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Glatiramer acetate inhibition of tumor necrosis factor-α-induced RANTES expression and release from U-251 MG human astrocytic cells

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Abstract

Glatiramer acetate is an approved drug for the treatment of multiple sclerosis (MS). RANTES is a β-family chemokine that manifests chemoattractant activity for T lymphocytes and monocytes/macrophages implicated in the pathogenesis of MS lesions. However, the effect of glatiramer acetate on the regulation of RANTES secretion in glial cells is unknown. In the present study, we demonstrate for the first time that treatment of human U-251 MG astrocytic cells with glatiramer acetate blocks tumor necrosis factor-α (TNF-α)-induced RANTES mRNA and protein in a dose- and time-dependent manner. This effect is attributed to inhibition of transcription and a 40% decrease in transcript stability. Furthermore, our electrophoretic mobility shift assays of nuclear extracts from TNF-α-treated cells reveal an increase in DNA-binding activity specific for the nuclear factor-kappa B (NF-κB) binding site, in the 5′-flanking promoter region of the human *RANTES* gene, and that this increase in NF-κB binding activity is prevented by pretreatment with glatiramer acetate or the NF-κB inhibitors. These findings suggest that glatiramer acetate may exert its therapeutic effect in MS partially through inhibiting NF-κB activation and chemokine production.

Keywords: astrocyte, glatiramer acetate, glia, nuclear factor-kappa B, RANTES, tumor necrosis factor-α.

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Multiple sclerosis (MS) is a primary inflammatory demyelinating disease of the CNS characterized clinically by relapses and remissions (McFarlin and McFarland 1982; Trapp *et al*. 1998). Pathologically, MS is characterized by acute and chronic inflammatory lesions of the brain and spinal cord in which demyelination is a major feature (McFarlin and McFarland 1982). Studies of acute MS lesions show that myelin damage is associated with infiltration of mononuclear cells of hematogenous origin (lymphocytes, monocytes, and macrophages; Prineas and Wright 1978; Traugott *et al*. 1981; Traugott *et al*. 1983; Prineas 1985), as well as activated glial cells (astrocytes and microglia). The mechanism for the migration of peripheral blood leukocytes into the MS lesions, however, is largely unknown. A growing number of reports show that chemokines may play an important role in this process (Baggiolini 1998; Luster 1998). RANTES (regulated upon activation, normal T-cell expressed and secreted) is a β-family proinflammatory chemokine, secreted from monocytes, lymphocytes, glial cells, endothelial cells, and other cells (Schall *et al*. 1988; Schall *et al*. 1990; Schall 1991). Recently, astrocytes have been shown to produce RANTES chemokine in response to stimulation with tumor necrosis factor-α (TNF-α) (Barnes *et al*. 1996; Janabi *et al*. 1998). This is important because TNF-α is produced by activated monocytes and macrophages in MS lesions

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Abbreviations used: Cop-1, glatiramer acetate (copolymer-1); EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IκB, cytoplasmic inhibitor of NF-κB; MBP, myelin basic protein; MHC, major histocompatibility complex; MS, multiple sclerosis; NF-κB, nuclear factor-kappa B; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF-α, tumor necrosis factor-α.

(Selmaj *et al*. 1991), and RANTES is a potent chemoattractant for both monocytes/macrophages and T lymphocytes (Schall *et al*. 1990; Schall 1991) that are implicated in the pathogenesis of MS lesions (Prineas and Wright 1978; Traugott *et al*. 1981; Traugott *et al*. 1983; Prineas 1985). However, the mechanism underlying regulation of RANTES production in glial cells remains poorly understood.

Nuclear factor-kappa B (NF-κB) is a nuclear protein of the Rel gene family involved in regulation of numerous genes encoding proteins, including RANTES (Grimm and Baeuerle 1993; Siebenlist *et al*. 1994; Moriuchi *et al*. 1997). NF-κB binding sites are frequently found in promoters or enhancers of genes that are inducible by a wide range of extracellular signals, most notably by TNF-α (Miyamoto *et al*. 1994; Krakauer *et al*. 1999). The DNA-binding subunits of NF-κB comprise six members in mammals: p50, p65 (Rel A), c-Rel, v-Rel, p52, and Rel B (Miyamoto & Verma 1995; Verma *et al*. 1995). Existing in the cytoplasm as an inactive form, NF-κB is stabilized by an inhibitory subunit IκB (Read *et al*. 1994). A variety of signals induce degradation of IκB, allowing free NF-κB dimers to migrate to the nucleus. The free NF-κB dimers then bind to κB recognition elements in a variety of genes, including elements in RANTES promoter (Nelson *et al*. 1993; Moriuchi *et al*. 1997), and activate gene expression (Siebenlist *et al*. 1994; Moriuchi *et al*. 1997). Although the role of NF-κB in the transactivation of RANTES in human glial cells is not completely elucidated, study of the RANTES expression in human T lymphocytic cells and promonocytic cells has indicated that NF-κB plays a pivotal role in cytokine-stimulated chemokine gene transcription (Moriuchi *et al*. 1997).

Glatiramer acetate (previously known as copolymer-1) is a synthetic basic copolymer of four amino acids (L-alanine, L-lysine, L-glutamic acid, and L-tyrosine) that was recently approved for use in the treatment of MS patients (Johnson *et al*. 1995; Johnson *et al*. 1998). Although its mechanism of action is not fully understood at this time, previous studies indicated that glatiramer acetate specifically inhibits the T-cell response to myelin basic protein (MBP), *in vitro,* by competing with it at the major histocompatibility complex (MHC) class II binding site and at T-cell antigen receptor (Teitelbaum *et al*. 1992; Fridkis-Hareli *et al*. 1994; Teitelbaum *et al*. 1996; Aharoni *et al*. 1998; Aharoni *et al*. 1999; Fridkis-Hareli *et al*. 1999a,b). Glatiramer acetate can also induce antigen-specific suppressor T-cells, *in vivo,* that prevent development of experimental autoimmune encephalomyelitis (EAE) and suppress MBP specific T-cell activation (Aharoni *et al*. 1993; Aharoni *et al*. 1997; Aharoni *et al*. 1998; Weiner 1999). The effect and mechanism of glatiramer acetate in RANTES expression in glial cells, however, have not been explored. Therefore, the present study was undertaken to investigate the regulation of RANTES production in human astrocytes by TNF-α and glatiramer acetate. We have shown in this paper that TNF-α and glatiramer acetate had opposite effects on RANTES chemokine secretion in human U-251 MG astrocytic cells. Our studies further suggest that these agents appear to exert their effects on the regulation of glial RANTES chemokine expression through an NF-κB-dependent mechanism.

Experimental procedures

Cell lines and cell culture conditions

The human astrocytoma cell lines U-251 MG and U-105 MG were kind gifts from Dr Darryl Bigner (Duke University, Durham, NC, USA) and Dr Suhayl S. Dhib-Jalbut (University of Maryland School of Medicine, Baltimore, MD, USA). The human astrocytoma cell line U-373 MG was obtained from Dr Moon L. Shin (University of Maryland School of Medicine, Baltimore, MD, USA). The characteristics of these cell lines and culture conditions have been described previously (Bigner *et al*. 1981; Noe *et al*. 1999).

Enzyme-linked immunosorbent assay (ELISA)

Human RANTES levels in the cell culture supernatants were measured by two-antibody ELISA using biotin–strepavidin–peroxidase detection. Briefly, polystyrene plates (Maxisorb; Nunc, Naperville, IL, USA) were coated with capture antibody in phosphate-buffered saline (PBS) overnight at 25°C. The plates were washed four times with 50 mM Tris, 0.2% Tween-20 (pH 7.0–7.5) and then blocked for 90 min at 25°C with assay buffer (PBS containing 4% BSA (Sigma, St Louis, MO, USA) and 0.01% Thimerosal, pH 7.2–7.4). The plates were washed four times and 50 μL assay buffer was added to each along with 50 μL of sample or standard prepared in assay buffer and incubated at 37°C for 2 h. The plates were washed four times and 100 μL of biotinylated detecting antibody in assay buffer were added and incubated for 1 h at 25°C. After washing the plate four times, strepavidin–peroxidase polymer in casein buffer (RDI) was added and incubated at 25°C for 30 min. The plate was washed four times and 100 μL of commercially prepared substrate (TMB, Dako, Carpinteria, CA, USA) were added and incubated at 25°C for approximately 10–30 min. The reaction was stopped with 100 μL 2M HCl and read at 450 nm on a microplate reader (Molecular Dynamics, Sunnyvale, CA, USA). A curve was fit to the standards using a computer program (SoftPro; Molecular Dynamics) and the chemokine concentration in each sample was calculated from the standard curve equation. Sensitivity of the ELISA for RANTES is 15.625 pg/mL.

Cell viability and proliferation assay

Cell viability and drug cytotoxicity was determined using the crystal violet staining assay, as previously described (Saotome *et al*. 1989; Yunmbam 1998; Li *et al*. 2001). Briefly, cells (1000 cells/well) were seeded in 96-well plates in a total volume of 100 μL/well in triplicate, and drug exposures were made the following day. Cells were preincubated with glatiramer acetate for 2 h at 10, 100, 500, 1000, 1500 or 2000 for U-251 MG, U-105 MG, or U-373 MG cells, and then with TNF-α for 0, 3, 6, 12, 24, or 48 h at concentrations ranging from 1 ng/mL to 100 ng/mL. Untreated cells and cells treated with TNF-α or glatiramer acetate, alone, were controls. At the end of each incubation, the spent medium was discarded, and

the cells were washed once with PBS and then stained with crystal violet (Fisher Scientific, Fair Lawn, NJ, USA) reagent for 15 min at room temperature. The dye was extracted from the intact cells with 0.1 M sodium citrate (Fisher Scientific) reagent (in 50% ethanol) and the optical density of the solution was measured spectrophotometrically at 540 nm wavelength in a Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, USA). Data were analyzed using the VV Deltasoft Program on a Macintosh computer, and percentage cell growth values were obtained.

Transcriptional analyses

Northern blots were performed as described previously (Chomczynski and Sacchi 1987; Li *et al*. 1998b; Li *et al*. 1999). Nuclear run-on transcriptional assays were performed as described (Raj and Pitha 1983; Li *et al*. 1998a; Zhang *et al*. 1998). The cDNA probes used were prepared as the following: A 0.37-kilobase (kb) cDNA probe for human RANTES was obtained from Dr Jeffery D. Hasday (University of Maryland School of Medicine, Baltimore, MD, USA). A 0.8-kb cDNA for human GAPDH was obtained from Dr Jeffery D. Hasday and Dr Mitchell Olman (University of California, San Diego, CA, USA). A GAPDH probe was also obtained commercially from Oncogene Research Products (Cambridge, MA, USA). cDNA inserts were excised using appropriate restriction enzymes, isolated by electrophoresis through 1% agarose gel (Gibco BRL, Gaithersburg, MD, USA) (Sambrook *et al*. 1989), and purified using the Geneclean II Kit (Bio 101, Inc., La Jolla, CA, USA). cDNA was labeled with ^{32}P using a commercial random primer kit (Gibco BRL) according to the manufacturer's instructions.

Measurement of mRNA stability

A standard technique for measuring the stability of labile transcripts was used (Belasco and Brawerman 1993; Li *et al*. 1998a,b). In brief, cells were incubated with medium containing TNF-α for 24 h. The medium was replaced with fresh medium containing α-amanitin (*5* μg/mL), or α-amanitin plus glatiramer acetate (Teva Pharmaceutical Industries, Ltd, Petah-Tiqva, Israel). Cells were harvested at the time of α-amanitin addition (0 h) or at varying time-points thereafter. Total cellular RNA was isolated and northern blot analysis was used to determine steady-state mRNA levels of RANTES and GAPDH.

Electrophoretic mobility shift assay (EMSA)

This assay was carried out as described earlier (Li *et al*. 1998a; Zhang *et al*. 1998; Li *et al*. 1999). The oligonucleotide sequence used in EMSA assay was based on sequence analysis of the 5′-flanking region of *RANTES* gene, as described previously (Nelson *et al*. 1993). Two duplex 20-bp oligonucleotides that encompassed a *RANTES* NF-κB site (5′-CCCCTTAGGGGATGCCCCTC-3′, between − 28 and − 47 from the transcriptional start site at + 1 in the *RANTES* promoter region) were synthesized by Lofstrand Labs Limited (Gaithersburg, MD, USA) and were purified by reverse phase cartridge chromatography. The double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP by phosphorylation with bacteriophage T4 polynucleotide kinase. Unincorporated precursors were removed using G-25 Sephadex columns (Boehringer Mannheim, Indianapolis, IN, USA). Oligonucleotides of 21–25-bp that contained the accepted consensus sequence for NF-κB and TFIID were obtained from Promega Corporation (Madison, WI, USA) and were used in the binding or competition studies described below.

Statistical analysis of data

The Student's *t*-test (Snedecor and Cochran 1967) was used to analyze the statistical significance of the differences in RANTES expression between control and treatment groups. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Induction of RANTES mRNA and protein expression by TNF-α

It has been shown recently that the proinflammatory cytokine TNF-α induces production of the RANTES chemokine in astrocytes (Barnes *et al*. 1996; Janabi *et al*. 1998). To establish a model system for studying the regulation of RANTES expression, *in vitro*, by cytokines in glia, we chose the human astroglioma cell line U-251 MG, as a model of human astrocytes, because more than 80% of the cells in this cell line are positive for glial fibrillary acidic protein (GFAP), a marker of astrocytes, and thus it is representative of native glial cells (Bigner *et al*. 1981). We found that TNF-α could stimulate RANTES mRNA and protein expression in this cell system. ELISA assay and northern blot analysis showed that no RANTES could be detected in the cell culture from the untreated cells. Treatment of U-251 MG cells with TNF-α produced an induction of RANTES, with peak levels occurring 48 h after addition of TNF-α (data not shown). Dose–response experiments showed that the effect of TNF-α was maximal at 50–100 ng/mL after 48-h incubation of U-251 MG cells with the cytokine (data not shown).

Glatiramer acetate blocks TNF-α-induced RANTES chemokine production

We next evaluated the effect of glatiramer acetate on RANTES production in our systems. U-251 MG cells were pretreated for 2 h with different concentrations (10–1500 μg/mL) of glatiramer acetate (Cop-1) and then treated with 50 ng/mL TNF-α for 48 h. Cell culture supernatants were collected and assayed for RANTES chemokine levels by ELISA. The results presented in Fig. 1 indicate that glatiramer acetate dose-dependently inhibited TNF-α-induced RANTES secretion in the cells, with a maximal effect at 1500 μg/mL. To determine the inhibitory kinetics of glatiramer acetate in RANTES release, both untreated and glatiramer acetate-pretreated cells were incubated with 50 ng/mL TNF-α for varying times, and then RANTES levels in the supernatants were determined by ELISA assay (Fig. 2). Glatiramer acetate dramatically abrogated TNF-α-mediated RANTES chemokine increase for all the time points examined, as compared with the controls for each time-point. We also tested the effect of glatiramer acetate on RANTES production by different concentrations of TNF-α.

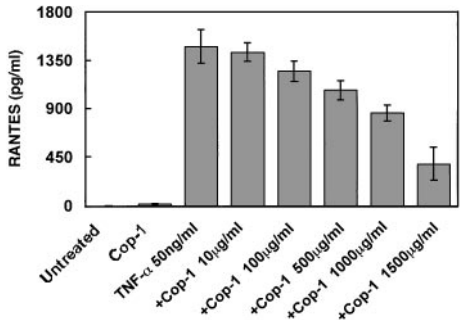

Fig. 1 Concentration-response relationship of glatiramer acetate on the inhibition of TNF-α-induced RANTES production in human astrocytic cells. U-251 MG cells were pretreated for 2 h with varying concentrations (10–1500 μg/mL) of glatiramer acetate (*Cop-1*), followed by a 48-h incubation with 50 ng/mL of TNF-α. After these treatments, cell culture supernatants were harvested and then assayed for RANTES chemokine levels, as described under 'Experimental procedures'. Cells treated with 50 ng/mL TNF-α or 1500 μg/mL glatiramer acetate alone, or left without treatment were controls. Data are mean ± SEM of triplicates and are representative of three separate determinations. *Cop-1*, glatiramer acetate.

For this, both untreated and glatiramer acetate-pretreated cells were incubated with various concentrations of TNF-α (1–100 ng/mL) for 48 h, and RANTES chemokine level in the supernatants was then measured by ELISA assay (Fig. 3). Although the induction of RANTES by higher concentrations of TNF-α was strong, glatiramer acetate (1500 μg/mL) almost inhibited it as efficiently as it did at lower concentrations.

To address the issue of whether this effect of glatiramer acetate is general or cell system-specific in astroglial cells, we also investigated the effect of glatiramer acetate on two additional human astrocytic cell lines, U-105 MG and U-373

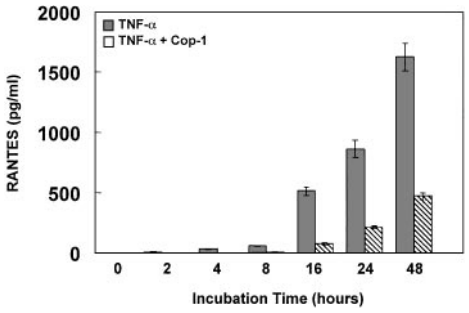

Fig. 2 Effect of glatiramer acetate on the kinetics of RANTES secretion by TNF-α in human U-251 MG astrocytic cells. Cells were incubated with 1500 μg/mL glatiramer acetate (*Cop-1*) for 2 h, treated with 50 ng/mL TNF-α for the times indicated, and then cell culture supernatants were sequentially harvested and measured for RANTES chemokine levels by ELISA assay. Cells treated with TNF-α alone were controls for each time-point. Data are mean ± SEM of triplicates and are representative of three separate experiments. *Cop-1*, glatiramer acetate.

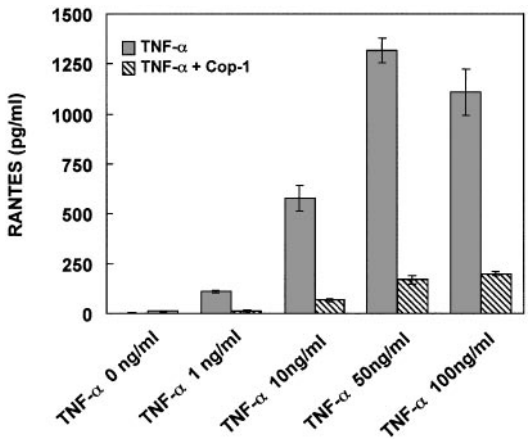

Fig. 3 Effect of glatiramer acetate on the production of RANTES induced by different concentrations of TNF-α in U-251 MG human astrocytic cells. Cells were incubated with 1500 μg/mL glatiramer acetate (*Cop-1*) for 2 h and then with the indicated concentrations of TNF-α, for 48 h. After these treatments, cell culture supernatants were harvested and quantitated for RANTES chemokine levels by ELISA assay. Data are mean ± SEM of triplicates and are representative of three independent determinations. *Cop-1*, glatiramer acetate.

MG. In each case, the glatiramer acetate effect was virtually identical to the effect seen in U-251 MG cells (data not shown). These results suggest that the down-regulation of RANTES expression appears to be a common effect of glatiramer acetate in different astrocytic cells.

To demonstrate that the inhibition of RANTES was specific and not merely due to a loss of cell viability, IL-8, IL-6 and GM-CSF were measured in the same samples. There was no reduction in the amounts of TNF-induced IL-8, IL-6 or GM-CSF after glatiramer acetate pretreatment (data not shown). These results have been reproduced in multiple independent experiments in our laboratory and show that glatiramer acetate has a specific inhibitory effect on the TNF-α-induced RANTES chemokine production in our systems.

Glatiramer acetate suppresses TNF-α-induced RANTES mRNA expression

Because glatiramer acetate blocked the TNF-α-dependent induction of RANTES protein, it was essential to determine whether this agent might have a similar effect on RANTES mRNA. Time course and dose–response experiments were conducted. Human astrocytic U-251 MG cells were pre-incubated for 2 h with different concentrations of glatiramer acetate and then incubated with TNF-α (50 ng/mL) for 48 h. Total cellular RNA was isolated and assessed for RANTES mRNA expression by northern blot analysis. As can be observed in Fig. 4, 1500 μg/mL of glatiramer acetate blocked most of the TNF-α-induced RANTES mRNA accumulation, and glatiramer acetate did not have any effect on the mRNA levels of the constitutively expressed

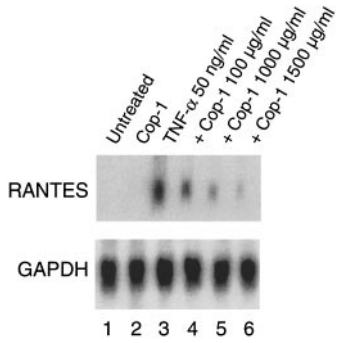

Fig. 4 Concentration-response of glatiramer acetate for the inhibition of TNF-α-dependent RANTES mRNA accumulation in human astroglial cells. U-251 MG cells were preincubated for 2 h with different concentrations (100–1500 μg/mL) of glatiramer acetate (*Cop-1*), followed by a 48-h incubation with 50 ng/mL TNF-α. After these treatments, total cellular RNA was extracted and then analyzed for RANTES mRNA levels by northern blot (lanes 4–6). Cells treated with 50 ng/mL TNF-α (lane 3) or 1500 μg/mL glatiramer acetate (lane 2) alone, or left without treatment (lane 1) were controls. Loading of RNA was monitored by rehybridization to a labeled *GAPDH* cDNA probe (lower). One representative experiment of three is shown. *Cop-1*, glatiramer acetate.

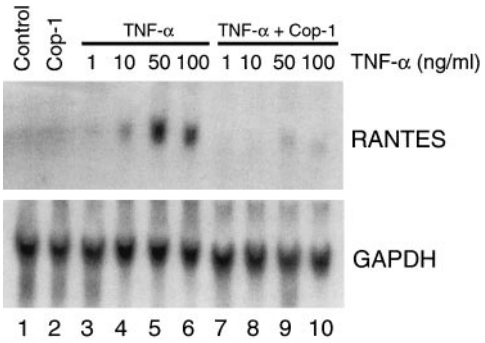

Fig. 6 Effect of glatiramer acetate on RANTES mRNA levels induced by different concentrations of TNF-α in U-251 MG human astrocytic cells. Cells were incubated with 1500 μg/mL glatiramer acetate (*Cop-1*) for 2 h and then treated with the indicated concentrations of TNF-α for 48 h (lanes 7–10). After these treatments, total cellular RNA was isolated and tested for RANTES mRNA abundance by northern blot (upper). Control cells were treated with either 1500 μg/mL glatiramer acetate (lane 2) or varying concentrations of TNF-α (lanes 3–6) alone, or left without treatment (lane 1). Equal loading of RNA was confirmed by probing the same membranes with a labeled *GAPDH* probe (lower). One representative experiment of three is shown. *Cop-1*, glatiramer acetate.

gene *GAPDH* in these samples. To determine the effect of glatiramer acetate on the kinetics of RANTES mRNA induction by TNF-α, both untreated and glatiramer acetate-pretreated cells were incubated in the presence of 50 ng/mL TNF-α for different times. The expression of RANTES steady-state mRNA up-regulated with increasing incubation time as shown by the increase in band intensity in non-glatiramer acetate-treated cells (Fig. 5). In contrast, the glatiramer acetate-pretreated cells showed a substantial decrease in RANTES mRNA levels, even up to 48 h of

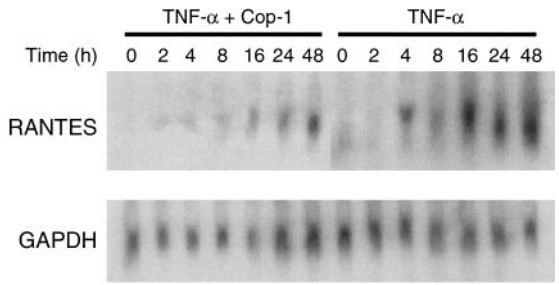

Fig. 5 Effect of glatiramer acetate on the kinetics of RANTES mRNA accumulation by TNF-α in human U-251 MG astrocytic cells. Cells were incubated with 1500 μg/mL glatiramer acetate (*Cop-1*) for 2 h, treated with 50 ng/mL TNF-α for the times indicated, and then total cellular RNA was sequentially isolated and measured for RANTES mRNA levels by northern blot analysis (upper). Cells treated with TNF-α only were controls for each time-point. Loading of RNA was monitored by hybridization to a labeled *GAPDH* cDNA probe (lower). One representative experiment of three is shown. *Cop-1*, glatiramer acetate.

TNF-α stimulation (Fig. 5). We then examined the effect of glatiramer acetate on RANTES mRNA levels induced by different concentrations of TNF-α. To determine this, both untreated and glatiramer acetate-pretreated cells were incubated with various concentrations of TNF-α (1–100 ng/mL) for 48 h, and RANTES mRNA was then analyzed by northern blotting. As shown in Fig. 6, glatiramer acetate at 1500 μg/mL completely abolished the increases in RANTES mRNA induced by all concentrations of TNF-α examined. Importantly, the time of incubation and the concentration of drug used in our studies had no effect on cell viability, as assessed by a previously described crystal violet staining assay (Saotome *et al*. 1989; Yunmbam 1998; Li *et al*. 2001) and by continued cell survival. Basically, neither TNF-α nor glatiramer acetate, alone, at the doses used in these experiments, was toxic to astroglial cells, and more than 90% to 95% of the cells were recovered when either one of the three cell lines was treated with both TNF-α and glatiramer acetate at their highest concentrations used in our studies. These results suggest that glatiramer acetate may exert a specific effect on the repression of *RANTES* gene expression induced by TNF-α in human glial cells.

Effect of glatiramer acetate on TNF-α-induced RANTES gene transcription

TNF-α treatment resulted in an up-regulation in mRNA coding for RANTES protein in U-251 MG human astrocytic cells, whereas glatiramer acetate caused a down-regulation in the steady-state levels of RANTES mRNA in a time- and concentration-dependent manner in these cells. These

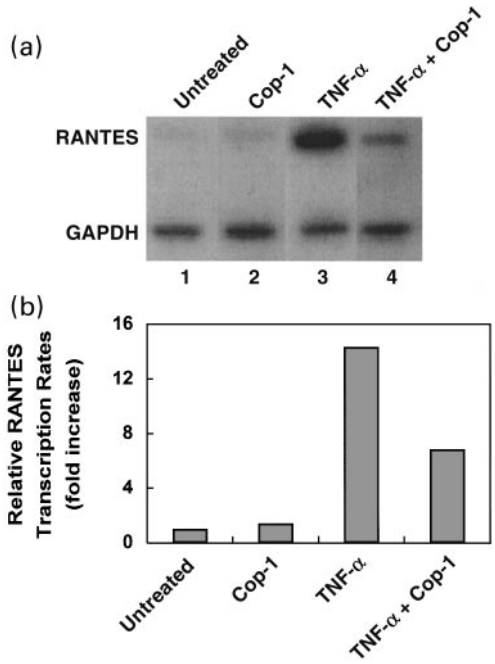

Fig. 7 Effect of glatiramer acetate on TNF-α-induced RANTES gene transcription in human astrocytic cells *in vitro* as assessed by nuclear run-on assay. U-251 MG cells were preincubated for 2 h with 1500 μg/mL of glatiramer acetate (*Cop-1*), followed by 8 h with 50 ng/mL of TNF-α (lane 4). After these treatments, nuclei were isolated and nuclear run-on reactions were performed, as described under 'Experimental procedures'. Run-on reaction mixtures were hybridized to immobilized *RANTES* cDNA probe on the membrane (a). Control cells were treated individually with 50 ng/mL TNF-α (lane 3) or 1500 μg/mL glatiramer acetate (lane 2) alone, or left without treatment (lane 1). *GAPDH* probe was used as a control for each sample. RANTES band densities were quantified by densitometry and expressed as a ratio to GAPDH, and these values are shown graphically (b). *Cop-1*, glatiramer acetate.

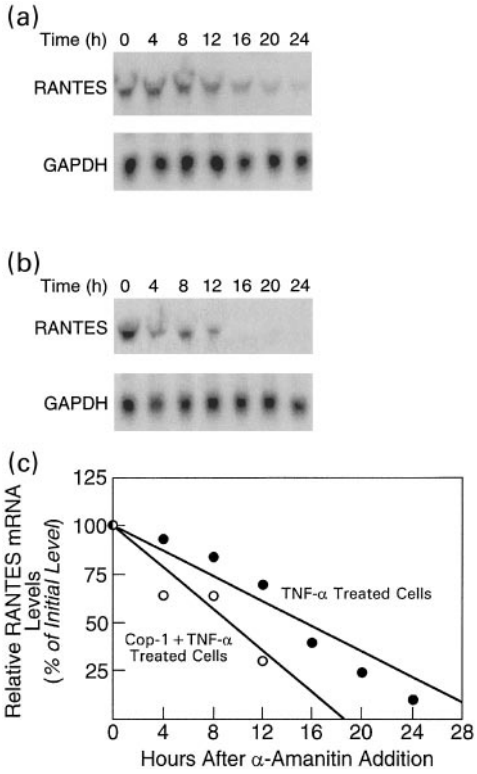

Fig. 8 Reduction by glatiramer acetate of the stability of TNF-α-induced RANTES mRNA in human astrocytic cells in culture. U-251 MG cells were stimulated with 50 ng/mL TNF-α for 24 h, and then replenished with fresh medium. Cells were treated either with 5 μg/mL α-amanitin (a) or with α-amanitin (5 μg/mL) plus glatiramer acetate (1500 μg/mL) (b). Total cellular RNA was isolated sequentially at the time of α-amanitin addition (0 h) or at different time-points thereafter, and RANTES mRNA was analyzed by northern blotting (upper). Loading of RNA was monitored by rehybridization to a labeled *GAPDH* cDNA probe (lower). RANTES band densities were quantified by densitometry and expressed as a ratio to GAPDH, and these values are shown graphically in (c).

observations prompted us to measure the transcription rate of the *RANTES* gene after treating cells with glatiramer acetate or TNF-α, alone, and after the combination treatment of the cytokine plus glatiramer acetate. The results of our experiments presented in Fig. 7 indicate that TNF-α (lane 3) alone induced *RANTES* transcription. By contrast, glatiramer acetate significantly reduced the transcription rate of the *RANTES* gene induced by TNF-α in U-251 MG cells (lane 4), as measured by *in vitro* transcript elongation (nuclear run-on) assays. However, glatiramer acetate by itself did not activate *RANTES* gene expression (lane 2).

Effect of glatiramer acetate on the turnover of RANTES mRNA

We also investigated whether the decrease in RANTES mRNA level could reflect an increased rate of mRNA degradation. This possibility is supported by the experiment depicted in Fig. 8, in which the TNF-α-induced RANTES mRNA transcripts were blocked by the transcriptional inhibitor α-amanitin. For this, U-251 MG cells were incubated with 50 ng/mL TNF-α for 24 h, after which the fresh medium was replaced. α-Amanitin (5 μg/mL) were then added to the cell culture in Fig. 8(a) to inhibit further RANTES mRNA accumulation, while both α-amanitin (5 μg/mL) and glatiramer acetate (1500 μg/mL) were added to the cells shown in Fig. 8(b). As can be seen, RANTES mRNA was more stable in the absence of glatiramer acetate shown in Fig. 8(a) (with a $t_{1/2}$ of 15 ± 1), as compared with that in the presence of the drug shown in Fig. 8(b) (with a $t_{1/2}$ of 9 ± 1), indicating that glatiramer acetate destabilizes the TNF-α-induced RANTES steady-state mRNA and causes an approximate 40% reduction in RANTES mRNA half-life in this cell system. These observations suggest that the suppressive effect of glatiramer acetate on RANTES

mRNA expression in human astroglial cells may be through decreased transcription of the *RANTES* gene and destabilization of the corresponding transcripts.

Glatiramer acetate blocks TNF-α-dependent NF-κB activation

The RANTES promoter contains several NF-κB binding sites (Nelson *et al*. 1993; Moriuchi *et al*. 1997), and recent evidence supports NF-κB-dependent regulation of *RANTES* (Nelson *et al*. 1993; Moriuchi *et al*. 1997). We therefore investigated the role of the NF-κB transcription factor in cytokine-mediated *RANTES* gene expression in glia. The binding of NF-κB to the *RANTES* NF-κB sequence following glial cell stimulation by TNF-α was examined. U-251 MG cells were incubated with 50 ng/mL TNF-α for about 2 h, and nuclear proteins were then extracted before performing electrophoretic mobility shift assays (EMSAs). Extracts from TNF-α-treated cells showed clear NF-κB binding to the 5′-flanking region of the *RANTES* gene (Fig. 9, lane 3), as compared with the extracts from the control, non-cytokine treated cells (Fig. 9, lane 1). Addition of an excess of cold probe specific for the *RANTES* NF-κB site blocked binding of the labeled probe (data not shown). In contrast, excess cold oligonucleotide specific for the TFIID binding site had no effect (data not shown), thus verifying the specificity of the binding reaction.

Having demonstrated that glatiramer acetate inhibits TNF-α-mediated RANTES mRNA and protein expression in U-251 MG cells, and that NF-κB is involved in the up-regulation of RANTES expression by TNF-α in these cells, we next examined the effect of glatiramer acetate on the activation of the nuclear transcription factor NF-κB by TNF-α. U-251 MG cells were preincubated for 2 h with 1500 μg/mL glatiramer acetate and then with 50 ng/mL TNF-α for an additional 2 h. Nuclear extracts were prepared and assayed for NF-κB activation by EMSA. The results presented in Fig. 9 indicate that glatiramer acetate decreased most of the TNF-α-induced NF-κB activity (lane 4) and that glatiramer acetate by itself did not activate NF-κB (lane 2). These results with glatiramer acetate are comparable with those of the known NF-κB inhibitors such as PDTC (50 μM) or isohelenin (50 μM) in this regard (data not shown). Importantly, in these experiments essentially all of the cells remained viable, as evaluated by trypan blue dye exclusion, and morphological signs of apoptosis (blebbing and cell condensation) were absent. These observations suggest that glatiramer acetate may inhibit TNF-α-induced *RANTES* gene expression in human astroglial cells by preventing activation of NF-κB.

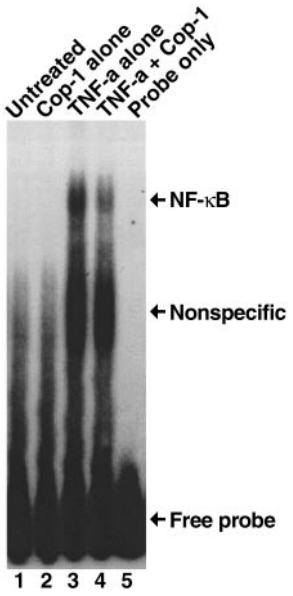

Fig. 9 Inhibitory effect of glatiramer acetate on TNF-α-induced NF-κB DNA-binding activity in human astroglial cells. U-251 MG cells were incubated for 2 h in the absence or presence of 1500 μg/mL glatiramer acetate (*Cop-1*), followed by exposure to TNF-α at 50 ng/mL for an additional 2 h. Prepared nuclear extracts were incubated with ^{32}P-labeled NF-κB oligonucleotide probe and analyzed by gel mobility shift assay, as described under 'Experimental procedures'. The NF-κB-specific band was inhibited by glatiramer acetate (lane 4). *Cop-1*, glatiramer acetate.

Discussion

Although glatiramer acetate was recently approved by the Food and Drug Administration (FDA) for the treatment of MS, the effect and the mechanism of the drug in the regulation of cytokine and chemokine expression are not well understood. In the current study, we demonstrate for the first time that glatiramer acetate blocks TNF-α-induced expression of RANTES mRNA and RANTES protein in U-251 MG human astrocytic cells. Our results also show that this inhibition of RANTES expression by glatiramer acetate is regulated at both the transcriptional and post-transcriptional levels. *In vitro* run-on assays in nuclei from control and glatiramer acetate-treated U-251 MG cells reveal that the decrease of RANTES mRNA occurred in the large part because of a reduction in the level of *de novo* transcription, suggesting that the most prominent action of glatiramer acetate on glial cell RANTES expression was by repression of RANTES gene transcription.

The NF-κB/Rel family is an important group of transcription factors responsible for the activation of a wide variety of genes in different cell types and tissues, which include those coding for inflammatory mediators and immunoregulatory molecules (Siebenlist *et al*. 1994; Barnes and Karin 1997). The proinflammatory cytokine TNF-α is the most potent NF-κB activator (Siebenlist *et al*. 1994; Barnes and Karin 1997; Krakauer *et al*. 1999), and this cytokine has been shown recently to induce RANTES

chemokine release from different cell types (Jose *et al*. 1990; Rathanaswami *et al*. 1993; Devergne *et al*. 1994; Pattison *et al*. 1994; Berkman *et al*. 1995; Marfaing-Koka *et al*. 1995; Barnes *et al*. 1996; Janabi *et al*. 1998). The results of our experiments in the present study indicate that NF-κB may be the transcriptional activator responsible for the TNF-α-induced RANTES expression in human U-251 MG astrocytic cells. Furthermore, we show in this work that the NF-κB transcription activating factor appears to be involved in the suppression of RANTES gene expression by glatiramer acetate. TNF-α-mediated NF-κB DNA-binding activity is substantially reduced by glatiramer acetate within 2 h. This glatiramer acetate-related NF-κB binding activity abrogation is followed by a reduction in the RANTES transcription rate, and decreases in the RANTES steady-state mRNA levels and RANTES chemokine protein levels. These results with glatiramer acetate are comparable with those of the known NF-κB inhibitors such as PDTC (Schreck *et al*. 1992; Pan *et al*. 1995), isohelenin (Hehner *et al*. 1998), or gliotoxin (Pahl *et al*. 1996) in these regards. These data collectively suggest that glatiramer acetate may block TNF-α-mediated RANTES expression in human astrocytic cells by decreasing the activity of NF-κB, and that the therapeutic effect of glatiramer acetate in MS patients may be due in part to its inhibitory effect on NF-κB activation and on chemokine release *in vivo*.

Although the physiological relevance of these findings could be questioned because the maximum effects of glatiramer acetate in our system required concentrations of 1500 μg/mL that are higher than those expected in the CNS, we believe that these effects may be important for several reasons. First, although the maximum effect was seen at 1500 μg/mL, inhibition was observed at lower concentrations (Figs 1 and 4). Second, there are at least two potential ways that systemically administered glatiramer acetate could access astrocytes in MS lesions: (i) glatiramer acetate could enter active MS lesions at points where the blood–brain barrier (BBB) has been opened by inflammatory mediators; and (ii) glatiramer acetate could be taken up by astrocytic foot processes that form part of the BBB. Lastly, because glatiramer acetate is administered peripherally at doses of 20 mg subcutaneously, local concentrations of the drug could be higher than 1500 μg/mL and might exert similar effects on lymphocytes or monocytes while in the periphery, thus altering the phenotype of the leukocytes that ultimately enter MS lesions (Aharoni *et al*. 2000).

However, the precise mechanism of glatiramer acetate in the modulation of NF-κB activity in glial cells is completely unknown, although the molecular basis of signal transduction pathways leading to the activation of NF-κB by TNF-α has been investigated in detail (Krakauer *et al*. 1999). It is reasonable to postulate that blockade of any step(s) in the TNF-α signal cascades, by glatiramer acetate, may ultimately interfere with the activation of NF-κB. Therefore, it is possible that glatiramer acetate may act on the cell surface receptors, the signal pathway starting sites for TNF-α, and shut down the entire downstream signal cascades from the very beginning. For example, glatiramer acetate may down-regulate TNF receptors by inhibiting the cytokine receptor expression, or cause internalization of the cytokine receptors, thus reducing the available cellular receptors for TNF-α. Otherwise, glatiramer acetate may induce the shedding of the cytokine receptors from the cell surface by activating enzyme(s) which cleave the cytokine receptor ectodomain and lead to shedding. Alternatively, glatiramer acetate may physically and/or chemically interfere with cytokine–receptor interactions, or glatiramer acetate may alter the conformation and configuration of the cytokine receptors, thereby reducing receptor binding affinities for their corresponding ligands. This physicochemical change in the three-dimensional structure of the cytokine receptors could contribute to the inability of drug-treated cells to transduce signals to NF-κB and possibly some other related nuclear transcriptional regulators.

Besides the possible interference with cytokine–receptor interaction, as a mechanism of blocking signal transduction, other possibilities may also exist. For instance, the effect of glatiramer acetate on RANTES could be due to inhibited activity of critical protein components of the signal cascades leading to NF-κB activation by TNF-α, or glatiramer acetate may function to desensitize cytokine receptor-dependent signal transduction. Alternatively, glatiramer acetate could modulate the activity of the NF-κB inhibitor IκB through the ubiquitin–proteasome degradation pathway, thereby regulating the activation of the transcription factor NF-κB, that, in turn, may influence, directly or indirectly, the transcription of the chemokine gene. Whether the mechanism of glatiramer acetate in RANTES expression is simply the result of an alteration in the cytokine–receptor interaction, modulation of the activity of the protein components in the signal transduction cascades leading to the activation of the RANTES gene, or a more specific effect of the biochemistry of this agent has yet to be determined.

While many questions remain unanswered, it is clear that a specific sequence of cellular and molecular events appears to take place after exposure to glatiramer acetate. Some of these events may greatly influence the biochemical cascades leading to the activation of the chemokine expression in human glial cells. First, TNF-α-induced RANTES chemokine production is greatly decreased, and this directly parallels a down-regulation in RANTES mRNA levels and transcription rates as well as NF-κB binding activity in the promoter region of the RANTES gene. Second, cytokine-mediated RANTES mRNA transcripts are destabilized, which leads to corresponding change in RANTES mRNA half-life in our system. Finally, the present study also documents an important effect of glatiramer acetate on suppression of the genes involved in leukocyte trafficking and

inflammation, in addition to its crucial immunomodulatory effects on antigen presentation (Teitelbaum *et al*. 1992; Fridkis-Hareli *et al*. 1994; Teitelbaum *et al*. 1996; Aharoni *et al*. 1998; Aharoni *et al*. 1999; Fridkis-Hareli *et al*. 1999a, b) and lymphocyte activation (Aharoni *et al*. 1993; Aharoni *et al*. 1997; Aharoni *et al*. 1998; Weiner 1999) in demyelinating diseases.

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