Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity *in vitro* and *in vivo*

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Summary

It is widely assumed that glatiramer acetate (GA), an approved agent for the immunomodulatory treatment of multiple sclerosis, acts primarily as an antigen for T lymphocytes. Recent studies, however, indicated that *in vitro*, GA directly inhibits dendritic cells, a rare but potent type of professional antigen-presenting cell (APC). To investigate whether these *in vitro* observations are relevant to the actions of GA *in vivo*, we studied the effects of GA on monocytes, the major type of circulating APC. In a first series of experiments, we investigated the effects of GA on monocyte reactivity *in vitro*. Monocytes were stimulated with ligands for Toll-like receptor (TLR)-2 (peptidoglycan and lipoteichoic acid), TLR-4 [lipopolysaccharide (LPS)] and TLR-5 (flagellin), as well as two proinflammatory cytokines (interferon-γ and granulocyte–monocyte colony-stimulating factor). Monocyte activation was measured by induction of the surface markers signalling lymphocytic activation molecule (SLAM), CD25 and CD69 (detected by cytofluorometry), and by production of monocyte-derived tumour necrosis factor (TNF)-α (detected by enzyme-linked immunospot assay). GA had a broad inhibitory effect on all measures of monocyte reactivity, regardless of which stimulator was used. It is unlikely that this reflects a simple toxic effect, because monocyte viability and CD14 expression were unaffected. In a second series of experiments, we investigated the properties of monocytes cultured *ex vivo* from eight GA-treated multiple sclerosis patients, eight untreated multiple sclerosis patients and eight healthy subjects. We found that LPS-induced SLAM expression and TNF-α production were significantly reduced in monocytes from GA-treated patients compared with controls. These results demonstrate for the first time that GA inhibits monocyte reactivity *in vitro* and *in vivo*, significantly extending the current concept of the mechanism of action of GA.

Introduction

Glatiramer acetate (GA, Copaxone), a random copolymer composed of alanine, glutamic acid, lysine and tyrosine, is an approved agent for the immunomodulatory treatment of multiple sclerosis. Clinical and magnetic imaging studies indicated that GA treatment reduces the activity of multiple sclerosis lesions (Johnson *et al*., 1995, 2000; Mancardi *et al*., 1998; Ge *et al*., 2000; Comi *et al*., 2001; Filippi *et al*., 2001; Wolinsky *et al*., 2001; Ziemssen *et al*., 2001). Over recent years, a number of studies have addressed the immunological basis of the clinical effects of GA in animal models and

human multiple sclerosis (reviewed in Aharoni *et al*., 2000; Neuhaus *et al*., 2001; Yong, 2002). These studies consistently showed that GA induces a 'TH1 to TH2 cytokine shift' in GA-reactive CD4^{+} T cells. In the immune system of untreated multiple sclerosis patients and healthy subjects, the majority of GA-reactive CD4^{+} T cells belong to the TH1 subset. These cells characteristically produce pro-inflammatory cytokines such as interleukin (IL)-2 and interferon-γ (IFN-γ) (Murphy and Reiner, 2003). During treatment with GA, the cytokine profile of the GA-reactive T cells shifts towards the TH2 type, which is characterized by production of anti-inflammatory 'TH2' cytokines, including IL-4 (Neuhaus *et al*., 2000; Farina *et al*., 2001; Dhib-Jalbut *et al*., 2002), IL-5 (Duda *et al*., 2000) and IL-13 (Wiesemann *et al*., 2003).

These observations laid the basis for the current concept of the mechanism of action of GA. It is thought that activated, GA-reactive TH2 T cells migrate across the blood barrier. In the CNS, they are re-activated locally by cross-reacting myelin auto-antigens (Neuhaus *et al*., 2001). After local re-stimulation, the GA-reactive TH2 cells release their anti-inflammatory cytokines, and also certain neurotrophic factors (Ziemssen *et al*., 2002). In this way, the recruited TH2 T cells can suppress neighbouring autoaggressive TH1 cells. This process is called 'bystander suppression' (Aharoni *et al*., 1998). In addition, the GA-reactive T cells can deliver brain-derived neurotrophic factor (BDNF) to neurons, which upregulate the corresponding full-length signalling tyrosine kinase receptor gp145 trkB in multiple sclerosis lesions (Stadelmann *et al*., 2002; Ziemssen *et al*., 2002).

The mechanism(s) of the therapeutically induced TH1 to TH2 cytokine shift of GA-reactive T cells is unknown. Theoretically, there are two (not necessarily exclusive) possibilities. First, GA might have a primary effect on T cells, for example by virtue of its properties as an 'altered peptide ligand', or by the special conditions of GA presentation in the skin. Secondly, GA might exert a primary effect on antigen-presenting cells (APCs), e.g. by altering their properties in such a way that they preferentially induce TH2 cells. Indeed, there is recent evidence that GA might affect the properties of APCs *in vitro* (Hussien *et al*., 2001; Vieira *et al*., 2003). In this regard, the findings reported by Vieira *et al*. (2003) are especially intriguing. Using *in vitro* cultures, these authors found that GA affects the T-cell-stimulating properties of dendritic cells (DCs). After *in vitro* treatment with GA, DCs have an impaired capacity to secrete TH1-polarizing factors, and therefore preferentially induce TH2 cells (Vieira *et al*., 2003). Whether these observations extend to other types of APC, and whether they are relevant *in vivo* presently is unknown.

The main aim of our present study was to search for evidence that GA affects the function of APCs *in vivo*. We focused our analysis on monocytes, because DCs constitute a very rare subset of APCs which can only be studied after prolonged expansion in culture, and which are therefore inaccessible for direct *ex vivo* analysis. In contrast, monocytes represent a major subset of professional APCs which can be easily obtained from peripheral blood and are readily accessible for *ex vivo* investigations.

In a first step of analysis, we performed a detailed series of experiments to identify the possible effects of GA on different patterns of monocyte activation *in vitro*. For monocyte activation, we used (i) four ligands for three distinct Toll-like receptors (TLRs); and (ii) the proinflammatory cytokines IFN-γ and granulocyte–monocyte colony-stimulating factor (GM-CSF). As markers of monocyte activation, we looked at (i) the expression of activation-related surface molecules including signalling lymphocytic activation molecule (SLAM) (CD150), CD25 and CD69; and (ii) production of tumour necrosis factor (TNF)-α. These experiments revealed that GA broadly affects monocyte activation by different ligands and pathways.

In the second step, we addressed the possible effects of GA on monocytes *in vivo*. To this end, we compared the properties of *ex vivo* monocytes from untreated and GA-treated subjects. '*Ex vivo*' means that the monocytes were exposed to GA only *in vivo*, but not *in vitro*. Based on the experiments from the first step, we chose lipopolysaccharide (LPS)-induced SLAM expression and TNF-α secretion as 'read-out' to test the monocyte stimulation thresholds *in vitro*. Our main observation is that monocytes cultured *ex vivo* from GA-treated patients were indeed significantly less susceptible to activation than monocytes from untreated patients and normal controls. These results demonstrate, we believe for the first time, that GA treatment *in vivo* leads to a systemic alteration of the properties of circulating monocytes, raising important new questions regarding the mechanism of action of GA.

Materials and methods
Subjects and cell samples
Blood was drawn from healthy individuals, GA-treated multiple sclerosis patients and untreated multiple sclerosis patients after their informed consent. This study has been approved by the local ethics commitee of the Ludwig Maximilians University of Munich. All patients had definite multiple sclerosis (McDonald *et al*., 2001). All GA-treated patients ($n = 8$) had a relapsing–remitting disease course. At the time of sampling, they had injected 20 mg of GA subcutaneously (s.c.) daily for at least 1 year, with a mean treatment duration of 34.4 ± 16.9 months [mean Expanded Disability Status Scale (EDSS) at time of sampling 1.75 ± 1.2; mean age 31.8 ± 8.4 years] (Table 1). The untreated group included four patients with relapsing–remitting multiple sclerosis, two with primary progressive multiple sclerosis and two with secondary progressive multiple sclerosis (five women and three men; mean EDSS 3.5 ± 1.9; mean age 47.1 ± 10.5 years). None of these patients was treated with immunosuppressive or immunomodulatory therapy during at least 3 months preceding the study. The group of healthy donors included four men and four women with a mean age of 35.1 ± 11.9 years.

Peripheral blood mononuclear cells (PBMCs) were isolated on a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). Viable cells were counted by trypan blue (Sigma-Aldrich) exclusion and resuspended in culture medium [RPMI

Table 1 *Characteristics of GA-treated MS patients and untreated MS patients.*

Subjects	Gender	Age (years)	Year of diagnosis	Disease course	EDSS*	GA treatment (months)*
GA-treated multiple sclerosis patients						
MS-GA-1	F	43	1981	RR	3.5	34
MS-GA-2	F	30	1991	RR	1.5	21
MS-GA-3	F	33	1995	RR	1.0	13
MS-GA-4	M	25	1988	RR	1.5	62
MS-GA-5	F	21	1999	RR	3.5	28
MS-GA-6	F	33	1999	RR	1.0	32
MS-GA-7	F	45	1993	RR	2	28
MS-GA-8	F	25	1993	RR	0	57
Untreated multiple sclerosis patients						
MS-NT-1	F	31	1996	RR	2.0	
MS-NT-2	F	36	2000	RR	2.5	
MS-NT-3	F	47	1997	RR	2.0	
MS-NT-4	M	39	1982	RR	1.0	
MS-NT-5	F	54	1975	SP	4.5	
MS-NT-6	M	56	1976	SP	6.5	
MS-NT-7	M	57	1996	PP	5.5	
MS-NT-8	F	57	2000	PP	4.0	

*At the time of sampling; MS-GA = GA-treated multiple sclerosis patient; MS-NT = untreated multiple sclerosis patient; RR = relapsing–remitting multiple sclerosis; SP = secondary progressive multiple sclerosis; PP = primary progressive multiple sclerosis.

1640 supplemented with 5% fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin; Gibco]. One batch of FCS was used throughout the study.

Reagents and antibodies

The following reagents were used: human IFN-γ (Roche, Mannheim, Germany), GM-CSF (R&D, Wiesbaden-Nordenstadt, Germany), flagellin from *Helicobacter pylori* (IBT, Reutlingen, Germany), peptidoglycan (PGN) from *Staphylococcus aureus* (Fluka, Sigma-Aldrich, Schnelldorf, Germany), lipoteichoic acid (LTA) from *Staphylococcus aureus*, and LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich). LPS-free reagents, water (BioWhittaker, Verviers, Belgium), phosphate-buffered saline (PBS; Gibco, Karlsruhe, Germany) and bovine serum albumin (BSA; Sigma) were used to prepare the aliquots. GA (Batch-No. 242992899) was from Teva Pharmaceutical Industries Ltd, Petah Tiqva, Israel.

The following antibodies were used for fluorescence activated cell sorting (FACS) analyses: phycoerythrin (PE)-labelled anti-SLAM, peridinin chlorophyll protein (PerCP)-labelled anti-human CD14, and fluorescein isothiocyanate (FITC)-labelled anti-human CD25 and CD69 (all from Becton Dickinson, Heidelberg, Germany). All the corresponding isotype controls were from Becton Dickinson.

FACS staining and analysis

The cells were labelled with the predetermined appropriate antibody dilution or with the corresponding isotype controls. FACS stainings were analysed on a FACScan using Cell-Quest software (Becton Dickinson). Monocytes were gated in forward/side scatter. The quadrants were set on the relative isotype controls. The *in vitro* experiments were repeated at least three times.

Enzyme-linked immunospot (Elispot) assay

96-well polyvinylidene difluoride plates (Millipore, Eschborn, Germany) were coated at 4°C overnight with the capture antibody (anti-TNF-α antibody; Mabtech, Nacka, Sweden). After the wells were washed and blocked with culture medium for 1 h at 37°C, the cells (1×10^3/well for the *in vitro* and 3×10^3/well for the *ex vivo* TNF-α assay) were seeded and stimulated for 18 h at 37°C and 5% CO_2. The experiments were performed in triplicate. After culture, the plates were washed and incubated first with the biotinylated detector antibody (Mabtech), then with streptavidin–alkaline phosphatase (Mabtech), and finally with BCIP/NBT (Sigma-Aldrich). The Elispot plates were analysed with an automated imaging system and appropriate computer software (KS ELISPOT automated image analysis system, Zeiss, Jena, Germany).

Statistical analysis

The GA dose dependence of the percentage of SLAM-positive monocytes and the frequency of TNF-α-producing cells were analysed with linear regression. The *t* test for independent samples was used to compare GA-treated multiple sclerosis patients, untreated multiple sclerosis patients and healthy controls. All *P* values given are two-sided and subject to a significance level of 5%.

Results
In vitro *culture with GA inhibits monocyte activation via different TLR ligands and inflammatory cytokines*

In previous experiments, we have characterized monocyte responses to (i) different bacterial TLR ligands (TLR-2 ligands PGN and LTA; TLR-4 ligand LPS; and TLR-5 ligand
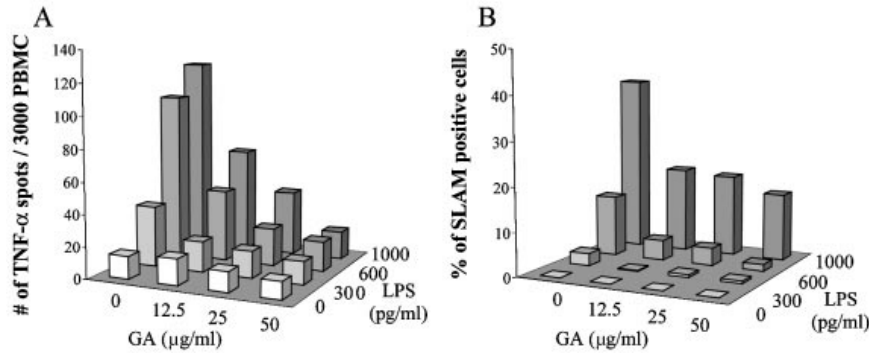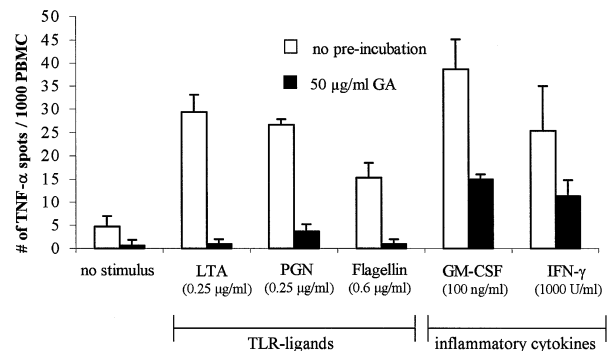
Fig. 1 GA-mediated inhibition of LPS-stimulated monocyte responses *in vitro* (dose–response analysis). In (**A**), monocyte reactivity was measured as LPS-induced TNF-α production by Elispot assay. In (**B**), monocyte reactivity was measured as LPS-induced SLAM expression by FACS.

flagellin), and (ii) inflammatory cytokines (IFN-γ and GM-CSF). Monocyte activation was measured by (i) induction of surface activation markers (CD25, CD69 and SLAM); and (ii) cytokine [TNF-α production (Farina *et al*., 2004)]. We found that SLAM was induced preferentially by stimulation with TLR ligands but not inflammatory cytokines, whereas CD25 and CD69 were induced by both TLR ligands and inflammatory cytokines. Further, TNF-α production was induced more strongly by TLR ligands than by IFN-γ and GM-CSF (Farina *et al*., 2004). These results laid the basis for our present study of the effects of GA on monocyte activation.

In a typical dose–response curve, a plateau of activation was reached with LPS concentrations >1000 pg/ml. At plateau, GA had no detectable inhibitory effect on monocyte reactivity. For optimal detection of the inhibitory effect of GA, we found that low to intermediate concentrations (150–1000 pg/ml) of LPS had to be used. PBMCs from healthy donors were pre-incubated for 1.5 h with four different concentrations of GA, and stimulated overnight with different concentrations of LPS. Figure 1 shows one of three representative experiments for TNF-α production (Fig. 1A) and SLAM induction (Fig. 1B). At each LPS concentration, the percentage of SLAM-positive monocytes and the frequency of TNF-α-producing cells was strongly reduced ($P < 0.05$ for TNF-α production by 300, 600 and 1000 pg/ml LPS; $P < 0.05$ for SLAM induction by 600 and 1000 pg/ml LPS). As shown in Fig. 1, the inhibitory effect of GA was dose dependent. We conclude that GA inhibits monocyte responses induced with the TLR-4 ligand LPS.

We also investigated the effects of GA on monocyte activation stimulated with the TLR-2 ligands PGN and LTA, and the TLR-5 ligand flagellin. As with LPS, GA had no detectable inhibitory effect at very high concentrations of these ligands. We measured dose–response curves for each ligand and determined the half-maximal stimulating concentrations. Again, the monocyte response was measured in terms of SLAM induction and TNF-α production by FACS and Elispot assay, respectively. After pre-incubation with

Fig. 2 GA-mediated inhibition of monocyte responses stimulated with different TLR ligands (LTA, PGN and flagellin) or inflammatory cytokines (GM-CSF and IFN-γ). Monocyte reactivity was measured as TNF-α production by Elispot assay.

50 µg/ml GA, SLAM induction was inhibited by 66.8 ± 37.4 (PGN stimulation), 80.4 ± 30.3 (flagellin) and $87 \pm 18.3\%$ (LTA) (average values $\pm$ SD; data not shown). Similarly, GA inhibited TNF-α production by monocytes stimulated with half-maximal doses of the different TLR ligands (Fig. 2; $P = 0.002$ for PGN, $P < 0.0001$ for LTA and flagellin).

GA inhibited not only TLR-stimulated, but also IFN-γ- and GM-CSF-stimulated TNF-α release (Fig. 2, $P = 0.001$ for IFN-γ and $P < 0.0001$ for GM-CSF), although in this case high concentrations of inflammatory cytokines were necessary for monocyte stimulation. In three different experiments, the inhibitory effect of GA on TNF-α production was consistently greater when TLR ligands were used as activators (87–97% inhibition), compared with stimulation with IFN-γ or GM-CSF (47–58% inhibition) (Fig. 2).

We also investigated the effects of GA on two additional markers of monocyte activation, CD25 and CD69. These molecules are newly induced on monocytes by stimulation with TLR ligands or inflammatory cytokines. Table 2 shows the results of one (out of three) representative FACS experiment. Pre-incubation with 50 µg/ml GA reduced the

fraction of activated monocytes expressing CD25 or CD69, regardless of which activator was used for stimulation (Table 2). Although the magnitudes of monocyte activation and of the inhibitory effect of GA varied between the different stimuli, GA consistently reduced the induction of CD25 and CD69 by all the different stimuli (CD69: 70% average inhibition for all activators; CD25: 70% average inhibition for LPS, LTA and PGN, 50% average inhibition for flagellin and IFN-γ, 30% inhibition for GM-CSF). In contrast, CD14, which is constitutively expressed on monocytes, was unaffected by GA (Fig. 3).

Treatment with GA in vivo *leads to a reduction of monocyte reactivity* ex vivo

The experiments described in the previous section indicated that GA inhibits monocyte activation by TLR ligands and inflammatory cytokines *in vitro*. To investigate whether *in vivo* treatment with GA also affects monocyte properties,

we assessed LPS-stimulated SLAM induction and TNF-α production by monocytes from GA-treated multiple sclerosis patients and controls. We compared PBMCs from eight GA-treated multiple sclerosis patients, eight untreated multiple

Table 2 *GA blocks induction of activation markers on monocytes*

	CD25*		CD69*	
	–GA	+GA**	–GA	+GA**
No stimulus	0.4%	1.2%	4.1%	2.4%
LPS 0.15 ng/ml	24.1%	0.8%	16.2%	0%
LTA 0.25 µg/ml	17.4%	7.8%	13.3%	3.1%
PGN 0.25 µg/ml	24.6%	6.7%	15.4%	2.4%
Flagellin 0.6 µg/ml	5.3%	3.3%	11.1%	1.4%
IFN-γ 1000 U/ml	5.9 %	3.5 %	13.6 %	5.9 %
GM-CSF 100 ng/ml	19.8 %	15.3 %	8.5 %	0.5 %

*The expression of the indicated activation markers on monocytes was determined by FACS and expressed as percentage of positive cells; **pre-incubation with 50 µg/ml GA.
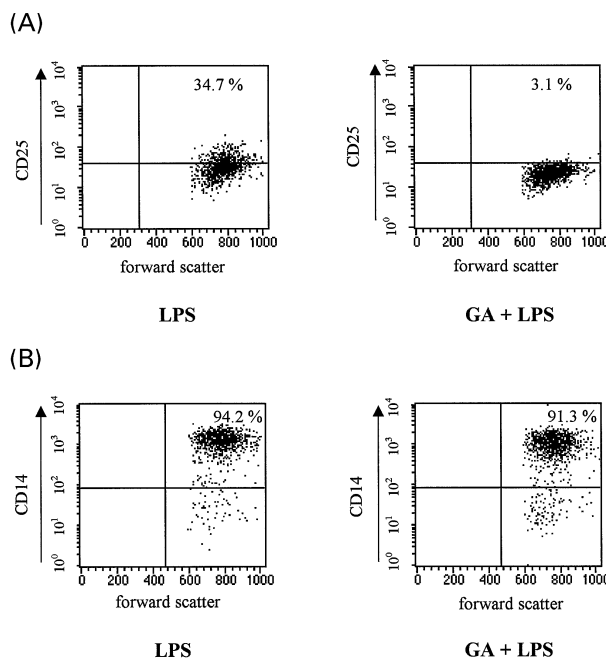
Fig. 3 GA-mediated effects on CD25 (**A**) and CD14 (**B**) expression by LPS-stimulated monocytes. LPS (0.15 ng/ml) and GA (50 µg/ml) were used. Upper panels (**A**) represent dot-blots of CD25 expression (ordinate) versus forward scatter (abscissa). GA reduced the proportion of CD25+ monocytes from 34.7% (without GA, upper left) to 3.1% (with GA, upper right). Lower panels (**B**) represent dot-blots of CD14 expression (ordinate) versus forward scatter (abscissa). There was no detectable change of CD14 expression in the presence of GA (lower right). The two markers were analysed in parallel in one experiment using PBMCs from one healthy donor.
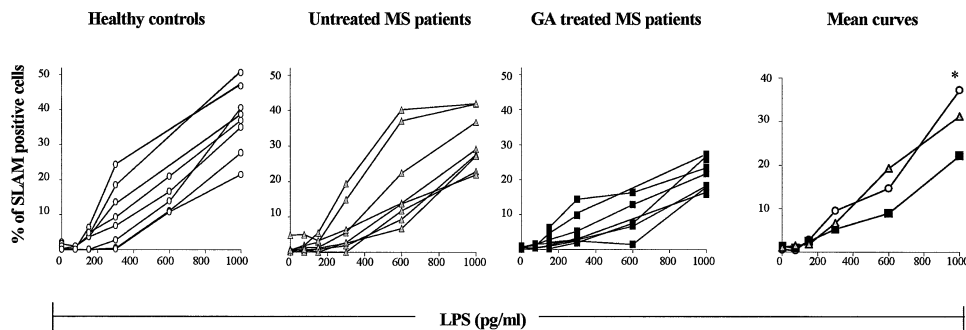
Fig. 4 LPS-stimulated SLAM induction on monocytes cultured *ex vivo* from eight healthy donors, eight untreated multiple sclerosis patients and eight GA-treated multiple sclerosis patients. SLAM induction was measured by FACS. The monocytes had been exposed to GA only during *in vivo* treatment, not *in vitro*. Monocytes from GA-treated patients showed significantly reduced responses. *P = 0.002 healthy controls versus GA-treated patients; P = 0.017 untreated patients versus GA-treated patients; P = 0.185 healthy controls versus untreated patients. The right panel shows means from each of the left panels. The P values at lower LPS concentrations were not significant.

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