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Therapeutic effect of transforming growth factor-β_2 on actively induced EAN but not adoptive transfer EAN

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SUMMARY

A possible effect of transforming growth factor type-β_2 (TGF-β_2) on autoimmune inflammation of the peripheral nervous system (PNS) was evaluated in experimental autoimmune neuritis (EAN) in Lewis rats, a disease model of the human Guillain–Barré syndrome. First, EAN was actively induced by immunization with a neuritogenic peptide corresponding to amino acids 53–78 of the bovine P2 protein. Intraperitoneal (i.p.) administration of 5 μg TGF-β_2 per day after onset of clinical disease shortened the duration and ameliorated the severity of EAN compared to sham-injected control animals. Inflammatory infiltration and demyelination was significantly reduced in sciatic nerves of TGF-β-treated animals, although expression of major histocompatibility complex (MHC) class II antigens was not down-regulated. Second, EAN was induced by adoptive transfer (AT) of activated P2-specific T-line cells (AT-EAN). Daily injections of 5 μg TGF-β_2 i.p., beginning on the day of first clinical signs, failed to modify the clinical course of AT-EAN, although the antigen-induced activation of the neuritogenic T-line cells used for induction of disease was found to be partially sensitive to the inhibitory effect of TGF-β *in vitro*. The experiments indicate that TGF-β_2 holds promise as a therapeutic agent to combat autoimmunity in the PNS. They also suggest that the therapeutic efficacy of TGF-β on rapidly developing disease such as AT-EAN is limited, as with other non-specific immunosuppressive drugs.

INTRODUCTION

Transforming growth factors of the β type (TGF-β) are multifunctional regulatory peptides for various cell types in an organism.[1] TGF-β_1 and TGF-β_2 are the best characterized homodimeric molecules of the TGF-β family. Their effects on the immune system are mainly suppressive.[2] Thus, TGF-β inhibits activation, growth and function of T and B lymphocytes.[3–10] Furthermore, TGF-β modulates the response of macrophages to various stimuli.[11–13] However, upon local injection, e.g. into joints, TGF-β has pro-inflammatory effects.[14]

The potential immunoregulatory actions of TGF-β *in vivo* have been evaluated in different disease models. At least in CD8-dependent T-cell-mediated experimental disease, such as lymphocytic choriomeningitis virus-induced central nervous system (CNS) disease, TGF-β inhibits the generation of antiviral cytotoxic T cells and delays the onset of disease.[15] This and other studies have established that TGF-β interferes with the initial antigen-specific immune response rather than with the effector phase.[16]

Experimental autoimmune neuritis (EAN), a model for the human Guillain–Barré syndrome[17] can be actively induced in Lewis rats by immunization with bovine P2 protein or a synthetic peptide containing its neuritogenic epitope.[18] Alternatively, EAN can be adoptively transferred (AT-EAN) by injection of activated P2-specific T lymphocytes.[19] Animals develop a monophasic disease with limp tail and paraparesis of the hind limbs due to multifocal inflammation and demyelination of peripheral nerves.

In this study TGF-β_2 was evaluated *in vivo* for a possible suppressive action on the inflammatory phase of both models of EAN.

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Abbreviations: APC, antigen-presenting cell; AT-EAN, adoptive transfer EAN; EAN, experimental autoimmune neuritis; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; P2 53–78, synthetic peptide encompassing the amino acids 53–78 of bovine P2 protein; PNS, peripheral nervous system; TGF-β, transforming growth factor type-β; TNF-α, tumour necrosis factor-α.

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MATERIALS AND METHODS

Animals

Female Lewis rats from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) were 7–8 weeks old and had a body weight of 140–170 g.

Reagents and antigens

Human recombinant TGF-β_2 was purified from supernatant of Chinese hamster ovary cells transfected with a TGF-β_2/glutathione-transferase (GTsF)-encoding expression plasmid.[5]

P2 protein was isolated from bovine intra- and extradural spinal roots as described by Kitamura *et al.*[20] A synthetic peptide (P2 53–78), representing the amino acids 53–78 of the bovine P2 protein was kindly provided by Dr Steven Brostoff (San Diego, CA).

Induction and treatment of actively induced EAN

Fourteen rats were immunized in the right hind footpad with 50 μl of a mixture of equal volumes of P2 53–78 in phosphate-buffered saline (PBS) and Freund's incomplete adjuvant oil (Gibco, Eggenstein, Germany), with addition of 0·5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI), resulting in a dose of 65 μg P2 53–78/rat.

TGF-β or sham treatment was started on the first or second day of clinical disease (days 9–14 after immunization). Rats were matched for clinical scores and were evenly distributed to the two groups. Animals in the treatment group received 5 μg TGF-β_2 intraperitoneally (i.p.) in a volume of 500 μl PBS with 0·1% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) every day. Sham-treated controls were injected with 500 μl PBS containing 0·1% BSA only.

Induction and treatment of AT-EAN

Culture conditions and the phenotype of the neuritogenic P2-specific T-cell line P2.48 used for *in vitro* and adoptive transfer experiments are described elsewhere.[21] For induction of disease, 3×10^5/ml washed line cells were restimulated with $1{\cdot}5 \times 10^7$/ml freshly isolated and irradiated (3000 rads) thymocytes as antigen-presenting cells (APC) and P2 protein (20 μg/ml). Seventy-two hours later activated T-line blasts were separated from cell debris by centrifugation on Ficoll (Nycomed AS, Oslo, Norway) gradients at 4°. Blasts from the interphase were washed twice and $4{\cdot}5 \times 10^6$ T-line cells/rat were injected intravenously (i.v.) in 1 ml Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany). Three days after cell injection all 10 animals developed clinical signs of EAN. On that day they were allocated to the sham- or TGF-β-treatment group and received injections as described above for 4 days.

Scoring of disease

Rats were weighed daily and inspected by two investigators for disease severity. Clinical scores were given according to the following scale: 0, normal; 1, reduced tone of the tail, hanging tail tip; 2, limp tail; 3, absent righting; 4, gait ataxia, abnormal position; 5, mild paraparesis of the hind limbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia of the hind limbs; 8, tetraparesis; 9, moribund; 10, death.

Histology

Nineteen and 20 days after immunization rats were anaesthetized with Narcoren^R (Iffa Merieux, Laupheim, Germany) and perfused through the left cardiac ventricle with Ringer solution (Fresenius, Bad Homburg, Germany) containing 20 000 U/l heparin, followed by 4% paraformaldehyde (Merck, Darmstadt, Germany) in a 0·1 M phosphate buffer, pH 7·4. Spinal cords with adjacent roots and right sciatic nerves were dissected and fixed for additional 14 hr. Material for the semi-quantitative assessment of histopathological changes was embedded in Araldit (Serva, Heidelberg, Germany) after postfixation in 2% osmium tetroxide for at least 4 hr. Semithin sections from the cauda equina and sciatic nerves were stained with toluidine blue. Areas surrounding intraneural vessels were examined by a blind-folded investigator. In sciatic nerves 30–48, and in spinal nerve roots 162–301, perivascular areas per animal were evaluated. The degree of pathological alterations was graded on the following scale: 0 was a normal perivascular area; 1, mild cellular infiltrate adjacent to the vessel; 2, cellular infiltration plus demyelination in immediate proximity to the vessel; 3, cellular infiltration and demyelination throughout the section.[22]

Material for immunohistological identification of T lymphocytes, major histocompatibility complex (MHC) class II molecules and monocytes/macrophages was embedded in paraffin. Deparaffinized 5-μm sections were treated with 0·03% H_2O_2 to quench endogenous peroxidase, and were preincubated with porcine serum to cover unspecific binding sites. Sections were indirectly stained by the avidin–biotin–peroxidase technique.[23]

Slides were incubated with the monoclonal antibodies W3/13, OX6 (both at a dilution of 1:50; Serotec, Crawley Down, UK) and ED1 (1:100; a kind gift of C.D. Dijkstra, Free University of Amsterdam, the Netherlands) overnight, followed by incubation with affinity-purified biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) that had been pre-absorbed with normal rat serum. The avidin–biotin–peroxidase complex reagent and diaminobenzidine were used as recommended by the manufacturer.

Proliferation assays

The sensitivity of the neuritogenic T-cell line P2.48 for TGF-β_2 was tested *in vitro* under serum-free conditions using Iscove's modified Dulbecco's medium supplemented with BSA (40 mg/ml), transferrin (30 μg/ml) and lecithin (12·5 μg/ml) (all from Gibco). For evaluation of TGF-β effects on antigen-induced T-line activation, $1{\cdot}5 \times 10^4$ resting P2.48 line cells were incubated for 3 days with $7{\cdot}5 \times 10^5$ irradiated syngeneic thymocytes, 20 μg P2/ml and graded concentrations of TGF-β_2 in a total volume of 120 μl/microtitre well. On the second day of restimulation 0·2 μCi [^{3}H]methyl-thymidine (Amersham, Braunschweig, Germany) per well was added and cells were harvested 16 hr later onto glass fibre filters by a Wallac 96-well harvester (Pharmacia, Freiburg, Germany). [^{3}H]Thymidine incorporation was measured by liquid scintillation counting (Wallac 1205 Betaplate; Pharmacia) and is expressed as mean c.p.m. ($\pm$ SD) of triplicate cultures.

The influence of TGF-β on interleukin-2 (IL-2)-dependent proliferation of the T-line cells was tested with 4×10^4 freshly P2-activated and Lymphoprep-isolated P2.48 T blasts/well in 200 μl supplemented Iscove's medium containing 7% supernatant of concanavalin A (Con A)-activated rat spleen cells. Four hours later cultures were labelled for 16 hr.

Statistical evaluation

Wilcoxon's rank sum test and Student's *t*-test were applied to compare clinical and histological scores, respectively, in the PBS/BSA-treated and TGF-β-treated groups. Student's *t*-test could be applied for comparison of histological scores[22] since

the high number of samples (perivascular areas) permitted assumption of a normal distribution according to the central limit theorem. In actively induced EAN one rat initially allocated to the control group did not show any signs of EAN on the following days. According to its clinical as well as to its histological score this animal was identified as an outler and removed from the study.

RESULTS

When P2 53–78-immunized rats developed symptoms of EAN, animals with similar clinical scores were assigned to the TGF-β- or sham-treated group (Fig. 1). Clinical signs worsened in the sham-treated group and five of six rats developed weakness of the hind limbs. In contrast to sham-treated rats, EAN did not deteriorate in any of the TGF-β-treated animals after the first TGF-β injection, none developed paresis of the hind limbs, and the mean clinical score of the seven animals decreased over time. Six days after initiation of TGF-β treatment all rats had recovered completely, whereas five of the six sham-injected animals still exhibited clinical signs of peripheral nerve disease.

Histological examination of the PNS of the rats 19 or 20 days after immunization demonstrated multifocal infiltration and demyelination. Pathology was more pronounced in sciatic nerves compared to spinal nerve roots (Fig. 2). Morphological changes were less severe in animals treated with TGF-β. To provide a representative assessment of the severity of histopathological alterations in control versus TGF-β-treated rats, the morphology of single perivascular areas in semi-thin sections was graded by a masked investigator. Therapy with TGF-β after the onset of clinical symptoms slightly and insignificantly diminished the number and extent of the histopathologically demonstrable lesions in spinal nerve roots (Fig. 2a). This effect was more pronounced and of statistical significance in sciatic nerves, which were more profoundly affected by the disease process than spinal nerve roots (Fig. 2b).

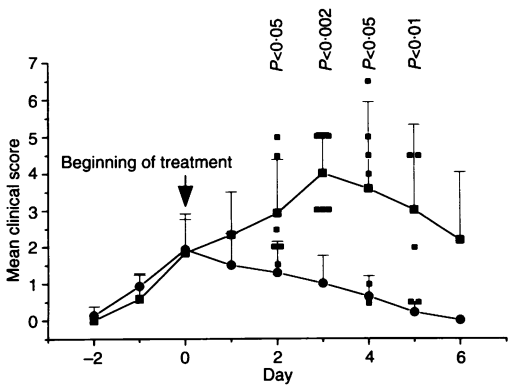

Figure 1. Treatment of P2 53–78-induced EAN with TGF-β_2. Lewis rats were immunized with 65 μg of the neuritogenic peptide P2 53–78 in complete Freund's adjuvant, as described in the Materials and Methods. Animals were inspected daily for the development of EAN. After the appearance of clinical signs animals with similar clinical scores were allocated to the sham- or TGF-β-treatment group, respectively, on day 0 of the normalized time scale. Five micrograms of TGF-β_2 in 0·5 ml PBS or PBS only was injected i.p. from days 0 to 5. Bars indicate SD. Smaller squares denote clinical scores of individual control rats. (■) Sham-treated rats (n = 6); TGF-β_2-treated rats (n = 7).

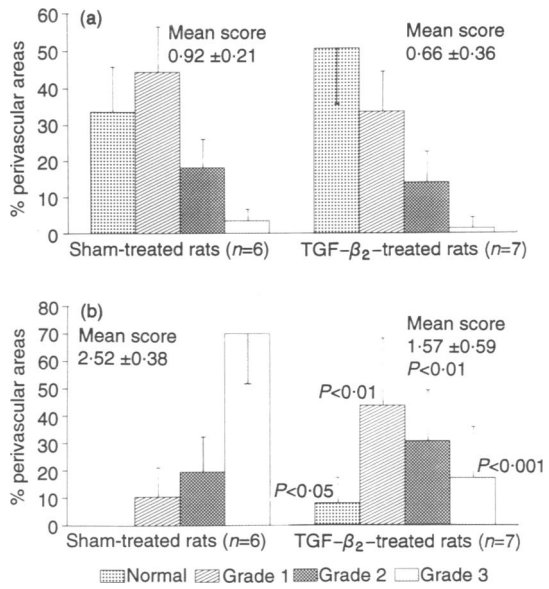

Figure 2. Semi-quantitative analysis of histopathological findings in P2 53–78-induced EAN. Semi-thin sections of sciatic nerves (a) and of spinal nerve roots (b) were inspected by a masked investigator. Severity of histological changes in perivascular areas was graded according to the scale described in the Materials and Methods. Per animal, 25–67 (mean 42) perivascular areas in sciatic nerves and 91–261 (mean 196) areas in spinal nerve roots were evaluated. Bars represent mean percentages (+ SD) of perivascular areas showing the indicated degree of pathology in that group. Framed mean scores give grand means of all scored areas in the respective group. TGF-β treatment caused a shift of the morphometric histogram towards lower degrees of inflammatory and demyelinative changes.

Immunohistochemical analysis of sciatic nerves of five animals from each group identified far less ED1-positive macrophages in the nerves of TGF-β-treated rats (Fig. 3A,B). In contrast to the numeric reduction of ED1-staining in TGF-β-treated animals, OX6 immunoreactivity, indicating MHC class II expression, showed a high variability and, in summary, neither an apparent reduction of focal nor parenchymal MHC class II expression was observed in sciatic nerves of TGF-β-treated rats (Fig. 3C,D).

As with actively induced EAN, TGF-β-treatment of AT-EAN induced by the P2-specific T-cell line P2.48 commenced on the day of first clinical signs. In all animals the first symptom was weakness of the tail, observed on day 3 after cell injection (day 0 in Fig. 4). In contrast to actively induced EAN, the clinical course of AT-EAN was not modified by injection of TGF-β_2 during the first 4 days of overt clinical disease.

To elucidate whether the lack of effect of TGF-β on AT-EAN was due to intrinsic resistance of the T-cell line P2.48 to TGF-β, P2.48 line cells were activated by the antigen P2 and APC *in vitro*, along with increasing concentrations of TGF-β. In the presence of $\geqslant$ 0·3 ng/ml TGF-β, maximal suppression of P2-induced T-line activation, as measured by [^{3}H]thymidine incorporation, ranged between 55% and 64% (Fig. 5), with comparable results in two additional experiments. Thus, antigen-induced activation of the neuritogenic T-cell line used

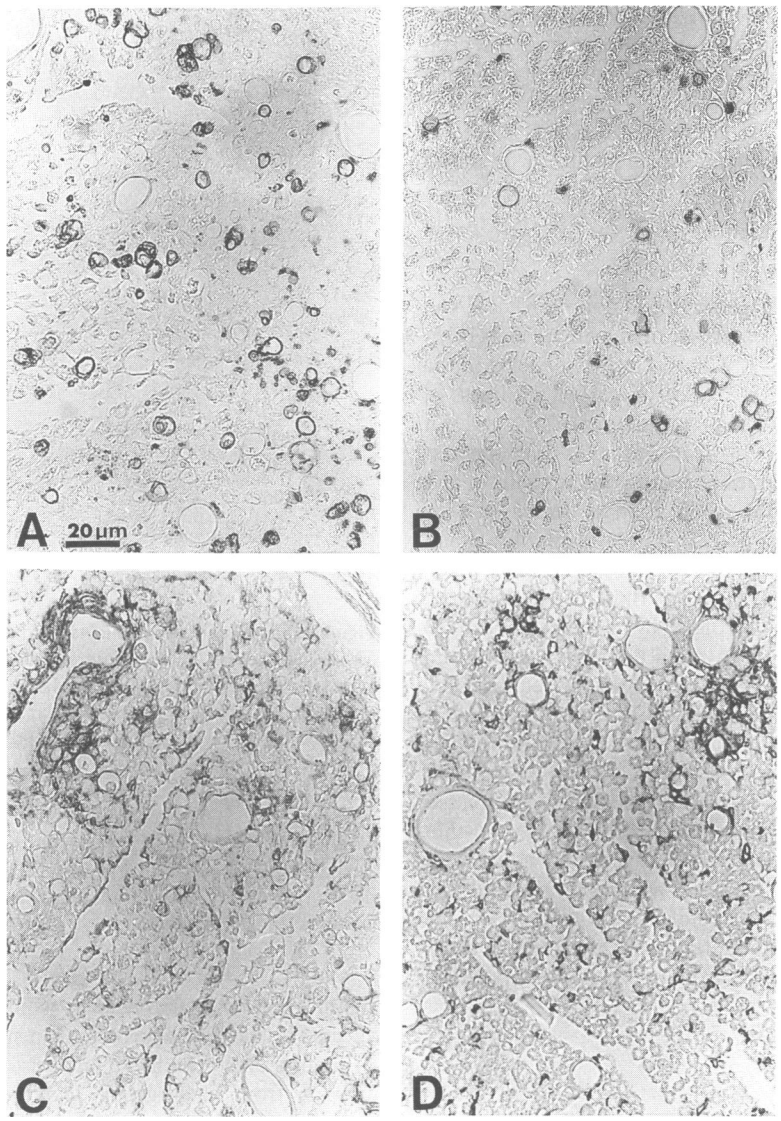

Figure 3. Immunohistochemical demonstration of macrophages and MHC class II molecules in sciatic nerves of P2 53–78-immunized rats. Representative sections of paraffin-embedded sciatic nerves of two P2 53–78-immunized animals that developed EAN on the same day and were PBS/BSA (A, C) or TGF-β (B, D) treated for 6 days after the appearance of clinical signs of EAN. Sections were stained with the monoclonal antibodies ED1 (for macrophages; A, B) or OX6 (for MHC II antigens; C, D) as described in the Materials and Methods. Note that there were fewer macrophages after TGF-β treatment (B versus A) but that MHC class II expression appeared unchanged (D versus C).

in the *in vivo* experiment appeared to be partly sensitive to the action of TGF-β.

Following earlier observations,[24] we tested the sensitivity of the IL-2-dependent proliferation of the line P2.48 using the supernatant of Con A-activated spleen cells as a source of IL-2. Compared to the inhibition of antigen-induced proliferation, IL-2-induced P2.48 proliferation was only minimally influenced by TGF-β_2, and maximal suppression did not exceed 20% (Fig. 5b) in three experiments.

DISCUSSION

Recent studies suggest that TGF-β produced by T cells[25,26] or by other yet unidentified cells[27,28] may play a role in limiting autoimmune inflammation. These observations raise the attractive possibility of treating autoimmune diseases with a cytokine that is physiologically involved as a negative feedback signal to limit the extent of T-cell activation.[7] Experimental allergic encephalomyelitis (EAE) and arthritis have been successfully prevented or ameliorated by the administration of TGF-β_1 before the onset of first clinical signs of disease.[29–33] Similarly, the incidence or severity of relapses in the chronic disease models was reduced by preceding treatment with TGF-β_1.[29,31,32] In virus-induced diseases of the nervous system TGF-β_2 was found to inhibit the inflammatory reaction in the brain parenchyma and the meninges.[5,15]

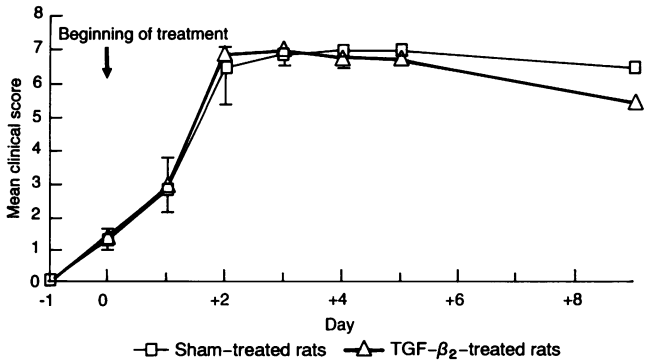

Figure 4. Treatment of AT-EAN with TGF-β_2. Lewis rats were injected with $4{\cdot}5 \times 10^6$ activated P2-specific T-line cells. Three days later 10 rats with incomplete paresis of the tail were divided into two groups on day 0 and received for 4 days either 5 μg TGF-β_2/day or the same volume of PBS/0·1% BSA i.p. EAN was scored as specified in the Materials and Methods.

Our experiments in EAN focused on the possible therapeutic utility of TGF-β_2 for the treatment of exacerbated acute autoimmune inflammation and demyelination in the PNS. In EAN induced by immunization with a neuritogenic peptide of P2 protein, therapy with TGF-β_2 stopped progression and shortened the duration of the disease, even if applied after onset of clinical disease. The extent of cellular infiltration, particularly by macrophages, and the degree of demyelination was reduced in the nerves of TGF-β-treated animals.

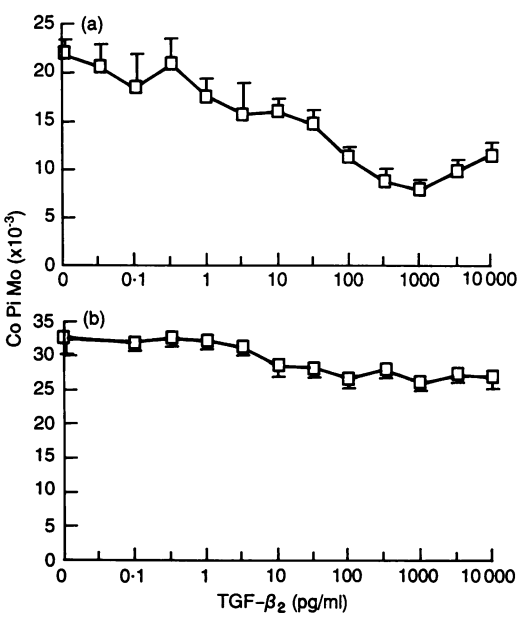

Figure 5. Sensitivity to TGF-β_2 of the P2-induced activation (a) and the IL-2-dependent growth (b) of the neuritogenic T-cell line P2.48 *in vitro*. (a) $1{\cdot}5 \times 10^4$ P2.48 line cells were activated in serum-free medium for 3 days with $7{\cdot}5 \times 10^5$ irradiated syngeneic thymocytes and P2 (20 μg/ml) in the absence or presence of indicated concentrations of TGF-β_2 in a total volume of 120 μl/microtitre well. Proliferation was measured by [^{3}H]thymidine incorporation during the last 16 hr of restimulation and is expressed as mean c.p.m. (± SD) of triplicate cultures. (b) 3×10^4 activated P2.48 line blasts/well were incubated for 24 hr in 200 μl Iscove's medium containing 7% supernatant of Con A-activated spleen cells. Proliferation was measured and expressed as in (a).

TGF-β is produced locally at sites of inflammation and exerts chemotactic effects on monocytes[34,35] and lymphocytes.[36] Therefore, systemic administration of TGF-β, as done in our experiments, may interfere with trafficking of macrophages and lymphocytes into the nerve by diminishing the gradient of TGF-β that focuses mononuclear cells into the site of inflammation. This mode of action may be supported by the inhibitory effect of TGF-β on adhesion of lymphocytes to endothelial cells.[37] The therapeutic effects of TGF-β on clinical signs of actively induced EAN suggest that TGF-β may not only reduce the number of macrophages invading the nerve but also inhibit the function of activated macrophages, which have already invaded peripheral nerves, to impair nerve conduction or cause myelin damage through the release of toxic mediators.[38] It is of note in this context that TGF-β abrogate the release of reactive oxygen[12] and nitrogen intermediates[13] as well as tumour necrosis factor-α (TNF-α) by activated macrophages.[12]

It was unexpected that the local expression of MHC class II molecules was not considerably reduced in sciatic nerves of TGF-β_2-treated rats with actively induced EAN. This result is in contrast to observations made in EAE and Borna virus-induced encephalomyelitis.[15,32] In these studies prophylactic application of TGF-β prevented disease, probably by inhibiting expansion of antigen-specific T-cells and diminishing subsequent T-cell-mediated induction of MHC class II antigens by interferon-γ (IFN-γ). In the present study, by contrast, TGF-β was administered only after manifestation of overt disease, a time-point at which up-regulation had already occurred. Alternatively, non-inflammatory events such as axonal degeneration could lead to expression of MHC class II antigens,[39] which may not be down-regulated by TGF-β.

In contrast to actively induced EAN, AT-EAN was not mitigated by therapy with TGF-β commencing at the day of first clinical signs. This may to some extent be due to an intrinsic resistance of a minor proportion of the T cells within the lymphocyte line P2.48 to TGF-β,[40,41] since P2-induced T-line activation was suppressed by maximally 64% in the presence of this cytokine. Moreover, IL-2-dependent growth of the line P2.48 *in vitro* was hardly affected by TGF-β (Fig. 5). This result contrasts with earlier observations made with murine T cells under serum-free conditions[24] but is in agreement with results obtained using autoreactive human or rat T-line cells in the presence of serum.[6,40]

It is not known whether adoptively transferred neuritogenic T cells that have entered the nerve have to be fully activated by APC displaying local autoantigen to exert their pro-inflammatory effect, or whether a reduced activation, as observed *in vitro* in the presence of TGF-β, is sufficient to set the immune cascade in motion. Concerning the specific activation of T cells within the nerve, one might also speculate that in active EAN T lymphocytes primed once by antigen in the popliteal lymph node are more sensitive to TGF-β than repetively P2-stimulated neuritogenic T-line cells used for induction of AT-EAN.

Based on recent results, we assume that the most likely explanation for our *in vivo* observations is that ongoing AT-EAN is quite refractory to any suppressive agent, possibly

due to its rapid progression. Thus, we observed that deoxyspergualine, a potent immunosuppressive molecule, given after onset of disease suppressed actively induced EAN but not AT-EAN (our unpublished observations) and that clinical signs of AT-EAN were inhibited by injection of an anti-IL-2-receptor antibody on the day of cell transfer but not on the following days.[22] Our findings with TGF-β_2 in AT-EAN are similar to those reported in another animal model, in which the acute phase of chronic relapsing EAE transferred by activated T cells was not significantly attenuated by TGF-β1 given after the onset of disease.[32]

The beneficial effect of TGF-β_2 on the course of actively induced EAN may have clinical implications for the treatment of inflammatory diseases of the PNS. It remains to be investigated whether modified TGF-β with a longer plasma half-life and different tissue distribution[42] may enhance its immunotherapeutic potential.

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