We tested for the presence of the GM-CSF mRNA by in vitro amplification of GM-CSF cDNA obtained by cDNA synthesis of polyA RNA isolated from intact and lesioned nerves (Fig. 3). GM-CSF mRNA was never detected in any one of five distinct polyA RNA isolations from intact nerves. It was detected in every one of five distinct isolations from 16 h in vitro degenerating nerves. It was detected in three out of six distinct isolations from 2–6 d in vivo degenerating nerves. We verified that the reaction product is indeed the GM-CSF cDNA by sequencing it, and by performing in vitro amplification of GM-CSF cDNA that was obtained by cDNA synthesis of poly A RNA isolated from the GM-CSF producing BMS2 cell line (not shown). It is not clear to us why it is that we could not demonstrate the GM-CSF mRNA in each one of the isolations from Wallerian degenerating nerves. One possible explanation could be the instability of the GM-CSF mRNA (32, 34, 37), which may account also, at least in part, for the variability in GM-CSF levels detected during the course of Wallerian degeneration (Fig. 2).

GM-CSF Activity Production in Lesioned Peripheral Nerves

GM-CSF produced by in vivo and in vitro degenerating nerves is biologically active. CM were tested for GM-CSF activity using the bone marrow-derived MP cell differentiation assay. We have used this system to test for GM-CSF

activity since GM-CSF supports the survival and differentiation of MP cells into mature adherent macrophages that express MAC-2, the molecule which is the focus of our interest (see Materials and Methods and Fig. 1). CM of 5 and 24 h in vitro degenerating nerve explants, and 5–7 d in vivo degenerating nerves led to the survival and differentiation of MP cells into MAC-2 positive macrophages in a dose dependent manner (Fig. 4 A). To quantify, we compared levels of activity produced by a standard amount of tissue (dilution 3 of CM) to levels of activity produced by a standard concentration of rGM-CSF (7.4 pg/ml) (Fig. 5 A). Levels of activity produced were largest for CM of 5–7 d in vivo degenerating nerves (59.1%), less for CM of 24 h in vitro degenerating explants (15.3%), and the least for CM of 5 h in vitro degenerating explants (2.6%).

GM-CSF activity blocking mAb repressed GM-CSF activity present in CM. The average levels of inhibition obtained were 58.5% of the activity of rGM-CSF (7.4 pg/ml), and 83.3 and 56.4% of the activities produced by CM (dilution 3) of 24 h in vitro degenerating explants and 5–7 d in vivo degenerating nerves, respectively (Fig. 5 B). In five inhibition experiments involving rGM-CSF (7.4 pg/ml), the number of surviving cells were counted: inhibition averaged $51.6\% \pm 2.5\%$ (SEM). Cell survival was also tested on two different samples of CM (dilution 3) produced by 5–7 d in vivo degenerating nerves: repression averaged 56.8%. The mAb thus inhibited the activities of rGM-CSF and CM to about the same extent with regard to MAC-2 expression and cell survival.

We tested the in vitro production of GM-CSF activity using an additional bioassay: the mixed granulocyte–macrophage (GM) colony formation assay (21). The uniqueness of GM-CSF is in its ability to induce single bone marrow precursor cells to form mixed GM colonies. Intact nerve explants were overlaid by bone marrow cells in soft agar. Mixed colonies were detected after 7–10 d of culture

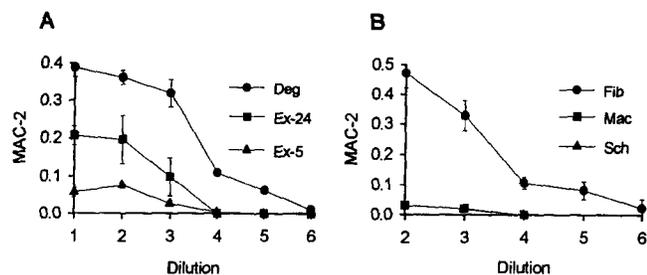


Figure 4. Lesioned peripheral nerves and nerve derived fibroblasts produce CM that like rGM-CSF (Fig. 1) induce MP cells to differentiate into macrophages expressing cell surface MAC-2 in a dose-dependent manner. (A) Examples of CM produced by 5–7 d in vivo degenerating nerves (*Deg*), and 5 (*Ex-5*) and 24 h (*Ex-24*) in vitro degenerating nerve explants. (B) Examples of CM produced by fibroblasts (*Fib*), Schwann cells (*Sch*), and macrophages (*Mac*). The values obtained for Schwann and macrophages are similar, and their symbols overlay. Levels of MAC-2 are presented in optical density units. Each dilution of CM represents a volume of medium conditioned by equal amounts of tissues (A) or equal numbers of cells (B), each dilution step was tested in quadruplicates and represents a threefold decrease in CM concentration. Error bars ± 1 SEM.

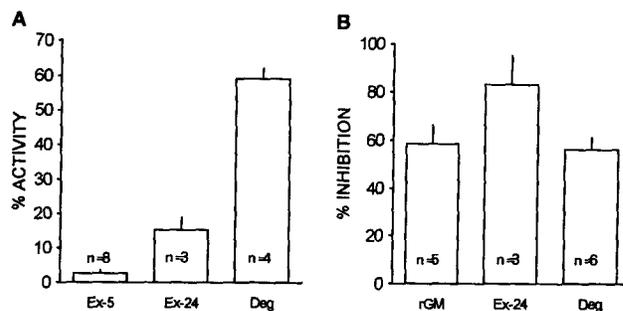


Figure 5. The ability of CM of lesioned nerves and of rGM-CSF to induce MP cells to differentiate into macrophages expressing MAC-2 is inhibited by a GM-CSF activity blocking monoclonal antibody. (A) 5 and 24 h in vitro degenerating nerve explants (*Ex-5* and *Ex-24*), and 5–7 d in vivo degenerating nerves (*Deg*) differ in the levels of GM-CSF activity they produce. Levels of activity were calculated from the ratio of cell surface MAC-2 expression induced by CM (dilution 3) over that induced by rGM-CSF (7.4 pg/ml). Averages differ significantly (*Ex-5* over *Ex-24* $P < 0.012$, *Ex-5* over *Deg* $P < 0.004$, by the two-tailed Mann Whitney U test). (B) A mAb raised against rGM-CSF inhibits the activity of CM (dilution 3) produced by 24 h in vitro degenerating explants (*Ex-24*) and by 5–7 d in vivo degenerating nerves (*Deg*) to the same extent as, or more than, it inhibits the activity of rGM-CSF (7.4 pg/ml). Bars represent averages, error bars ± 1 SEM, n = number of experiments. Four nerves were examined in each one of the experiments that were done in quadruplicates.

thus indicating the presence of GM-CSF activity in the medium.

The time course of GM-CSF production by in vitro degenerating nerves is phasic. We examined the time course of GM-CSF production during in vitro degeneration by the MP differentiation assay at 24 h time intervals. Nerve explants were incubated in fresh medium for time periods alternating between 5 and 19 h for 5 d. CM obtained during the first 5 h of each day were tested. GM-CSF activity production was about threefold higher during the second day of in vitro degeneration than during the first day. As of the third day and through the fifth day it declined to about the same or lower levels than those produced during the first. This phasic in vitro production of GM-CSF was verified by determining GM-CSF in CM by ELISA.

GM-CSF Production by Nerve-derived Fibroblasts, Macrophages, and Schwann Cells

Nerve derived fibroblasts but neither Schwann nor macrophages produce significant levels of GM-CSF. The non-neuronal cell population of intact nerves and intact nerve explants are primarily Schwann and fibroblasts. Macrophages are scarce, but increase in number following injury, consequent to their recruitment from the circulation (28, 31, 35). Fibroblast cell cultures were established either from intact nerves or 6 d in vivo degenerating nerves. Fibroblast cultures were first obtained 2 d after the beginning of cell dissociation. Thereafter, fibroblasts were allowed to condition medium for 16 h. Fibroblasts CM were tested by the MP cell differentiation assay and found to contain significant levels of GM-CSF activity (Fig. 4 B). In three experiments involving CM (dilution 1) of fibroblasts obtained from intact nerves, activity averaged $66.6\% \pm 6.3\%$

(SEM) of the activity of rGM-CSF (7.4 pg/ml). In two experiments involving fibroblasts obtained from 6 d *in vivo* degenerating nerves, activity averaged $101\% \pm 2\%$ (SEM) of the activity of rGM-CSF (7.4 pg/ml). Inhibition of activity by the activity blocking GM-CSF mAb was tested in one experiment and reached 55%. The production of GM-CSF activity by fibroblasts was further verified using the mixed GM colony formation assay. Monolayers of fibroblasts originating from 6 d *in vivo* degenerating nerves were overlaid by bone marrow cells in soft agar. Mixed GM colonies were detected 7–10 d there after, indicating the presence of GM-CSF activity. The GM-CSF molecule was further detected by ELISA in CM of nerve derived fibroblasts that were grown in regular medium or medium supplemented by rIL-1 β and rTNF α (see below), and medium supplemented by 1 μ g/ml LPS (not shown).

Nerve derived macrophages produced minute levels of GM-CSF. The MP differentiation assay was used to test CM of macrophages obtained from 5–7 d freeze-damaged nerves. In such preparations the normal nonneural cell population that had been destroyed by freezing is replaced, primarily, by recruited macrophages, and only to a lesser extent by fibroblasts (28). We were unable to detect GM-CSF activity in either one of three experiments in which macrophage CM were tested (Fig. 4 B). Freeze-damaged nerve derived macrophages were allowed to produce CM for 24 h in medium supplemented by 1 μ g/ml LPS, which amplifies GM-CSF production (see below). CM were assayed by ELISA and GM-CSF levels detected were $0.25 \text{ pg}/5 \times 10^5 \text{ cells} \pm 0.11$ (SEM; six experiments). In comparison, thioglycolate elicited peritoneal macrophages that were exposed to LPS as above produced $25.30 \text{ pg GM-CSF}/5 \times 10^5 \text{ cells} \pm 0.33$ (SEM, three experiments). The failure of nerve derived macrophages to produce significant levels of GM-CSF was also deduced from six experiments in which we could not detect GM-CSF in media conditioned for 24 h by 6 d freeze-damaged nerve prepara-

tions neither by ELISA nor by the MP cell differentiation assay. Determined by ELISA, freeze-damaged nerve preparation exposed to 50 μ g/ml LPS for 24 h in culture produced $0.92 \pm 0.05 \text{ pg GM-CSF}/\text{mg wet weight tissue}$ (SEM; three experiments). To test for the effectiveness of LPS, we exposed 6 d Wallerian degenerating nerves to LPS, as above, and detected production levels of $62.18 \pm 7.65 \text{ pg GM-CSF}/\text{mg wet weight tissue}$ in 24 h (SEM, six experiments). GM-CSF production by Wallerian degenerating nerves is amplified by LPS (compare to Fig. 3).

Schwann cells did not produce GM-CSF. The MP differentiation assay was used to test CM of Schwann cells obtained from 5–7 d *in vivo* degenerating nerves. We were unable to detect GM-CSF activity in either one of four experiments in which Schwann cells CM were tested (Fig. 4 B). The failure of cultured Schwann cells to produce GM-CSF activity was further verified in three mixed GM colony formation assays that gave negative results. We were further unable to detect GM-CSF by ELISA in CM of Schwann cells that were either or not exposed to 1 μ g/ml LPS for 24 h.

GM-CSF Production Is Up-regulated by IL-1 β and TNF α

IL-1 β and TNF α are the first cytokines to be produced in response to tissue damage and, in turn, they initiate a cascade of inflammatory and immune related events (8, 9), one of which is the induction of GM-CSF production in macrophages, fibroblasts and endothelial cells (3, 5, 11, 16, 17, 33, 38). We tested, therefore, whether IL-1 β and TNF α can increase GM-CSF production in lesioned nerves and nerve derived fibroblasts as well. Intact nerve explants and nerve derived fibroblasts were exposed to rIL-1 β and rTNF α for 48 h and CM assayed for GM-CSF content by ELISA. rIL-1 β and rTNF α significantly increased GM-CSF production by nonneural cells in nerve explants, and by nerve derived fibroblasts in culture (Fig. 6).

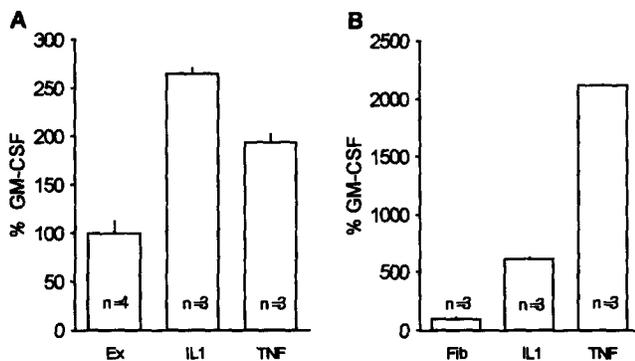


Figure 6. IL-1 β and TNF α induce an increase in GM-CSF production by nerve explants (A) and nerve-derived cultured fibroblasts (B). (A) Nerve explants were incubated in medium (Ex) and medium supplemented by 100 U/ml of rIL-1 β (IL1) or 40 ng/ml of rTNF α (TNF) for 48 h. (B) Nerve derived fibroblasts were incubated in medium (Fib) and medium supplemented by 100 U/ml of rIL-1 β (IL1) or 100 ng/ml of rTNF α (TNF) for 48 h. CM were assayed for GM-CSF production by ELISA. The results are expressed as percent of production by nerve explants and fibroblasts incubated in regular medium. Bars represent averages, error bars ± 1 SEM, n = number of experiments.

GM-CSF Up-regulates MAC-2 Expression in Schwann Cells, Macrophages, and Peripheral Nerve Tissue

Nerve injury is followed by a dramatic increase in MAC-2 expression by Schwann cells that normally do not display the molecule at all (28). GM-CSF can induce the up-regulation of surface expression of MAC-2 in Schwann cells. Schwann cells dissociated from intact nerves were seeded on laminin coated 96-well plates. Adherent Schwann cells were exposed to rGM-CSF for 4 d. A maximal increase in surface MAC-2 expression was obtained at a concentration of 66 pg/ml of the cytokine, reflecting a 2.7-fold increase over levels of expression by Schwann cells that were cultured in regular medium (Fig. 7 B). No increase in surface levels of MAC-2 were obtained when cultures were exposed to 600 pg/ml of GM-CSF. GM-CSF thus has a dual effect on Schwann cell surface MAC-2 expression. It should be noted that levels of MAC-2 expressed by cultured Schwann cells that were not exposed to GM-CSF do not reflect levels of MAC-2 expression by Schwann cells residing in intact nerves, which is undetectable (28). MAC-2 expression by these cultured cells is, most probably, the result of them being in the presence of the GM-CSF producing fibroblasts (see above) for at least 3 d,

which is the earliest before Schwann cells are separated from fibroblasts during the course of cell dissociation.

The vast majority of macrophages that reside in Wallerian degenerating nerves express MAC-2. Macrophage cell cultures were established from nerve segments that were allowed to degenerate *in vivo* for 5 d. Cells were stained immunohistochemically to visualize surface associated MAC-2. Of 1350 cells that were randomly sampled, 98% displayed MAC-2 on their surface. In contrast, only 60–70% of blood monocytes, the pool from which tissue macrophages are drawn from, display surface MAC-2 (24). Since recruited macrophages become activated, they are expected to up-regulate MAC-2 expression (10). To test whether GM-CSF could have been involved in the up-regulation of surface expression of MAC-2 in tissue macrophages, we examined peritoneal macrophages before and after exposure to rGM-CSF. We chose peritoneal macrophages because they arise from the same pool of blood monocytes as do nerve macrophages, they display low levels of MAC-2 on their surface, and are easy to obtain. Resident peritoneal macrophages (obtained by peritoneal lavage) were seeded in 96-well plates (15×10^4 cells/well) and allowed to adhere to the dish for 2–4 h. Non adherent cells were washed, and adherent macrophages exposed to rGM-CSF for 4 d. Cell surface levels of MAC-2 were determined by ELISA. Three and fivefold increases in surface levels of MAC-2 expression were obtained at a concentration of 66 pg/ml and 600 pg/ml of the cytokine, respectively (Fig. 7 A).

We further tested the ability of rGM-CSF to up-regulate MAC-2 production in the peripheral nerve tissue. We first determined, by ELISA, the time course for the “inherited” lesion induced increase in MAC-2 expression during *in vivo* and *in vitro* degeneration. *In vivo* degenerating nerves were examined in three experiments. MAC-2 contents (percentages of MAC-2 content in 5 d degenerating nerves ± 1 standard error), were 1.6 ± 0.2 in intact nerves, and 4.2 ± 0.4 in 1 d, 12.9 ± 1.6 in 2 d, and 70.6 ± 6.5 in 3 d *in vivo* degenerating nerves. *In vitro* degenerating nerves were assayed at 6 h time intervals. MAC-2 contents (percentages of MAC-2 content in 6 d degenerating nerves ± 1 standard error), were 1.5 ± 0.42 in intact nerves, and $1.3 \pm$

0.25 in 6 h, 2.2 ± 0.25 in 12 h, 2.4 ± 0.08 in 18 h, and 4.5 ± 0.85 in 24 h degenerating nerves. Thus, the initial small but significant increase in MAC-2 content in *in vitro* degenerating nerves occurred after 12 h of degeneration. Note that the initial increase in GM-CSF production occurred within the first 5 h after lesion and thus precedes the initial increase in MAC-2 production that occurs 12 h after injury. We have used the time window of the first 2 d after injury to test for the additional effect of rGM-CSF on the “inherited” increase in MAC-2 content during *in vitro* degeneration. Intact nerve explants that were exposed for 48 h after explantation to 10 ng/ml rGM-CSF expressed significantly higher levels (1.7-fold increase) of MAC-2 than nerve explants that were not exposed to rGM-CSF (Fig. 7 C).

Discussion

In the present study we provide evidence for a novel cascade of molecular events and cellular interactions that take place during the course of Wallerian degeneration of mice peripheral nerves. Nerve injury induces non neuronal cells, primarily fibroblasts, to produce GM-CSF. GM-CSF, in turn, induces macrophages and Schwann cells to up-regulate surface expression of MAC-2. The injury induced production of GM-CSF is mediated, at least in part, by IL-1. We further suggest that the up-regulation of MAC-2 expression is significant to myelin removal and, in turn, to regeneration for either one or two reasons. One, MAC-2 may be directly involved in mediating myelin phagocytosis. Second, MAC-2 marks activated phagocytic macrophages regardless of the type of phagocytic mechanism they use.

GM-CSF Production

It is most probable that nonneuronal cells that reside in intact nerves do not produce GM-CSF. This conclusion is based on two observations. One, the lack of detection of the GM-CSF mRNA in intact nerves. Second, the GM-CSF molecule was not detected by two site sandwich ELISA in CM produced by intact nerve explants during the first 3 h after removal from animals. In contrast, non-

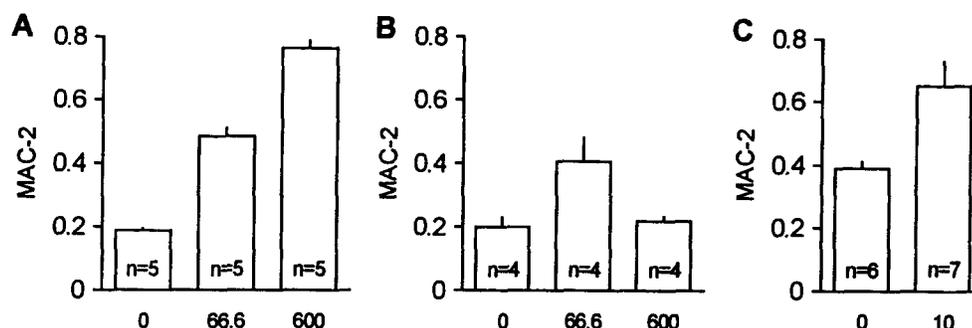


Figure 7. GM-CSF up-regulates MAC-2 expression by isolated peritoneal macrophages, isolated Schwann cells, and Schwann cells residing in nerve explants. (A) Peritoneal macrophages exposed to rGM-CSF (pg/ml as indicated) for 4 d express significantly higher levels of surface MAC-2 than macrophages cultured in regular medium ($P < 0.008$). (B) Schwann cells derived from

nerve explants were exposed to rGM-CSF (pg/ml as indicated) for 4 d. rGM-CSF has a dual effect on cultured Schwann cells. At a concentration of 66.6 pg/ml, Schwann cells express significantly higher levels of surface MAC-2 than cells cultured in regular medium ($P < 0.028$). At a concentration of 600 pg/ml surface levels of MAC-2 do not differ from those in cells cultured in regular medium. (C) Intact nerve explants exposed to rGM-CSF (ng/ml as indicated) for 48 h express significantly higher levels of MAC-2 than explants cultured in regular medium ($P < 0.014$). Bars represent averages, error bars ± 1 SEM, n = number of experiments. Two-tailed levels of significance by the Mann Whitney U test. In C, four nerves were examined in each one of the experiments that were done in quadruplicates.

neuronal cells that reside in *in vitro* and *in vivo* degenerating nerves produce GM-CSF. This conclusion is based on four independent lines of observations. First, GM-CSF mRNA was detected in *in vitro* degenerating and *in vivo* degenerating nerves. Second, the identification and detection of the GM-CSF molecule by two site sandwich ELISA in CM of *in vitro* and *in vivo* degenerating nerves. Third, the detection of GM-CSF bioactivity in CM by the mixed granulocyte-macrophage colony formation and the MP cell differentiation assays. Fourth, the inhibition of GM-CSF bioactivity in CM by GM-CSF activity blocking monoclonal antibodies.

In vivo, the up-regulation of GM-CSF production is rapid and continuous. It was detected as early as of the fourth h after nerve injury, and continued for the entire 2 wk after lesion period tested. The *in vitro* production is phasic. It subsided to very low levels 1–2 d after injury. Levels of GM-CSF production by *in vivo* degenerating nerves were further augmented by the exposure to LPS, thus suggesting that infection and other inflammatory stimuli can amplify GM-CSF production already induced by trauma.

The nonneuronal cells that compose intact and *in vitro* degenerating nerves are primarily Schwann cells, fibroblasts, and endothelial cells. Macrophages that are scarce, become numerous during *in vivo* Wallerian degeneration, following their recruitment from the circulation (28, 31, 35). Fibroblasts can account for GM-CSF production in the *in vitro* and *in vivo* degenerating nerves. This conclusion is based on the detection of GM-CSF production by the nerve derived fibroblasts (by ELISA, MP differentiation assay, and GM colony forming assay). Our results are in accord with previous observations demonstrating GM-CSF production by fibroblasts that reside in other tissues (16, 38). We could not detect GM-CSF in CM of cultured Schwann cells. We could detect very little GM-CSF production, if any, by macrophages that were present in or dissociated from freeze-damaged nerves. If the conduct of dissociated Schwann cells and macrophages *in vitro* is a reflection of their behavior *in vivo*, then neither cell type adds significantly to the production of GM-CSF during the course of *in vivo* Wallerian degeneration. Of particular interest is the finding of the poor production of GM-CSF by nerve derived macrophages, since activated macrophages that reside in other tissues (peritoneal, for example, in the present study) produce high levels of GM-CSF. Vascular endothelial cells can also produce GM-CSF (3, 5, 33). Since these cells are present in peripheral nerves, the possibility that they may have contributed to the production of GM-CSF in degenerating nerves remains unresolved.

It is very likely that IL-1 mediates the up-regulation of GM-CSF production during the course of Wallerian degeneration. This suggestion is supported by three observations. One, that IL-1 β and TNF α are the first cytokines to be produced in response to tissue damage (8, 9). Second, our present observation that rIL-1 β increased the production of GM-CSF in nerve explants and nerve derived fibroblasts. Third, our detection of IL-1 activity production in 5 h *in vitro* and 5–7 d *in vivo* degenerating mice nerves (29). We presently demonstrate that TNF α can also up-regulate GM-CSF production. We have little evidence that TNF α does so during the course of Wallerian degeneration

since we barely were able to detect TNF α , by ELISA at a sensitivity of 10 pg/ml, in some of the same samples of CM in which we were able to detect GM-CSF (unreported results).

GM-CSF Up-regulates Schwann and Macrophage Cell Surface Expression of MAC-2

GM-CSF that is produced during *in-vivo* Wallerian degeneration up-regulates cell surface expression of MAC-2 by Schwann cells that normally do not express MAC-2 (28). Three lines of observation support this conclusion. One, rGM-CSF increased cell surface expression of MAC-2 by cultured Schwann cells. Second, GM-CSF activity production preceded the increase in MAC-2 expression during the course of both *in vitro* and *in vivo* degeneration by several hours. Third, the addition of rGM-CSF to nerve explants during their first 48 h in culture added significantly to the “inherited” lesion induced increase in MAC-2 expression by Schwann cells.

GM-CSF that is produced during *in-vivo* Wallerian degeneration up-regulates cell surface expression of MAC-2 by recruited macrophages. This suggestion is based on the following observations. We presently show that 98% of macrophages that reside in Wallerian degenerating nerves express MAC-2, whereas only 60–70% of blood monocytes, the source of macrophage recruitment, do so (23, 24). We further demonstrate that cell surface expression of MAC-2 is up-regulated by GM-CSF in non activated peritoneal macrophages that originate from blood monocytes as do macrophages that reside in Wallerian degenerating nerves. Our observations and suggestions are in agreement with previous findings. One, that the percentage of macrophage that display cell surface MAC-2 and levels of MAC-2 expressed by individual cells are subject to modulation by changing environmental conditions (12, 23, 24). Second, that GM-CSF activates macrophages and concomitantly up-regulates MAC-2 expression (10).

It is most likely that the concentrations of GM-CSF that we have used to test for its effect on macrophages and Schwann are within physiological range. Taking a GM-CSF production rate of 10 pg/mg/5h, for example (Fig. 2), and assuming a volume of 1 μ l for 1 mg tissue weight, the above production rate will build up a concentration of 10 ng/ml. This is a rough estimate which is not taking into consideration that the extracellular volume of the tissue is much smaller, thus suggesting that the concentration of the cytokine may reach much higher levels. We have demonstrated that GM-CSF modulates macrophage precursor cell survival and surface MAC-2 expression in the concentration range of 1 pg/ml to 1,000 pg/ml (Fig. 2), macrophage and Schwann surface expression of MAC-2 in the concentration range of 60–600 pg/ml (Fig. 7), and MAC-2 content in an *in vitro* degenerating nerve at a concentration of 10 ng/ml (the effective concentration is likely to be lower because of diffusion barriers for a protein into a tissue).

Conclusion

Our data suggest the following sequence of events to take place during the course of Wallerian degeneration. Nerve lesion is followed by GM-CSF production which is medi-

ated, at least in part, by IL-1. Fibroblasts are a major source for GM-CSF production. GM-CSF activates macrophages and concomitantly induces the expression of the galactose specific lectin MAC-2 on the surfaces of macrophages and Schwann. MAC-2 thus marks activated phagocytic macrophages, and as a cell surface associated molecule may also be instrumental in lectin-mediated phagocytosis of myelin.

In the present study we have focused on GM-CSF as a macrophage activator and an up-regulator of MAC-2 expression. GM-CSF could play additional roles in Wallerian degeneration. In support of this notion, GM-CSF levels remain high after phagocytosis is complete. Examples of such additional roles could include the modulation of IL-1 activity present in injured nerves (29) by increasing IL-1 receptor antagonist production (15), and thus affect, indirectly, nerve growth factor synthesis (19). GM-CSF could also affect the endothelial cell wall of capillaries that supply blood to peripheral nerves by inducing endothelial cell proliferation and migration (6, 14).

Our present findings further indicate that fibroblasts, by producing GM-CSF and thereby affecting Schwann cell and macrophage function, play a significant role in the cascade of molecular events and cellular interactions that characterize Wallerian degeneration. This notion is further supported by previous findings of ours that nerve resident fibroblasts produce apolipoprotein E (30) and interleukin-6 (27).

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