Brain (2002), **125,** 2381–2391

Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy

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Summary

The clinical effects of glatiramer acetate (GA), an approved therapy for multiple sclerosis, are thought to be largely mediated by a T-helper 1 (TH1) to T-helper 2 (TH2) shift of GA-reactive T-lymphocytes. Current theories propose that activated GA-reactive TH2 cells penetrate the CNS, release anti-inflammatory cytokines such as interleukin (IL)-4, IL-5 and IL-10, and thus inhibit neighbouring inflammatory cells by a mechanism termed 'bystander suppression'. We demonstrate that both GA-specific TH2 and TH1 cells produce the neurotrophin brain-derived neurotrophic factor (BDNF). As the signal-transducing receptor for BDNF, the full-length 145 tyrosine kinase receptor (trk) B, is expressed in multiple sclerosis lesions, it is likely that the BDNF secreted by GA-reactive TH2 and TH1 has neurotrophic effects in the multiple sclerosis target tissue. This may be an additional mechanism of action of GA, and may be relevant for therapies with altered peptide ligands in general. To demonstrate that GA-reactive T cells produce BDNF, we selected four GA-specific, long-term T-cell lines (TCLs), which were characterized according to their cytokine profile by intracellular double-fluorescence flow cytometry. Three TCLs (isolated from a normal subject) had the phenotypes TH1, TH1/TH0, and TH0; the fourth, derived from a GA-treated patient, had the phenotype TH2. To demonstrate BDNF production, we used a combination of RT-PCR (reverse transcription-polymerase chain reaction) and two specially designed techniques for BDNF protein detection: one was based on ELISA (enzyme-linked immunosorbent assay) of supernatants from co-cultures of GA-specific TCLs plus GA-pulsed antigen-presenting cells, and the other on the direct intracellular staining of BDNF in individual T cells and flow cytometric analysis. The different assays and different TCLs yielded similar, consistent results. All four GA-specific T-cell lines, representing the major different TH phenotypes, could be stimulated to produce BDNF.

Keywords: multiple sclerosis; altered peptide ligand (APL); immunotherapy; neuroprotection; glatiramer acetate

Abbreviations: APL = altered peptide ligand; APC = antigen presenting cell; BDNF = brain-derived neurotrophic factor; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorter; FITC = fluorescein isothiocyanate; GA = glatiramer acetate; IL = interleukin; MBP = myelin basic protein; PBMC = peripheral blood mononuclear cell; PMA = phorbol 12-myristate 13-acetate; RT-PCR = reverse transcription-polymerase cell reaction; TCL = T-cell line; TCR = T-cell receptor; TH1 = T-helper 1; TH2 = T-helper 2; trk = tyrosine-receptor kinase

Introduction

Glatiramer acetate (GA, copolymer 1, Copaxone®) is a heterogeneous but standardized mixture of synthetic polypeptides consisting of L-glutamic acid, L-lysine, L-alanine and L-tyrosine (average molecular mass, 6400 Da). GA has been known for a long time to have suppressive and protective effects in experimental autoimmune encephalomyelitis, which can be induced in different species by various encephalitogenic antigens (Teitelbaum *et al*., 1972; Webb *et al*., 1975; Teitelbaum *et al*., 1996; Sela, 1999). More recently, GA has also been shown to have beneficial effects

on the clinical course and MRI-defined brain lesions of patients with multiple sclerosis. As a result, GA is now approved for use in the immunomodulatory therapy of relapsing-remitting multiple sclerosis (Teitelbaum *et al*., 1997; Comi *et al*., 2001; Sela *et al*., 2001; Ziemssen *et al*., 2001)

Among potential mechanisms, the initial T-helper 1 (TH1) type response of GA-treated patients was found to gradually shift to a T-helper 2 (TH2) type response (Miller *et al*., 1998; Duda *et al*., 2000; Gran *et al*., 2000; Neuhaus *et al*., 2000; Qin *et al*., 2000). TH1 cells characteristically produce a spectrum of 'proinflammatory' cytokines such as interferon (IFN)-γ, interleukin (IL)-2 and IL-12. In contrast, TH2 cells produce TH2-type 'anti-inflammatory' cytokines, i.e. IL-4, IL-5, IL-6 and IL-13 (Paul and Seder, 1994; Mosmann and Sad, 1996; Allen and Maizels, 1997). An intermediate type of T cell, called a TH0 cell, produces both TH1- and TH2-type cytokines. Current theories propose that the GA-specific TH2 cells, which are induced and constantly activated during treatment, migrate into the CNS and release their TH2-like cytokines locally (Aharoni *et al*., 2000). These cytokines are thought to have beneficial effects on the local inflammatory milieu and to inhibit the action of encephalitogenic T cells by 'bystander suppression' (Neuhaus *et al*., 2001).

Although plausible, this scenario may actually be an oversimplification as two unexpected and intriguing findings suggest. Human immune cells including T-lymphocytes, B-lymphocytes and monocytes can produce brain-derived neurotrophic factor (BDNF) (Besser and Wank, 1999; Kerschensteiner *et al*., 1999)—a potent neurotrophin that has profound effects on neuronal survival and repair (Thoenen, 1995; Barde, 1997). Moreover, the receptor for BDNF, gp145TrkB, is expressed in neurones and astrocytes in multiple sclerosis brain lesions (Stadelmann *et al*., 2002). These findings prompted us to ask the following questions: (i) can GA-reactive T lymphocytes produce BDNF, and if so, (ii) do TH1-type and TH2-type GA-specific T cells differ in their capacity to produce BDNF?

To answer these questions, we first had to overcome two major technical obstacles: (i) adapting our culture system to prevent added GA from affecting the BDNF enzyme-linked immunosorbent assay (ELISA) and (ii) optimizing the intracellular detection of BDNF in individual T-lymphocytes. This enabled us to demonstrate formally that GA-specific TH1, TH2 and TH0 cells all have the capacity to produce BDNF. We therefore postulate that the beneficial effects of not only TH2-type, but also TH1-type GA-reactive T cells, might, at least partly, be due to their release of BDNF in multiple sclerosis lesions.

Material and methods

Subjects

Blood samples were drawn from a GA-treated patient (B.K.) and a healthy donor (T.Z.) after their informed consent was given. The patient, a 47-year-old woman, had been diagnosed in 1993 to have relapsing-remitting multiple sclerosis. Her current Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) is 1. She has been essentially free of exacerbations since GA treatment was started in December 1998. Her human leukocyte antigen (HLA) class II phenotype is DR2/DR4. The HLA class II phenotype of the healthy volunteer (T.Z.), a 28-year-old postdoctoral fellow, is DR8/DR13. HLA typing was kindly performed by Drs E. Albert and S. Scholz, Department of Immunogenetics, University of Munich, Germany.

Selection and culture of GA-specific T-cell lines (TCLs)

GA-specific CD4+ TCLs were selected from peripheral blood mononuclear cells (PBMCs) using a split-well technique (Kitze *et al*., 1988; Pette *et al*., 1990; Neuhaus *et al*., 2000). GA (batch 242992899, average molecular mass 6400 Da) was obtained from Teva Pharmaceutical Industries, Petah Tiqva, Israel.

Four GA-specific CD4+ TCL representatives of the phenotypes TH1 (TZ-COP-1), TH1/0 (TZ-COP-3), TH0 (TZ-COP-5) and TH2 (BK-M6-COP-7) were used (Neuhaus *et al*., 2000). The protocols for fluorescence-activated cell sorter (FACS) phenotyping of the TCLs are described below. The TCLs TZ-COP-1, TZ-COP-3 and TZ-COP-5 were obtained from the healthy untreated subject T.Z. The TH2-type TCL, BK-M6-COP-7, was obtained from the GA-treated patient B.K. This TCL was originally described by Neuhaus *et al*. (2000).

Stimulation of GA-specific TCL with GA-pulsed antigen presenting cells (APCs)

In pilot experiments, the GA in the culture supernatants was occasionally observed to interfere with the BDNF ELISA, especially in the low range of BDNF concentrations (M. Kerschensteiner, W. Klinkert and T. Ziemssen, unpublished data). We therefore established a rigorous antigen-pulsing protocol to minimize these soluble GA concentrations. This protocol was used in all the experiments reported here. Thrombocyte-depleted APCs, X-irradiated with 40 Gy (Stabiloplan 2; Siemens, Erlangen, Germany), were incubated ('pulsed') with GA at a final concentration of 400 μg/ml for 4 h. The GA-pulsed APCs were washed twice before being used to stimulate the GA-specific TCLs. The same protocol was used for parallel proliferation assays. Proliferation was measured by [^{3}H]thymidine uptake as described previously (Neuhaus *et al*., 2000).

For proliferation and BDNF secretion (ELISA) assays, 10^5 washed GA-specific TCL cells were stimulated in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin (all from Gibco BRL, Gaithersburg, MD, USA) with 9×10^4 GA-pulsed

APCs. Supernatants were removed after 72 h and analysed for BDNF concentrations by ELISA (see below). For proliferation assays, parallel cultures were labelled after 48 h with [^{3}H]thymidine (0.2–0.5 μCi per well; Amersham Buchler, Braunschweig, Germany; 1 μCi = 37 kBq) and harvested 16–18 h later. [^{3}H]thymidine incorporation was measured with a direct β-counter (Matrix TM 96; Packard, Frankfurt, Germany). For reverse transcription-polymerase chain reaction (RT-PCR) analysis of BDNF transcription, RNA was extracted (see below) from cell pellets after 24 h of incubation with GA (50 μg/ml).

Phenotypic characterization of the GA-specific TCL by flow cytometry

TCLs were stained with monoclonal antibodies directed against CD3 (mouse IgG1, biotinylated; Immunotech, Marseille, France) plus streptavidin-phycoerythrin (PE) (PharMingen, San Diego, CA, USA), CD4 (mouse IgG1, PE-labelled; PharMingen) and CD8 (mouse IgG1, fluorescein isothiocyanate (FITC)-labelled; Becton Dickinson, San Jose, CA, USA) or the corresponding non-immune isotype controls [mouse IgG1, biotin- or FITC-labelled (PharMingen); PE-labelled (Becton Dickinson)]. The T-cell receptor (TCR) Vβ (variable region) repertoire was analysed using monoclonal antibodies that recognize the following subfamilies: Vβ2, Vβ3, Vβ3.1, Vβ5.3, Vβ7, Vβ7.1, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ23 (Immunotech) and Vβ5a, Vβ5b, Vβ6.7 (T-cell Diagnostics, Woburn, MA, USA). Monoclonal antibodies and isotype controls [mouse IgG1 (Becton Dickinson); mouse IgG2a and IgG2b (Cymbus, Chandlers Ford, Hampshire, UK)] were visualized with a FITC-labelled goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). The stained cells were analysed using a FACScan (Becton Dickinson).

Intracellular flow cytometry analysis of cytokine profile and BDNF production

Intracellular flow cytometry of the TCLs was performed 8–10 days after restimulation in the absence of viable APCs. GA-specific TCL were stimulated with phorbol 12-myristate 13-acetate (PMA, 2.0 μg/ml) and ionomycin (250 pg/ml) (both from Sigma, St Louis, MO, USA) for 3 h (cytokine profile) or 12 h (BDNF production); the last 2 h (cytokine profile) or 6 h (BDNF production) in the presence of monensin (2 μmol/l; Sigma). The T cells were then washed with phosphate-buffered saline (PBS) fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) and permeabilized with 0.1% saponin/PBS (Sigma). For the characterization of the cytokine profile, the T cells were then stained using appropriate concentrations of monoclonal antibody directed against IL-4 (mouse IgG1, PE-labelled; PharMingen) and IFN-γ (mouse IgG1, FITC-labelled; PharMingen) or the corresponding isotype controls [mouse IgG1, PE-labelled (Becton Dickinson); mouse IgG1, FITC-labelled (Immunotech)]. For the detection of intracellular BDNF production, activated and non-activated T cells were stained with a chicken IgY antibody against human BDNF or, as an isotype control, with a chicken control immunoglobulin IgY (both Promega, Madison, WI, USA). IgY, the 180 kDa chicken IgG homologue, can be produced in chickens against certain biological antigens that fail to elicit a humoral immune response in other mammals due to species relatedness. The antibody is highly specific for BDNF. A rabbit anti-chicken Ig antibody (FITC-labelled; Promega) was used as secondary antibody.

The untransfected murine ecotropic packaging line GP+E86 was used as a negative control for intracellular BDNF FACS staining. The packaging line transfected with the retroviral vector pLXSN into which BDNF cDNA was cloned (kindly provided by R. Kramer, Max-Planck-Institute of Neurobiology, Martinsried, Germany) served as a positive control (Flugel *et al*., 2001).

The stained cells were analysed using a FACScan (Becton Dickinson). On a dot plot showing forward and side scatter, lymphoid cells were gated for further analysis. Dead cells were excluded by gating.

Quantification of BDNF protein secretion in culture supernatants by ELISA

BDNF protein concentrations were determined in duplicate using a sensitive sandwich ELISA as described previously (Kerschensteiner *et al*., 1999). In brief, 96-well flat-bottomed plates were coated with the chicken anti-human BDNF IgY antibody (Promega) in 0.025M $NaHCO_3$ and 0.025M Na_2CO_3 (pH 8.2). Recombinant human BDNF (used as standard; Research Diagnostics, Flanders, PA, USA) was used in serial dilutions and cell supernatants in 1 : 2 dilutions. Bound BDNF was detected by incubating plates with a mouse anti-human BDNF antibody (Research Diagnostics) followed by peroxidase-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany). The plates were developed using a 3,3′,5,5′-tetramethyl-benzidine liquid substrate system (Sigma); the optical density was determined at 450 nm.

RT-PCR analysis of BDNF transcription

Total cellular RNA was extracted using the RNA extraction system of Qiagen (Hilden, Germany) with DNase digestion. The RNA (1 μg) was transcribed with oligo(dt) primers, Superscript® Reverse Transcriptase (both Gibco BRL) and dNTP (MBI Fermentas, St Leon-Rot, Germany). All PCR reactions were carried out in a total volume of 50 μl containing 2 U *Taq* polymerase (Qiagen), 200 μM of each dNTP, and 15 pmol of each primer for 35 PCR cycles with an annealing temperature of 60°C. The correct size of the bands

was determined by comparison with a DNA mass standard (SM0403, MBI Fermentas). RNA samples incubated in the absence of reverse transcriptase were used as negative controls to exclude genomic contamination. The primer sequences were as follows: BDNF forward 5′-AGCGTGAATGGGCCCAAGGCA-3′ (position 208–228); BDNF reverse 5′-TGTGACCGTCCCGCCCGACA-3′ (position 570–551); β-actin forward 5′-CCTCGCCTTTGCCGATCC-3′ (position –8 to 9); and β-actin reverse 5′-GGATCTTCATGAGGTAGTCAGTC-3′ (position 623–604).

Results

Phenotypic characterization of GA-specific TCLs

To analyse the production of BDNF by GA-specific T cells after antigen challenge *in vitro*, four GA-specific long-term TCLs were used—one TCL obtained from the GA-treated multiple sclerosis patient (B.K.) and three TCLs from the healthy donor (T.Z.). The TCLs were selected with the split-well cloning technique (Kitze *et al*., 1988; Pette *et al*., 1990). FACS analysis showed that all GA-specific TCLs had a $CD3^+CD4^+CD8^-$ phenotype (data not shown). The TCL from the multiple sclerosis patient (BK-M6-COP-7) was previously characterized as part of another study (Neuhaus *et al*., 2000). The other three TCLs were analysed as to their TCR Vβ usage. A panel of 23 different anti-TCR-Vβ antibodies was used to demonstrate the oligoclonal nature of the TCLs. As characteristically seen with TCLs selected with the split-well protocol (Kitze *et al*., 1988; Pette *et al*., 1990), each line reacted predominantly with one monoclonal antibody from the panel of anti-TCR Vβ antibodies (Fig. 1). The three investigated TCLs used different Vβ elements. The fact that the TCL populations predominantly stained positive for only one of the 23 Vβ elements indicated that they were oligoclonal (Fig. 1).

Fig. 1 shows the cytokine profile of the TCLs for IL-4 and IFN-γ. Whereas the TCLs TZ-COP-1 and TZ-COP-3 displayed a TH1 or TH1/TH0 phenotype, TZ-COP-5 cells produced both IL-4 and IFN-γ (TH0 phenotype). As described previously (Neuhaus *et al*., 2000), the TCL from the GA-treated multiple sclerosis patient (BK-M6-COP-7) had a stable TH2 cytokine profile (Fig. 1).

GA-induced proliferation and BDNF protein secretion by GA-specific TCLs

To demonstrate the ability of GA-specific T cells to secrete BDNF upon stimulation, we quantified the amount of BDNF in supernatants of GA-stimulated T cells and, in parallel, assessed the proliferative response to GA (Fig. 2). All GA-specific TCLs showed a specific proliferative response to GA with stimulation indices ranging between 6.5 and 21.0 over several re-stimulations. None of the tested GA-specific TCLs showed a proliferative response or cytokine production when challenged with myelin basic protein (MBP) as was previously reported for murine and human GA-specific T cells (data not shown) (Aharoni *et al*., 1998; Neuhaus *et al*., 2000). Irradiated APCs alone did not proliferate, regardless of whether they were pulsed with GA or not (Fig. 2). When T cells were added to unpulsed APCs, there was a small background proliferation that was at least six times lower than the proliferation in the presence of GA-pulsed APCs (Fig. 2, right columns).

The results of the BDNF ELISA indicate that the irradiated APCs (that is, PBMCs containing monocytes, T cells and B cells) produced small but clearly detectable amounts of BDNF (Fig. 2, left columns in left panels), although they did not proliferate (Fig. 2, left columns in right panels). There was a tendency for higher BDNF production by GA-pulsed APCs alone. All GA-specific TCLs showed an increased BDNF production after incubation with GA-pulsed APCs (Fig. 2, right columns in left panels).

RT-PCR analysis of BDNF transcription in GA-specific TCL

The transcription of BDNF mRNA in GA-specific T-cells was analysed by RT-PCR. In contrast to the BDNF protein secretion assay, the cells were harvested after a culture period of 24 h for RNA extraction and reverse transcription. The expression of BDNF was examined using RT-PCR in comparison with the housekeeping gene β-actin. There was no detectable contamination by genomic DNA, i.e. there were no bands in the negative BDNF and β-actin controls using RNA samples processed in the absence of reverse transcriptase (Fig. 3). Consistent with the BDNF protein data shown in Fig. 2, RT-PCR revealed weak bands in the absence of GA and stronger bands in the presence of GA (Fig. 3).

Detection of BDNF protein in GA-specific T-cells by intracellular flow cytometry

To confirm that the GA-specific T-cells are the source of the GA-induced BDNF release, we developed a new intracellular staining technique suitable for flow cytometry and FACS analysis of intracellular BDNF production. This method allows the analysis of BDNF production by individual unstimulated und stimulated T cells. Since the analysis was performed at least 8–10 days after the last restimulation with antigen and irradiated APCs, the cultures contained only T cells in the absence of viable APCs. Before intracellular staining and FACS analysis, the T cells were stimulated with ionomycin and PMA; this mode of stimulation does not require the presence of APCs (Dayton *et al*., 1994). A BDNF-transfected and non-transfected murine retroviral packaging line was used as the positive and negative controls (Fig. 4, top panels).

This new method was able to detect BDNF produced by the GA-specific TCLs, even without stimulation (left panels in

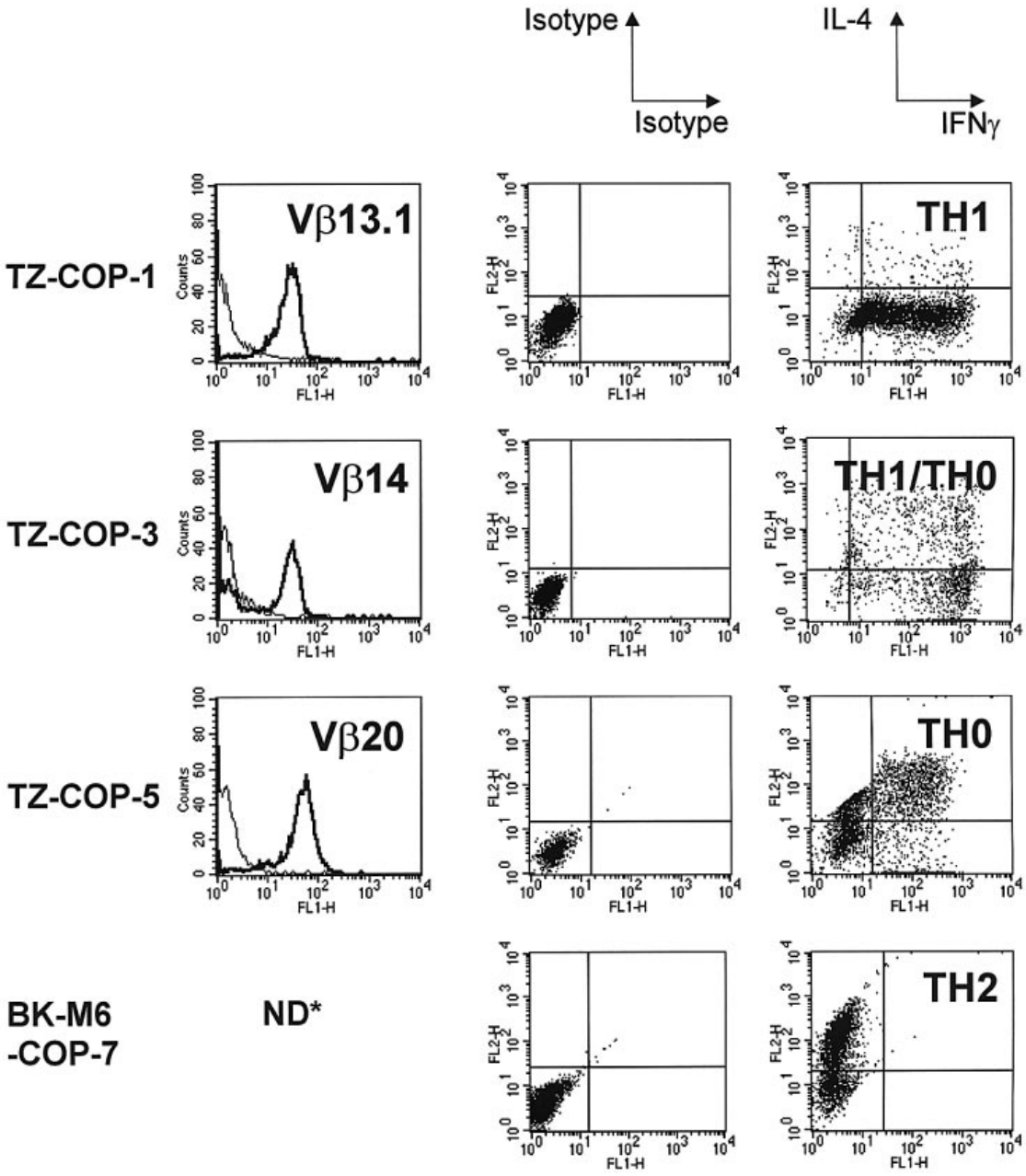

Fig. 1 Phenotypical characterization of the GA-specific TCLs (TZ-COP-1, TZ COP-3, TZ-COP-5, BK-M6-COP-7) as to their Vβ TCR usage (*left panels*: fine lines represent isotype controls and bold lines represent the indicated TCR Vβ antibodies) and their cytokine secretion profile analysed by intracellular double-fluorescence cytometry (*middle panels* with isotype controls; *right panels* with the cytokine profile). ND* = not done; the Vβ TCR usage of the published GA-specific T cell line BK-M6-COP-7 was not determined (Neuhaus *et al*., 2000).

Fig. 4; fine lines represent isotype controls). After stimulation with ionomycin and PMA, there was an increase of intracellular BDNF production (Fig. 4, right panels). The results of this intracellular assay of BDNF expression are consistent with the results of the ELISA and RT-PCR analysis.

Discussion

Technical aspects of the study

We clearly show that GA-specific, activated TH0, TH1 and TH2 cells produce the neurotrophic factor BDNF—not only at the transcriptional (mRNA) level by RT-PCR, but also at the protein level—using the newly developed assays for

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