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Inhibition of Ras attenuates the course of experimental autoimmune neuritis

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Abstract

EAN induced in Lewis rats by immunization with peripheral bovine myelin was treated by the Ras inhibitor farnesylthiosalicylate (FTS). Treatment from day 0 with FTS (5 mg/kg intraperitoneally twice daily) attenuated peak clinical scores (mean ± S.E., 2.5 ± 0.5 compared to 4.1 ± 0.5 in saline treated controls, $p = 0.018$, t-test) but not recovery. Treatment from day 10 with FTS attenuated peak disability (2.5 ± 0.6, $p = 0.032$ compared to saline treated controls) and improved recovery (0.84 ± 0.42, untreated controls 2.4 ± 0.6, $p = 0.028$ by repeated measures ANOVA). Effects were confirmed by rotarod and nerve conduction studies. An inactive analogue, geranylthiosalicylate, had no clinical effect. Inhibition of Ras is of potential use in the treatment of inflammatory neuropathies.
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1. Introduction

Experimental autoimmune neuritis (EAN) is a T cell mediated acute demyelinating inflammatory disease of the peripheral nervous system (PNS) that can be induced in susceptible animals by active immunization with PNS tissue, purified PNS myelin proteins or synthetic P2 peptide (Kadlubowski and Hughes, 1979; Shin et al., 1989; Waksman and Adams, 1955). EAN is an established animal model of human acute inflammatory demyelinating polyradiculoneuropathy (AIDP) (Hahn, 1996; Notterpek and Tolwani, 1999), a major cause of acute neuromuscular paralysis (Parry, 1993). Guillain–Barré syndrome (GBS) is the most common form of AIDP with a yearly incidence of 2 : 100,000 (Soffer et al., 1978). The severe form of GBS requires prolonged mechanical ventilation and intensive care in patients who, however, may eventually make a complete recovery. EAN is a useful tool for studies of pathogenic mechanisms and novel therapeutic strategies for the treatment of AIDP. Animals, like humans, develop a monophasic disease with ascending paresis and electrophysiological evidence for demyelinating neuropathy (Cragg and Thomas, 1964; Wietholter et al., 1988). The pathogenesis of EAN comprises breakdown of the blood–nerve barrier, infiltration of peripheral nerves with macrophages and activated T lymphocytes and focal demyelination of the peripheral nerves (Hahn, 1996; Rostami et al., 1984). In addition to immunological findings, which indicate enhanced lymphocyte activation (Hartung and Toyka, 1990), the established induction of EAN by passive transfer of myelin reactive T lymphocytes (Linington et al., 1984) and the efficiency of various T cell directed

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therapies in preventing the disease (Jung et al., 1992, 1996), established the importance of the cellular arm of the immune system in the pathogenesis of EAN. The crucial role of lymphocytes in the disease pathogenesis makes them an attractive target for new therapeutic strategies.

Activation of lymphocytes requires parallel stimulation of several signal transduction pathways (Downward et al., 1990; June, 1991). One of these pathways involves the activation of the GTP-binding protein Ras, and therefore inhibition of Ras may result in suppression of T lymphocyte activation (Downward et al., 1990; Henning and Cantrell, 1998; Pastor et al., 1995; Woodrow et al., 1993). Ras-dependent signaling requires not only that Ras be GTP bound, but also that it be associated with the inner leaflet of the cell membrane (Lowy, 1993). Specific anchorage of Ras proteins in the cell membrane is promoted inter alia by their carboxy terminal *S*-farnesyl cysteine (Cox et al., 1992; Kato et al., 1992). A recently developed farnesyl analogue, *S-trans-trans*-farnesylthiosalicylic acid (FTS), destabilizes the attachment of Ras to the cell membrane and thus potently inhibits Ras-mediated signal transduction (Aharonson et al., 1998; Egozi et al., 1999; Gana-Weisz et al., 1997; Haklai et al., 1998; Marciano et al., 1995; Marom et al., 1995). FTS appears to act on specific membrane domains that associate with Ras, since the compound and its analogues present distinctive structure–function relationships with respect to dislodgment of Ras from the cell membrane (Aharonson et al., 1998; Haklai et al., 1998; Niv et al., 1999) and inhibition of Ras-dependent signaling (Aharonson et al., 1998; Egozi et al., 1999; Gana-Weisz et al., 1997). FTS with its C15 farnesyl moiety and its C20 geranylgeranyl analogue (GGTS), but not its C10 geranyl analogue (GTS), were found to be potent Ras antagonists. Because FTS is structurally similar to the *S*-farnesyl cysteine common to all Ras proteins, it has been proposed that it may interfere with anchorage sites that recognize the *S*-prenyl moiety of Ras (Bolton et al., 1997; Marciano et al., 1995; Marom et al., 1995). FTS exhibits significant selectivity towards Ras in its active, GTP-bound form (Haklai et al., 1998; Kloog et al., 1999). Importantly, FTS has not been found to induce total depletion of cellular Ras (Niv et al., 1999). These findings may explain why FTS is not cytotoxic for cells and distinguish it from other Ras inhibitors.

Many of the conventional immunosuppressive treatments have no effect in GBS. Effective therapies, such as intravenous immunoglobulins (IVIg) and plasmapheresis, have a relatively fast onset of action. GBS and EAN may be amenable to inhibitors of lymphocyte activation which may have an even faster onset of action. Since preliminary experiments have shown that FTS has an effect on in vivo lymphocyte activation within 2 days, we examined the therapeutic potential of this compound in EAN.

2. Materials and methods

2.1. Animals

Female Lewis rats were purchased from Harlan Jerusalem at 2 months of age and were housed in the animal facility at the Tel-Aviv University Medical School. They were kept under standard conditions of 23 ± 1 °C, 12 h light/dark cycle and ad libitum access to food and water. The animal welfare committee of Tel Aviv University approved all experimental procedures and measures to avert pain and suffering to the rats.

2.2. Induction of EAN

Rats weighing 175–210 g were immunized by injection into both hind footpads of 200 μl of inoculum containing 10 mg of bovine peripheral myelin (BPM, prepared according to the method of Kadlubowski et al. (1980)) and 4 mg *Mycobacterium tuberculosis* (strain H37RA; Difco) emulsified in 100 μl saline and 100 μl complete Freund's adjuvant (CFA, Difco). Rats, which served as controls without EAN, were immunized with an inoculum containing the *M. tuberculosis* emulsified in saline and CFA only.

2.3. Treatment protocol

FTS and GTS were stored in chloroform, which was evaporated when needed under a stream of nitrogen. The powder was dissolved in absolute ethanol and diluted to the desired concentration in sterile saline made basic with NaOH. 1 ml of carrier solution containing 1–2 mg FTS or GTS (5 mg/kg) was injected intraperitoneally into each rat twice a day. Sham solution was made by the same method starting with chloroform alone. Three separate experiments were performed: In the first experiment rats were divided into 5 groups: (1) 10 EAN rats were treated with FTS from day 0 (after the immunization) to day 15 post immunization (PI) and with sham solution from day 16 to day 28 PI (short early treatment). (2) 10 EAN rats were treated with FTS from day 0 to day 28 PI (extended early treatment). (3) 10 EAN rats were treated with FTS from day 10 to day 20 PI and with sham solution from day 0 to day 9 and from day 21 to day 28 PI (late treatment). (4) 10 EAN rats were treated with sham solution from day 0 to day 28 PI (sham treatment). (5) 5 rats were immunized with CFA and *M. tuberculosis* only, and were treated with sham solution from day 0 to day 28 PI (CFA group). In the second experiment EAN was induced in all rats which were then divided into 3 groups all treated from day 10 to day 20 PI: (1) 8 rats were treated with FTS, (2) 8 rats were treated with GTS, and (3) 8 rats were treated with sham solution. A third experiment was carried out in order to perform lymphocyte proliferation assays in which rats were divided into 4

groups of 6 rats each. In the first 3 groups EAN rats were treated with either FTS from the day of immunization, FTS from day 10 PI, or sham solution from the day of immunization. A fourth group was immunized with CFA and *M. tuberculosis* only, and was treated with sham solution from the day of immunization until day 14 PI.

2.4. Clinical evaluation

Clinical signs were assessed immediately before immunization, daily for the subsequent 40 days PI and thereafter twice a week up to day 80 PI. At day 135 PI all the rats were assessed again. Severity of weakness was graded as follows: 0—undetected, 1—limp tail, 2—abnormal gait, 3—mild paraparesis, 4—severe paraparesis, 5—paraplegia, 6—paraplegia with forelimb involvement, 7—paraplegia with forelimb involvement and respiratory distress, 8—moribund or dead (Hoffman et al., 1980).

2.5. Rotarod test

Motor performance was assessed by means of a rotarod test. The rats were pre-trained before immunization to run on the rod, which rotated at a fixed speed of 13 turns per minute. After immunization all the rats were assessed every 1–4 days for 80 days. The rats were allowed to run for up to 1 min in each trial, or until they fell off. The mean of the three consecutive trials was recorded for each rat.

2.6. Electrophysiological studies

Electrophysiological studies of the tail were performed before immunization and on days 23, 43 and 135 PI. We have recently found this method to have advantages over the commonly used sciatic nerve–plantar muscle preparation (Kafri et al., 2002). The rats were anesthetized with phenobarbital (intraperitoneal, 24 mg/kg). Two pairs of monopolar needle electrodes were used to stimulate the tail nerves. Stimulating cathodes were inserted to a depth of 4–5 mm at the base of the tail, and 4 cm distally. An anode at each location was inserted into the skin 1 cm proximal to the cathode. A ground electrode was placed between the distal stimulating electrode and the active recording electrode. The stimulator (model SD9, Grass Medical Instruments, USA) delivered single square wave pulses of 0.2 ms duration. EMG recordings of responses to proximal and distal stimuli were made from the muscles of the tail using a pair of ring electrodes coated with electrode jelly and placed 1 cm distally to the distal stimulating electrode. The tail skin was cleaned carefully with alcohol before the electrodes were placed. The electromyographic (EMG) responses were displayed on a dual beam storage oscilloscope (Tektronix, USA) and then recorded by a Polaroid camera (model DS-34, USA) for further analysis. Both proximal and distal latencies were measured using time intervals from the stimulus artifact to the first deflection from baseline. To calculate the motor nerve conduction velocity (MNCV), the distance between stimulating cathodes was divided by the latency difference. Amplitudes of the compound muscle action potential (CMAP) from both proximal and distal stimulation were measured from the negative to the positive peaks. The reduction of the proximal CMAP compared to distal CMAP was calculated by the equation: (distal CMAP − proximal CMAP)/distal CMAP × 100 and was defined as the *R* ratio. Temperature differences were minimized by conducting the study as soon as the anesthesia had taken effect and warming the tail with a heating lamp.

2.7. Lymphocyte proliferation assay

Three rats from each group were killed by overdose of phenobarbital at days 10 and 14 PI, and popliteal and inguinal lymph nodes were removed under aseptic conditions. Cell suspensions were prepared by grinding the lymph nodes through a nylon mesh. The cells were suspended in Dulbecco's phosphate buffered saline (DPBS) and centrifuged at 1100×*g* for 7 min. Erythrocytes were lysed by a 7-min incubation in 0.83% (weight/volume) ammonium chloride, and cells were immediately washed three times with DPBS. Cells were suspended to a concentration of 3×10^6 cells/ml in RPMI-1640 medium (Biological Industries, Israel) containing 5% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells suspended in 200 μl aliquots were cultured in 96-well flat-bottomed microculture plates at a cell density of 1.25×10^6 cells/ml. For lymphocyte stimulation, 20 μl aliquots of either BPM, concanavalin A (Con A) and lipopolysaccharide (LPS) were added to the cultures at a final concentration of 10 μg/ml, 1 μg/ml and 1 μg/ml, respectively. For assessment of spontaneous proliferation, the same amount of incubation medium was added. After 72 h of incubation in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C 110 μl of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) solution containing XTT reagent and reaction solution (Biological Industries, Israel) in a 50 : 1 ratio were added to the wells, and the cultures were further incubated for 6 hours. The plates were read at 490/630 nm. The results are expressed as the mean absorbance value of each group ± S.E.

2.8. Statistical analysis

Differences between clinical scores, body weight and time on rotarod of each treatment group were assessed by ANOVA with repeated measures prospectively comparing each treatment group to the sham treated controls. Separate analyses were performed for the two distinct phases of the disease, deterioration to the peak and recovery from this peak. All animals were included in the analysis of the deterioration phase including those that died with their last

observation carried forward. In the recovery phase only the surviving animals were included in the analysis. A single maximal clinical score and minimum walking time on the rotarod from each animal was used to compare each treatment group to the sham treated controls by a one-way ANOVA followed by an LSD post hoc test. The lymphocyte proliferation and electrophysiological data were compared at each time point by independent group t-tests (one-tailed).

3. Results

3.1. FTS effect on clinical measures of EAN

3.1.1. Experiment 1: timing of treatment

Results of clinical scores, rotarod test and weights of the various groups are presented, in the interest of clarity, in Figs. 1 and 2. Fig. 1 summarizes the data from EAN groups treated with FTS from day 0 (early treatment) with sham treated controls. Early treatment was continued until day 15 (short early treatment) or day 28 (extended early treatment). Fig. 2 summarizes data from EAN rats treated from day 10 to 20 (late treatment) with sham treated controls and non-EAN CFA immunized controls.

3.1.1.1. Clinical score. The EAN group showed the first clinical signs at day 10 PI followed by rapid deterioration. The peak of the clinical deficit was at day 17 PI. After a short plateau, a slow recovery was observed. By day 80 PI, 87% of the rats were fully recovered and by day 134 PI, all surviving rats were fully recovered. As can be seen in Fig. 1A, early FTS treatment inhibited the clinical deterioration relative to sham treated controls ($p = 0.03$ for the short early treatment and $p = 0.002$ for the extended early treatment,

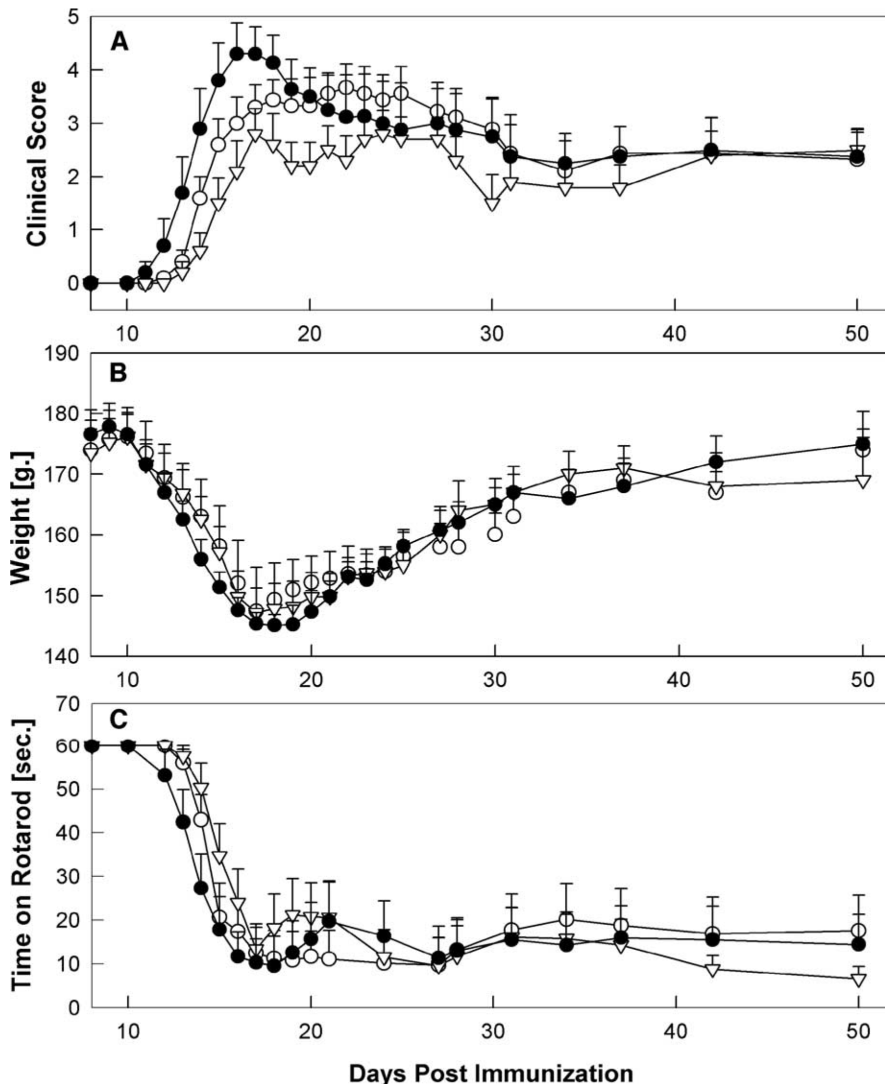

Fig. 1. The clinical score (A) body weight (B) and mean time on the rotarod apparatus (C) in 10 EAN rats treated with FTS from 0 to 15 days post immunization (short early treatment (○)) or in 10 EAN rats treated with FTS from 0 to 28 days post immunization (extended early treatment (▽)) or in 10 EAN rats treated with sham solution (●). Bars denote standard errors.

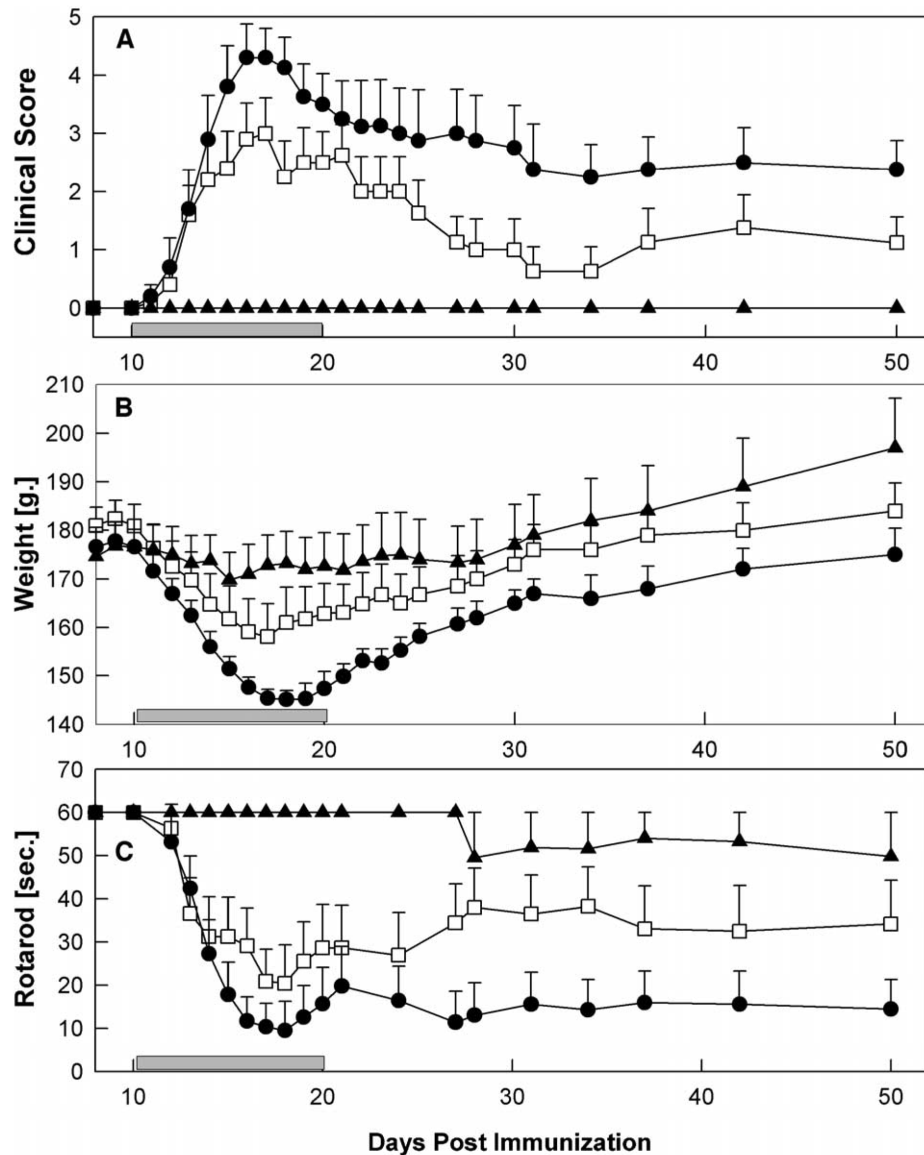

Fig. 2. The clinical score (A) body weight (B) and mean time on the rotarod apparatus (C) in 10 EAN rats treated with FTS from 10 to 20 days post immunization (late treatment (□)) or in 10 EAN rats treated with sham solution (●) and in 5 CFA control rats treated with sham solution (▲). Bars denote standard errors.

repeated measures ANOVA days 10 to 17 PI with LSD post hoc test). These results indicate slower deterioration in the treated groups that did reach a similar peak of disease to sham treated animals: The mean ± S.E. maximal clinical score was 4.70 ± 0.54 in sham treated controls, 4.00 ± 0.33 in the short and 4.00 ± 0.15 in the extended early treatments (NS by one-way ANOVA). During the recovery phase the late treatment groups were not significantly different from the sham treated controls and moreover, the extended early treatment seemed to recover very little from the peak of disease (Fig. 1).

As can be seen in Fig. 2A, the late treatment suppressed the severity of the disease as compared to sham treatment (mean ± S.E. maximal clinical scores: 3.50 ± 0.45 and 4.70 ± 0.53, respectively, $p = 0.015$, one-way ANOVA with post hoc LSD test) and allowed faster recovery of the rats ($p = 0.01$, repeated measures ANOVA from day 18 to 80 PI).

Mortality was 3/10 for the sham and for both short and extended early treatments, 2/10 for the late treatment and 0/5 for the CFA controls.

3.1.1.2. Rotarod. EAN rats treated with sham solution demonstrated decreased mean rotarod walking time from day 12 PI. The minimal mean rotarod walking time was on day 18 PI, paralleling the clinical scores noted above (Figs. 1A and 2A). The short and extended early treatments delayed the motor deterioration, but did not significantly affect the worse motor performance of the rats (Fig. 1C). As can be seen in Fig. 2C, the late treatment suppressed the motor deterioration (20.4 ± 8.9 sec mean ± S.E. minimal

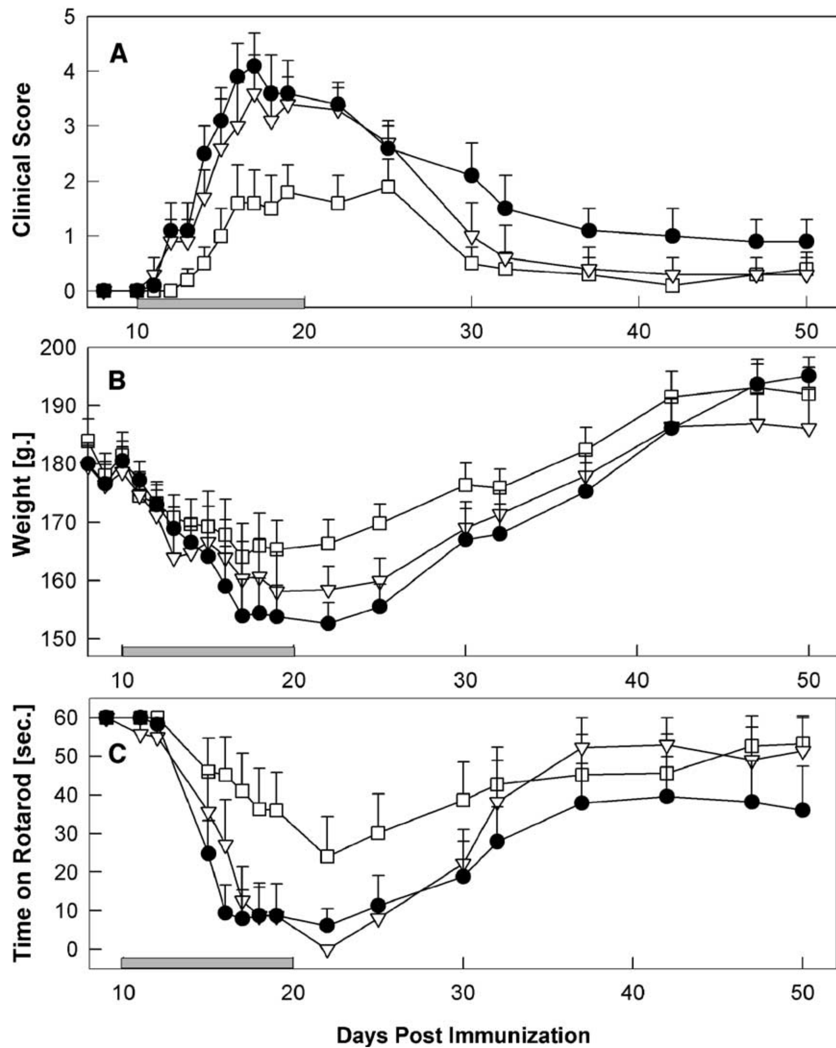

Fig. 3. The clinical score (A) body weight (B) and mean time on the rotarod apparatus (C) in 8 EAN rats treated with FTS from 10 to 20 days post immunization (□) or in 8 EAN rats treated with GTS from 10 to 20 days post immunization (▽) or in 8 EAN rats treated with sham solution (●). Bars denote standard errors.

walking time on rotarod) as compared to the sham treatment (9.5±6.8 sec), although not significantly ($p=0.15$). During recovery, late treatment did significantly ameliorate the motor deficits of the rats ($p=0.04$, repeated measures ANOVA for days 18 to 80 PI, Fig. 2C).

3.1.1.3. Body weight. EAN rats treated with sham solution suffered weight loss with the appearance of clinical signs and this became most severe during the peak of the disease (Fig. 1B). Early treatment with FTS resulted in a small non-significant loss in body weight during the first few days of the treatment before the onset of clinical disease. Both short and extended early FTS treatments did not affect the changes in body weight during the course of the disease. The late treatment with FTS inhibited the loss in body weight associated with EAN and was associated with a significantly higher body weight during the recovery phase ($p=0.04$, repeated measures ANOVA for days 18 to 30 PI, Fig. 2B).

3.1.2. Experiment 2: specificity of the effects to Ras inhibition

Results of clinical score, weight and rotarod test of the EAN rats treated from day 10 to 20 with FTS, GTS and sham solution are presented in Fig. 3.

3.1.2.1. Clinical score. FTS treatment delayed the first clinical signs as compared to sham treatment ($p=0.016$, repeated measures ANOVA for days 10 to 17 PI), suppressed the severity of the disease as compared to GTS treatment and sham treatment (mean±S.E. maximal clinical score: FTS=2.8±0.5, GTS=4.8±0.8, $p=0.02$ and 0.05, respectively, one-way ANOVA with post hoc LSD test), and accelerated the recovery of the rats ($p=0.01$, repeated measures ANOVA from day 18 to 80 PI as compared to sham treatment). GTS treatment had no significant effect on the clinical course of the disease though there was a trend to a faster rate of recovery.

3.1.2.2. Rotarod. FTS treatment ameliorated the motor deterioration as compared to sham treatment ($p=0.02$, repeated measured ANOVA from days 10 to 17 PI) while GTS had no beneficial effect. During the recovery phase there were no significant differences between the FTS, GTS and sham groups.

3.1.2.3. Body weight. FTS treatment resulted in a trend to higher body weight during the acute phase of the disease. GTS treatment did not affect the changes in body weight during the disease.

Mortality was minimal in this experiment, one rat each in the sham and GTS groups.

3.1.2.4. Treatment with FTS improved the long-term outcome of nerve conduction. Nerve conduction studies were performed at days 23 and 134 PI on the rats from Experiment 1: At day 23 PI all the EAN rats demonstrated severe pathology in the EMG parameters, without significant differences between the FTS treated groups and the sham treated group. At day 134 PI, the MNCV of the late FTS treatment group was significantly higher as compared to the sham treated group (32.6±2.4 m/sec. and 24.5±1.4 m/sec, respectively, $p=0.01$, Fig. 4A). The R ratio in all the EAN groups resumed normal values as compared to the CFA group ($p=0.3$ for short early treatment, $p=0.4$ for extended early treatment, $p=0.3$ for late treatment and $p=0.07$ for sham treatment). Both the extended early treatment and the late treatment with FTS had significantly smaller R ratio values as compared to the sham treatment group ($p=0.01$ for the extended early treatment and $p=0.02$ for late treatment, Fig. 4B). The delayed FTS treatment also showed a beneficial effect on the proximal CMAP amplitude, which was significantly higher compared to the sham treated group (11.1±1.9 mV and 7.1±1.7 mV, $p=0.04$, t-test).

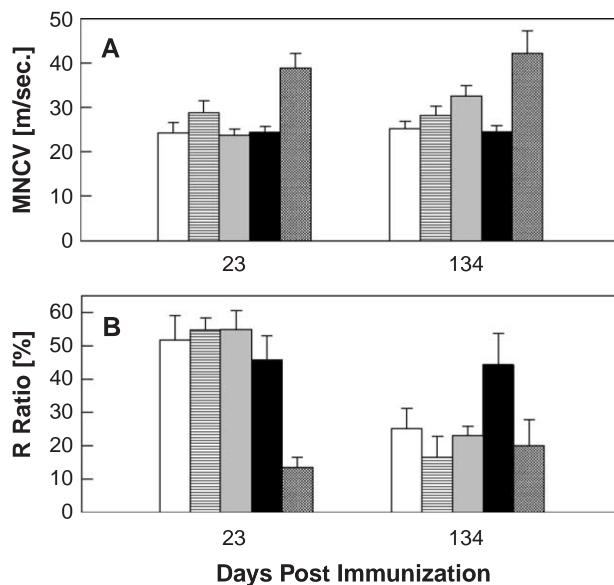

Fig. 4. Mean motor nerve conduction velocity (A) and R ratio (B) in the tail nerves at 23 and 134 days post immunization in EAN rats treated with FTS from 0 to 15 days post immunization (open bars) or from 0 to 28 days post immunization (horizontal lines) or from 10 to 20 days post immunization (gray bars) or treated with sham solution (black bars) or in naive rats treated with sham solution (diagonal crosshatch). $N=9$, 10, 8, 8 and 5, respectively, at the 23rd day and $n=7$, 7, 8, 7 and 3, respectively, at the 134th day.

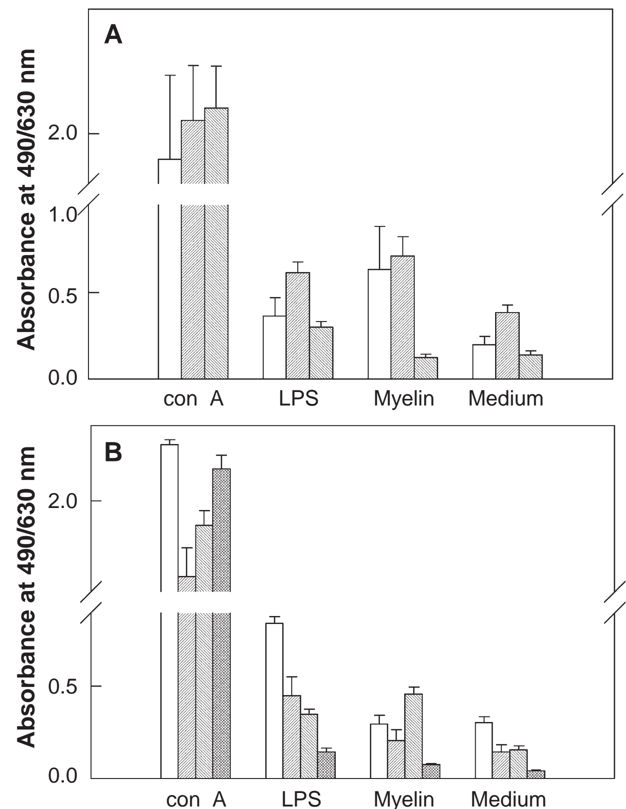

Fig. 5. The proliferation response of lymphocytes to Con A, LPS, myelin or in the absence of mitogen (medium) were measured by using XTT assay as described under Methods. The levels of proliferation are represented as mean absorbance values. The data shown are the mean absorbance values 10 days post immunization (A) in EAN rats treated with FTS from 0 to 10 days post immunization (empty bars) or with sham solution (horizontal lines) or in control rats treated with sham solution (diagonal crosshatch) and 14 days post immunization (B) in EAN rats treated with FTS from 0 to 14 days post immunization (empty bars) or from 10 to 14 days post immunization (gray bars) or with sham solution (horizontal lines) or in control rats treated with sham solution (diagonal crosshatch).

3.1.2.5. Lymphocyte proliferation to myelin antigens. As can be seen in Fig. 5A, at day 10 PI the early treatment with FTS decreased the spontaneous lymphocyte proliferation ($p=0.005$) and the reactivity to LPS ($p=0.03$), but did not affect the proliferation reaction to Con A and to myelin. Fig. 5B presents the proliferation reaction at day 14 PI. The late treatment with FTS from day 10 to 14 PI inhibited the reactivity to the specific antigen myelin ($p=0.001$) but did not affect the spontaneous lymphocyte proliferation and the reactivity to the non-specific mitogens Con A and LPS. The early treatment with FTS caused an increase in the spontaneous proliferation ($p=0.02$), and in the reactivity to the non-specific mitogens Con A ($p=0.05$) and LPS

($p<0.001$), as opposed to a decrease in the reactivity to myelin ($p=0.02$).

4. Discussion

GBS is a severe, potentially lethal disease. The therapies currently used for GBS, plasmapheresis and IVIg (Group, 1985; van der Meche and Shmitz, 1992, Korczyn and Nisipeanu, 1996), while clearly effective, can only accelerate recovery but do not increase its likelihood. Because of the costs and possible adverse events (and in the case of plasmapheresis also technical difficulties), these treatments may not be given at an early stage and are frequently reserved for relatively severe disease. Moreover, their efficacy is doubted in the more severe, axonal type of GBS. There is therefore an obvious need for more efficacious and acceptable therapies. The mechanisms of action of IVIg and of plasmapheresis are unknown but probably involve neutralization or removal of circulating antibodies. Considerable evidence is available, however, to suggest that GBS is T-cell – rather than humorally – mediated. The approach used in the present study is a novel one, attempting to neutralize activated lymphocytes by utilizing a recently described blocker of activated Ras.

In order to examine our hypothesis we have employed a well-established EAN model of GBS. The sham-treated controls developed a severe disease beginning at day 10 PI and suffered severe paralysis followed by spontaneous recovery in survivors. The effects of Ras inhibition by FTS were evaluated by a number of disease measures including quantitative clinical observations, a quantitative assessment of motor strength (the rotarod test) and electrophysiological tests. In all of these measures FTS was found to be effective in ameliorating EAN. The rotarod test was best at assessing mild to moderate disease corresponding to clinical scores 1–4, while the clinical score was more sensitive to change in more severely affected animals. Electrophysiological data supported the results in regards to long-term outcome but not in the early phase of disease. There is a known delay between clinical and electrophysiological findings in EAN and GBS and the time points chosen for the conduction tests in the present study may not have been optimal to detect an effect. The long term results are compatible with long term protection against denervation which may indicate the long term value of Ras modulation in the treatment of autoimmune neuritis.

The trend to a transient loss of weight observed in rats treated with FTS before the onset of EAN (early treatment groups) is similar to our previous observations in MRL mice and may be a side effect of this treatment. During the clinical phase of EAN, FTS treatment tended to ameliorate weight loss. There was no mortality in the FTS treated groups before the onset of EAN and no excess mortality after the appearance of clinical signs. Though the results of the present study suggest that FTS treatment is relatively safe, more extensive toxicology studies are necessary before this compound can be evaluated in human subjects. The lack of overt toxicity of FTS may be related to its relative specificity to the active form of Ras (Ras-GTP (Haklai et al., 1998; Kloog et al., 1999)). The beneficial effect of delayed FTS treatment in the EAN model correlates with its preferential inhibition of myelin reactive lymphocytes. In contrast, prolonged FTS treatment resulted in an increased proliferation response to non-specific mitogens but not to myelin, possibly reflecting activation of compensatory pathways. The delayed treatment protocol was included in this study because of its similarity to the real life clinical situation in which treatment is initiated only after the onset of symptoms. The greater efficacy of FTS given by this protocol is encouraging. In contrast, prolonged treatment with FTS may have inhibited or delayed the recovery from paralysis. While the mechanism of this effect is unclear, and may be due to inhibition of the proliferation or activation of anti-inflammatory T cells (TH2 lymphocytes) (Bai et al., 1997; Hartung and Toyka, 1990; Zhu et al., 1998), it draws attention to the delicate balance between different, and sometimes antagonistic actions of immunomodulatory drugs. It is therefore critical to evaluate the optimal dosage and timing at which Ras inhibitors are given before attempting such therapy in human autoimmune diseases.

The lack of clinical and immunological efficacy of GTS, an FTS analogue that does not affect Ras, argues against non-specific factors such as abdominal inflammation due to repeated IP injections and response to lipid solvents being responsible for the effect of FTS. The differences between late and early treatments also argue against such an effect.

FTS compares well with other published therapies for EAN which include immunologically active substances such as sodium fusidate (Marco et al., 1999), linomide (Zhu et al., 1999), cyclosporin (Hartung et al., 1987), IL-10 (Bai et al., 1997), β-interferon (Zou et al., 1999), nasal tolerance to myelin peptides (Zou et al., 1998) and antibodies to CD-2 receptors (Jung et al., 1996) and ICAM-1 (Archelos et al., 1993). The standard therapies for GBS in humans are plasmapheresis (Group, 1985; Korczyn and Nisipeanu, 1996), which has not been reported in animal models, and IVIg which has produced equivocal results (Enders et al., 1997; Gabriel et al., 1997; Miyagi et al., 1997). We have performed one preliminary experiment with IVIg in parallel with the FTS experiments and found no beneficial effect of this therapy. GBS has an acute monophasic course which does not enable medications to be started early and this may explain the inefficacy of steroid and other standard immunosuppressive therapy in this disease (Hughes et al., 1981; Parry, 1993). FTS acts almost immediately on cells in culture without affecting their viability (Katzav et al., 2001) and is therefore a potential new possibility for therapy in GBS.

In our in vitro experiments we have demonstrated that FTS inhibited the proliferation of myelin-reactive lymphocytes, presumably by inhibiting the active form of Ras

(Ras-GTP) (Haklai et al., 1998; Kloog et al., 1999). The effect on the response to myelin is much larger than on other non-specific mitogens (LPS and Con A), similar to the results we have previously found in EAE (Karussis et al., 2001). This response is consistent with the animal data and provides support for the efficacy of FTS through a presumed immune mechanism in EAN. It is interesting to note that early treatment with FTS from day 0 to 14 PI caused an increase in the proliferation to non-specific mitogens. This is in line with studies that found that early treatment of EAN with immunosuppressive drugs such as cyclophosphamide is detrimental (Kallen et al., 1986). It is also important to note that the present results do not exclude effects of FTS other than those demonstrated in lymphocytes. For instance, Ras is known to influence activation of macrophages by chemokines (Buscher et al., 1995; Kieseier et al., 2000; Li et al., 1995), apoptosis of T cells (Downward, 1998; Gomez et al., 1997) and cell migration (Joneson and Bar-Sagi, 1997; Tanka, 2000). The onset of the clinical effect of delayed treatment with FTS is within 1–2 days. At this stage the immune reaction has shifted into the nerve where the effect of FTS may be mediated by Ras inhibition of lymphocyte migration, other immune cells such as macrophages and mast cells and intrinsic components of the nerve such as Schwann cells, all of which depend on Ras signaling pathways (Downward, 1998; Harrisingh and Lloyd, 2004). Future studies are necessary to assess which of the potential effects of FTS are significant in ameliorating EAN.

There are a number of clinical implications of the present study that deserve mention. The modulation of intracellular signaling pathways is a novel approach in both EAN and GBS and may offer the advantage of faster onset and reversibility. This well-defined temporal action of FTS will probably require finding precise timing for the most efficient treatment protocols in human studies. Such issues may also be relevant to the delicate balance of immunomodulatory effects induced by currently used therapies, for instance the paradoxical effects of steroids in GBS as well as the opposing effects of agents such as cyclophosphamide given at various time points during EAN.

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