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The neurotrophic analogue of ACTH(4–9), Org 2766, protects against experimental allergic neuritis

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SUMMARY

Demyelinating diseases such as multiple sclerosis or the Guillain–Barré syndrome originate from an autoimmune response resulting in the degradation of myelin and impaired neuronal function. Prophylactic administration of the neurotrophic peptide, H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH [an ACTH(4–9) analogue], to rats with experimental allergic neuritis, a model for the Guillain–Barré syndrome, markedly suppresses the clinical symptoms, protects against loss of motor coordination and prevents the degeneration of myelinated axons in the affected peripheral nerve. Therefore, this peptide may provide a new approach to the therapy of peripheral demyelinating polyneuropathies.

INTRODUCTION

Demyelinating diseases are an important group of neurological disorders that await effective pharmacotherapy. The underlying pathogenesis of these diseases, which include multiple sclerosis (central nervous system) and the Guillain–Barré syndrome (peripheral nervous system), involves a cellular autoimmune response directed against specific components of myelin (reviewed in Hughes, 1990; Arnason and Soliven, 1993). Myelin breakdown is accompanied by severe deficits in neuronal function (Madrid and Wisniewski, 1977; Hahn *et al.*, 1988). Current therapeutic strategies, such as treatment with corticosteroids in multiple sclerosis (Goodin, 1991) and immunoglobulin therapy or plasmapheresis in Guillain–Barré syndrome (van der Meché *et al.*, 1992; Guillain–Barré Syndrome Study Group, 1985), aim to modulate or suppress the autoimmune response (Hughes, 1990; Oksenberg and Steinman, 1991). However, non-specific inactivation of the immune system may render the patient more susceptible to concomitant infections and possible relapses of these demyelinating syndromes (Hughes, 1990). As immune-based therapies so far have yielded limited results, we hypothesized that a therapy based on the application of a neurotrophic factor that improves the function of the compromised neuron could be an important asset in the amelioration of the symptoms of these demyelinating syndromes. To test this hypothesis we studied the possible beneficial effect of a neurotrophic adrenocorticotrophic hormone (ACTH)-derived neuropeptide, the degradation resistant ACTH(4–9) analogue Org 2766, on the development of experimental allergic neuritis (EAN).

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Experimental allergic neuritis is an animal model of the human, primary demyelinating disease of the peripheral nervous system known as the Guillain–Barré syndrome (Waksman and Adams, 1955; Arnason, 1993). Experimental allergic neuritis in Lewis rats is widely accepted as an animal model of the human Guillain–Barré syndrome. In EAN, the underlying pathological mechanism appears to be a cell-mediated autoimmune response to peripheral nervous system myelin, in particular to the myelin P_2 protein (Leibowitz and Hughes, 1983; Brosnan *et al.*, 1988; Arnason and Soliven, 1993). Experimental allergic neuritis can be induced by injection of Complete Freund's Adjuvant together with a homogenate of whole peripheral nerve (Waksman and Adams, 1955; Smith and Hofmann, 1979), purified myelin extract (Kadlubowski *et al.*, 1980), myelin P_2 protein (Brostoff *et al.*, 1972; Kadlubowski *et al.*, 1980), myelin P_0 protein (Milner *et al.*, 1987; Linington *et al.*, 1992) or fragments of myelin P_2 protein (Brostoff *et al.*, 1980; Rostami and Gregorian, 1991) or by intravenous transfer of activated P_2 specific T cells (Hughes *et al.*, 1981; Linington and Wekerle, 1984).

The clinical profile of EAN consists mainly of ataxia which sometimes progresses to paralysis. In the rat, symptoms are first observed at the tip of the tail (loss of tail tip reflex), eventually resulting in a flaccid tail. Eventually the hind legs are involved, leading to paresis and paralysis. There is often a striking loss of body weight at the onset of the disease. Electrophysiological studies have shown a decreased conduction velocity, both afferently and efferently, in animals with EAN. Compound action potentials of reduced amplitude and enhanced dispersion with conduction block of some fibres at the site of demyelination are also observed in EAN (Heininger *et al.*, 1986; Wiethölter *et al.*, 1988). Characteristically the histological picture consists of randomly distributed perivenular cellular infiltration of the peripheral nerve by lymphocytes and macrophages, accompanied by segmental demyelination. Dorsal and ventral roots, dorsal root ganglia and the peripheral nerves are all affected by EAN (Powell *et al.*, 1983; Simmons *et al.*, 1988).

The neurotrophic peptide used in this study, Org 2766, is a synthetic analogue of ACTH(4–9) (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH). Adrenocorticotrophic hormone fragments, including ACTH(4–10) and ACTH(4–9), and the analogue Org 2766 are neurotrophic peptides devoid of corticotrophic and melanotrophic activity (De Wied and Jolles, 1982). This permits investigation of the potential neuroprotective and neurotrophic role of these peptides in neurological disorders. In previous animal studies we documented the trophic effect of this peptide in peripheral neuropathies induced by neurotoxins (acrylamide, cisplatin) and caused by metabolic disorders (diabetes mellitus; van der Hoop *et al.*, 1988, 1990; Van der Zee *et al.*, 1989; Edwards *et al.*, 1991). An important difference between these neuropathies and the Guillain–Barré syndrome is that the latter is a nerve disorder of which the primary deficit is not usually neuronal or axonal but involves primary inflammatory demyelination. In this article we show that Org 2766, an ACTH(4–9) analogue, exerts a neuroprotective effect on clinical, functional and histological parameters on animals with experimentally induced allergic neuritis.

METHODS

Animals

Female Lewis rats of an inbred strain (DLA, central department for laboratory animals, University of Limburg, Maastricht, NL), weighing 160–170 g at the beginning of the experiment were used throughout the

experiments. Upon arrival the rats were allowed to acclimatize for 2 weeks. All animals were housed individually in Macrolon cages (RUCO, NL) on sawdust bedding and had free access to commercial rat chow and water. A dark–light cycle of 12 h was maintained, with lights on from 06.00 to 18.00 h. Lewis rats were used because these rats are highly susceptible to the induction of EAN and in most cases develop a severe form of neuritis (Smith *et al.*, 1979; Brosnan *et al.*, 1988).

Myelin isolation

The cauda equina was obtained from humans post-mortem (in collaboration with the Department of Neurology, Academic Hospital, Utrecht, NL). The tissue was not affected by neurological disease and was removed within a few hours after death and stored at −20°C until needed. The period of storage varied from a few days up to 3 weeks.

Myelin from the cauda equina was purified by the method descibed by Luijten (Luijten *et al.*, 1984). All tissues were cleaned carefully by removing fat, connective tissue (including meninges) and blood. The cauda equina was homogenized in a 0.88 M sucrose solution (5% homogenate). The homogenate was overlaid with 0.32 M sucrose solution and centrifuged (30 min, 75 000 g_{av}). The crude myelin layer at the interface was collected, resuspended in 0.88 M sucrose solution and centrifuged again. The crude myelin fraction was suspended in bidistilled water and stirred for 60 min at 0°C. Subsequently, the suspension was centrifuged for 20 min at 29 000 g_{av} in a fixed angle centrifuge. The myelin pellets were collected, lyophilized and the purity and composition of the myelin was assessed by a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (15% SDS–PAGE gels). The protein content of the myelin preparations was determined according to Bradford (1976).

Induction of experimental allergic neuritis

In the present experiments we induced EAN with subcutaneous injections of an emulsion containing Complete Freund's Adjuvant and myelin. The emulsion was prepared by mixing, on a 1:1 (v/v) basis, the purified peripheral myelin solution (each rat 5 mg freeze-dried myelin in 100 μl PBS, pH 7.3) with Complete Freund's Adjuvant (CFA, 100 μl each rat). Complete Freund's Adjuvant was prepared by adding 4 mg of *Mycobacterium tuberculosis* (H37RA, Difco) per millilitre of Incomplete Freund's Adjuvant (Difco Laboratories, Detroit, Michigan, USA).

Rats were anaesthetized with Hypnorm© (Janssen Pharmaceutica, Tilburg, NL), containing fluanisone 10 mg/ml and fentanylcitrate 0.315 mg/ml, dose 0.8 mg/kg body weight administered subcutaneously and received four subcutaneous injections of 50 μl of myelin–CFA emulsion in the proximal part of the dorsum of each paw. Control animals received four subcutaneous injections of 50 μl of PBS–CFA emulsion.

Peptide treatment

Org 2766, a degradation resistant ACTH(4–9) analogue (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH, a gift from Organon Int. Bv.) was dissolved in 0.1% BSA/0.05 M HCl and diluted with 0.9% NaCl. Each rat received 75 μg/kg Org 2766 in 0.5 ml saline by subcutaneous injection in the neck every 48 h, starting immediately after inoculation with the myelin emulsion. Characteristically, Org 2766 displays a bell-shaped (neurotrophic) dose-response curve, with optimal doses of 7.5–75 μg/kg body weight each 48 h (Van der Zee *et al.*, 1988; Gispen, 1990). Control rats were given 0.5 ml of 0.9% NaCl per rat every 48 h.

Clinical symptoms

After injection of the myelin suspension, the animals were examined daily for clinical symptoms, such as flaccid tail, paraparesis and ataxia. The severity of the symptoms was graded on a scale of 0 to 3 as follows: grade 0, no clinical symptoms; grade 1, impaired tail tip reflex, limp tail; grade 2, mild to moderate paraparesis with some locomotor problems and ataxia; grade 3, severe paraparesis with severe disturbance of walking and muscle atrophy; paraplegia; death. Animals that died were given a score of 3 throughout the rest of the experiment. This method of describing symptoms is a simple and reproducible parameter in the evaluation of the severity of EAN.

Walking pattern analysis

The walking pattern of the rats was analysed as described by de Medinaceli *et al.* (1982) and modified by de Koning and Gispen (1987) as follows. After dipping the hind paws of the rat in photographic developer,

the rat walked over photographic paper placed on the bottom of a 50 cm long confined walkway with a dark box at the end. From the track records, several different parameters can be measured (Fig. 1): the step length (SL), print length (PL), toe spreading (TS) and inter-toe spreading (IT) and the toe-spreading index (TSI) calculated (de Koning and Gispen, 1987). This represents the change in the motor performance of the rats.

$$TSI = [(TS_{experimental} - TS_{control})/TS_{control}] + [(IT_{experimental} - IT_{control})/IT_{control})] * 100$$

Since both sciatic nerves and therefore motor function in both feet are afflicted in this model, we used the footprints of the age-matched control group as a reference to the footprints of EAN animals.

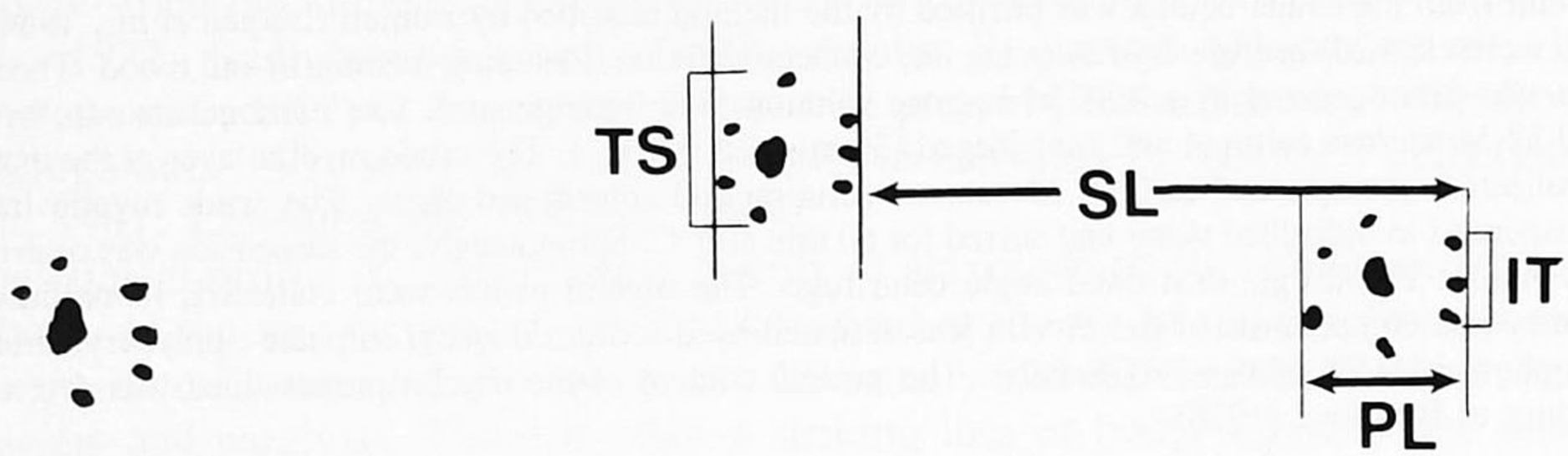

FIG. 1. Walking pattern analysis. The step length (SL), print length (PL), toe spreading (TS) and inter-toe spreading (IT) were measured from the footprint traces and used to calculate the toe-spreading index.

Rotarod performance test

Rotarod performance was analysed as described by Kaplan and Murphy (1972) with minor modifications according to Gipon *et al.* (1977). A horizontal wooden rod (7.5 cm in diameter) was rotated 35 cm above a grid floor at a rotation speed of 8 rev/min. The animals had been trained to remain on the rotating rod for 2 min by use of negative reinforcement. Rotarod performance was measured daily. Each rat was submitted to three sessions of 2 min on each day of the experiment, beginning at day 0 (day of inoculation). Rotarod performance for a given day is given as the mean of these three measured values.

Histology

On day 24 post inoculation (10 days after exacerbation), 10 rats of each treatment group were chosen at random and sacrificed for histological analysis. The right sural and sciatic nerves were removed and fixed in a glutaraldehyde (2%)/sodium cacodylate solution for 5 h, post-fixed in osmium tetraoxide (1%) for 2 h, and subsequently embedded in EPON©. The sural nerve was cut 3 cm below the sciatic notch (which served as a reference point during dissection). Semi-thin (1 μm) transverse sections of the sural nerve were prepared for morphological examination. Sections were stained for myelin, with paraphenylene diamine (1%), and photographed (magnification $\times 792$). In most animals, the sural nerve consisted of two fascicles (19 out of 30 nerves). The myelinated fibres from all fascicles of the nerve section were counted on the photomicrographs (Bijlsma *et al.*, 1983). In order to obtain more detailed information about changes in perimeter distribution as a result of EAN or peptide treatment, further examination of the sural nerve sections was performed by computer-assisted image analysis (TIM system, DIFA, Breda, NL). With this method we measured the perimeter of each myelinated fibre (i.e. axon including the myelin sheath) of four areas per section chosen at random (four fields of 50×35 μm).

Statistics

The investigators were blind to the treatments as well as the codes of the rats and tissue samples. These codes were broken only after group analyses had been carried out. The data are expressed as the mean per day ($\pm$SEM). The results were analysed using an analysis of variance for repeated measures (MANOVA) followed

by supplemental t tests (P values are shown in the figures). Rotarod performance scores were analysed by using a Kruskal–Wallis test followed by supplemental Mann–Whitney U tests. Clinical scores were analysed by using a non-parametric χ^2 test. $P < 0.05$ was considered statistically significant. The SPSS Statistics package (SPSS Inc., Chicago) was used for all of these tests.

Outline of the two experiments

Experiment 1. In this experiment 51 female Lewis rats were divided at random into two groups of 19 rats and one group of 13 rats (control group). The rats of the age-matched control group were inoculated with CFA/PBS emulsion and received subcutaneous injections of saline every 48 h ($n = 13$). Experimental allergic neuritis was induced in rats of the second and third groups as described above. One group received the ACTH(4–9) analogue (Org 2766, 75 μg/kg) subcutaneously every 48 h ($n = 19$), whereas the other group received saline injections ($n = 19$). Starting 1 day after immunization, all animals were submitted to several functional tests to assess their clinical state and motor function. On day 24 post inoculation, randomly chosen animals were sacrificed. The sural nerves of these animals were examined for possible changes in the number of myelinated fibres as a result of EAN and peptide treatment. The clinical status and functional performance of the remaining rats were studied until the animals had recovered completely (day 38 post inoculation).

Experiment 2. The walking pattern was assessed in a separate experiment but under identical conditions as Experiment 1 with regard to the CFA–myelin injections and peptide treatment. Each treatment group in this walking pattern experiment consisted of 10 female Lewis rats (total 30 rats).

RESULTS

Clinical score

As illustrated in Fig. 2, the first symptom, i.e. an impaired tail tip reflex, became apparent on day 14 post inoculation. Approximately 24 h later, several animals developed a paresis and by day 16 post inoculation all animals (Groups 2 and 3) had developed neurological symptoms of EAN (i.e. limp tail, paresis). Severely affected animals became apathetic and developed complete paralysis of the hind legs. Rats with EAN reached maximal clinical scores on day 22 post inoculation. Even after 36 days, symptoms were still evident in some of the EAN rats (four out of 17 EAN rats). As illustrated in Fig. 2, treatment with the ACTH(4–9) analogue did not delay the onset of the EAN syndrome but significantly reduced the severity of the EAN. Peptide treatment ameliorated the clinical scores significantly on days 16, 17, 19, 20 and 23 post inoculation ($P < 0.05$, χ^2 test). Throughout the experiment the median scores of the EAN–saline group were higher than those of the peptide-treated EAN animals. During the experiment only one animal died as a result of the induced allergic neuritis (*see* Fig. 1). This animal was given a score of 3 throughout the remnant of the experiment. As expected, age-matched control animals showed no neurological symptoms at all.

Body weight

Experimental demyelination induced a severe weight loss in the Lewis rats. Weight loss was most evident on day 23 post inoculation. Org 2766 treatment significantly diminished weight loss on day 20 to day 23 post inoculation [MANOVA: day 20 to day 23 post inoculation: $F(36,1) = 4.13$, $P = 0.05$]. On day 23 post inoculation, EAN animals treated with saline had lost 12.4% of their body weight whereas the peptide-treated EAN animals had lost only 5.0% of their body weight compared with the age-matched control group (data not shown).

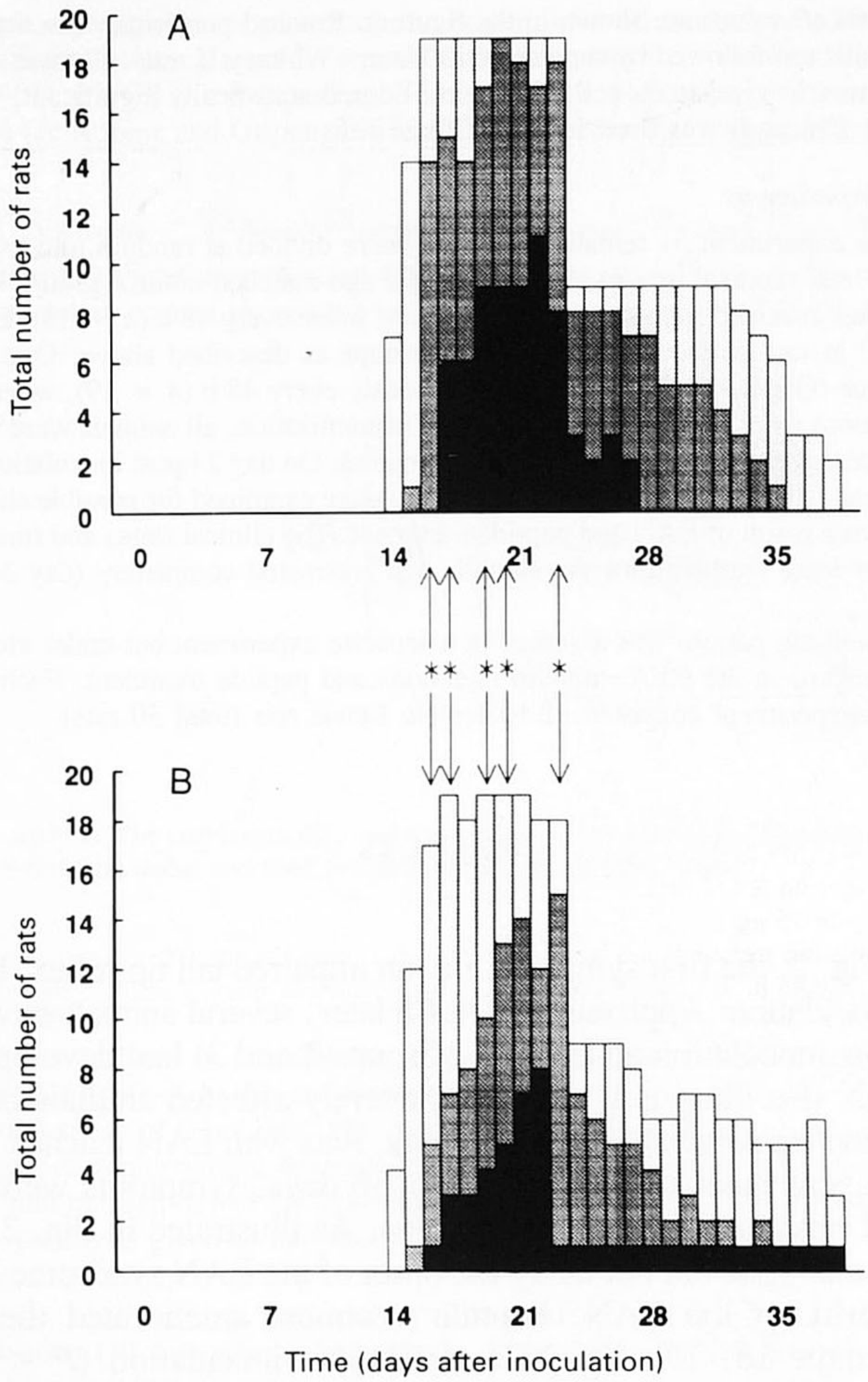

FIG. 2. Clinical grading. The severity of the EAN in Lewis rats (Central Department for Laboratory Animals, Limburg University, Maastricht, NL, weight 160–170 g) was graded on a scale of 0–4 as follows: grade 0, no visible neurological signs or symptoms; grade 1, limp tail; grade 2, mild paraparesis/ataxia; grade 3, severe paraparesis/muscle atrophy/tetraparesis/death. Data are shown using stacking bars rather than by using mean ± SEM because this grading system does not guarantee linear coherence. The bars depict the number of rats with a score of 1, 2 or 3: light shading = grade 1; medium shading = grade 2; dark shading = grade 3. Experimental allergic neuritis was induced by subcutaneous injection of purified, human peripheral myelin (5 mg freeze-dried myelin/rat) in CFA (4 mg/ml *M. tuberculosis*). After dissection, the number of animals were reduced to nine per group (day 24 post inoculation). A, clinical grading distribution of the group of rats with EAN treated with 0.5 ml saline injections every 48 h (n = 19). B, clinical grading distribution of the group of rats with EAN treated with 75 μg Org 2766/kg body weight in 0.5 ml saline (n = 19). Scores were compared by using a non-parametric χ^2 test. $^*P < 0.05$ was considered statistically significant.

Walking pattern analysis.

As illustrated in Fig. 3, the toe-spreading index of saline-treated EAN rats had decreased significantly by day 10 post inoculation (Fig. 3). In 6 days the toe-spreading index was reduced with a minimum of −83.3 ± 6.51. The decline in the toe-spreading index correlated

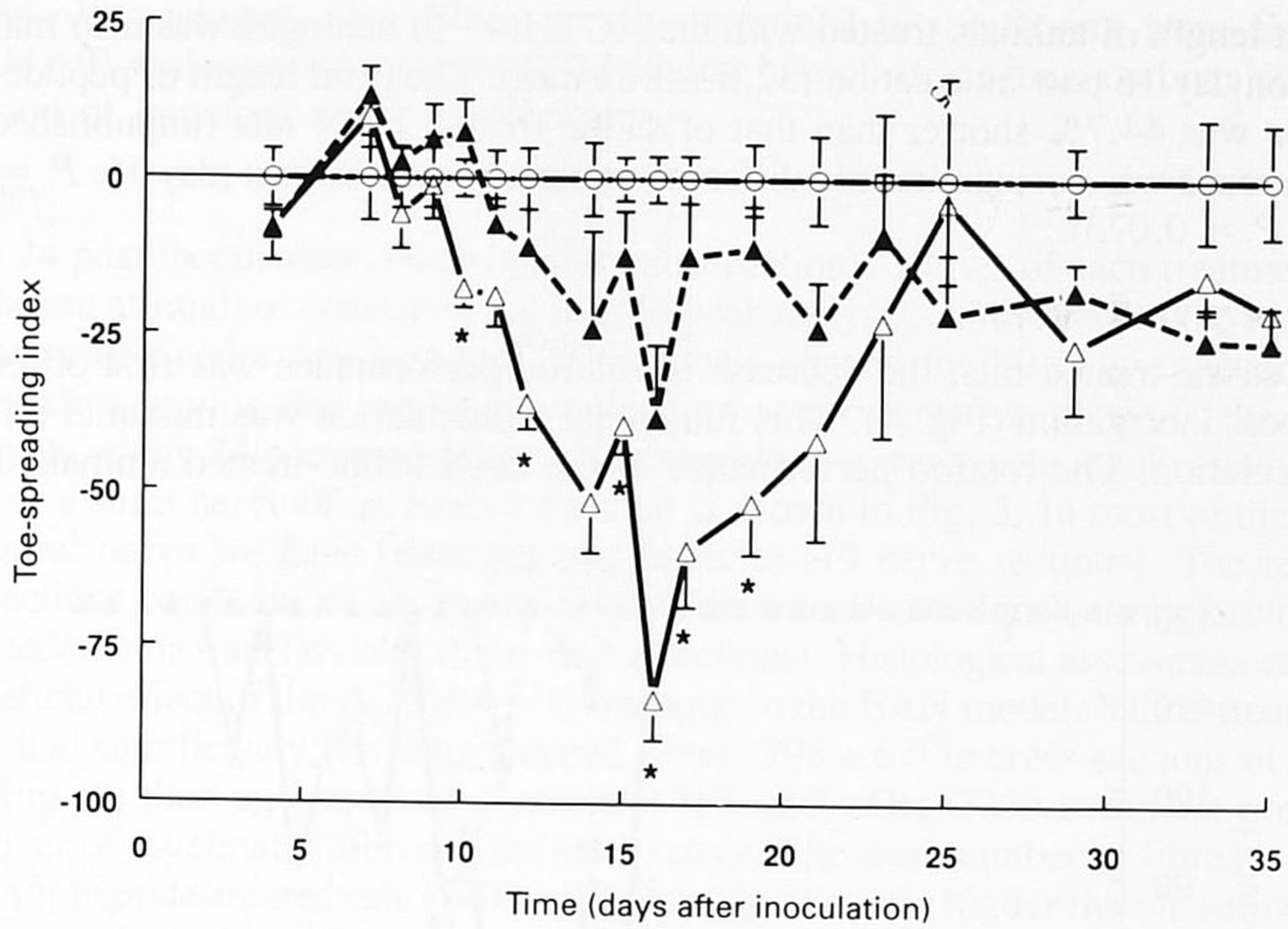

FIG. 3. Toe-spreading index derived from toe-spreading and inter-toe-spreading distance. Solid line (open triangle, n = 10) represents Lewis rats with EAN treated with 0.5 ml saline; dashed line (closed triangle, n = 10) represents Lewis rats with EAN treated with 75 μg Org 2766/kg body weight in 0.5 ml saline; dashed line with open circles represents age-matched control Lewis rats treated with 0.5 ml saline (n = 10). Saline and peptide were administered by subcutaneous injections in the neck every 48 h. The toe-spreading index is presented as mean ± SEM. Statistics: analysis of variance for repeated measurements, day 10 to day 19 post inoculation: $F(2,23) = 38.36$, $P < 0.0005$; followed by Student's t tests comparing saline- to peptide-treated rats with EAN, $^{*}P < 0.05$.

well with the clinical score (data not shown). The index returned to normal values on day 23, but stayed low compared with that of control rats (toe-spreading index on day 35 post inoculation: −20). The toe-spreading index of the peptide-treated animals began to decrease on day 12 post inoculation and the maximal decline was seen on day 16 post inoculation, when the toe-spreading index was −38.3 ± 11.84 (SEM), 54% higher than the toe-spreading index of saline-treated EAN rats. Treatment with ACTH(4–9) analogue induced a highly significant ameliorative effect on the toe-spreading index throughout the experiment [Fig. 3, MANOVA day 10 to day 19 post inoculation: $F(14,1) = 18.55$, $P = 0.001$]. The Org 2766-treated animals recovered by day 17 post inoculation. The age-matched control rats showed no impairment of toe-spreading index whatsoever and were used as a reference for EAN rats.

Other parameters derived from the walking pattern analysis were the step length and print length (Fig. 1). Animals suffering from EAN showed a slightly impaired step length. However, this impairment of step length was not statistically different from that of the age-matched control group. There was no difference between the step lengths of peptide-treated EAN animals and saline-treated EAN animals (data not shown). Print length was increased in rats with EAN, 12 days post inoculation, and was maximally affected 16 days post inoculation [41.72 ± 3.99 mm (SEM)] compared with the age-matched control group (21.6 ± 0.95 mm). Treatment with the peptide did not improve the print length.

The print length of animals treated with the ACTH(4−9) analogue was also maximally affected on day 16 post inoculation (32.6 ± 3.84 mm). The print length of peptide-treated EAN rats was 44.7% shorter than that of saline treated EAN rats (unpublished data). This difference was not significantly different, using a Student's *t* test (day 16: $P = 0.127$; day 17: $P = 0.073$).

Rotarod performance test

In EAN saline-treated rats, the decrease in rotarod performance was first observed on day 17 post inoculation (Fig. 4). This functional motor deficit was maximal on day 20 post inoculation. The rotarod performance of the EAN saline-treated animals declined

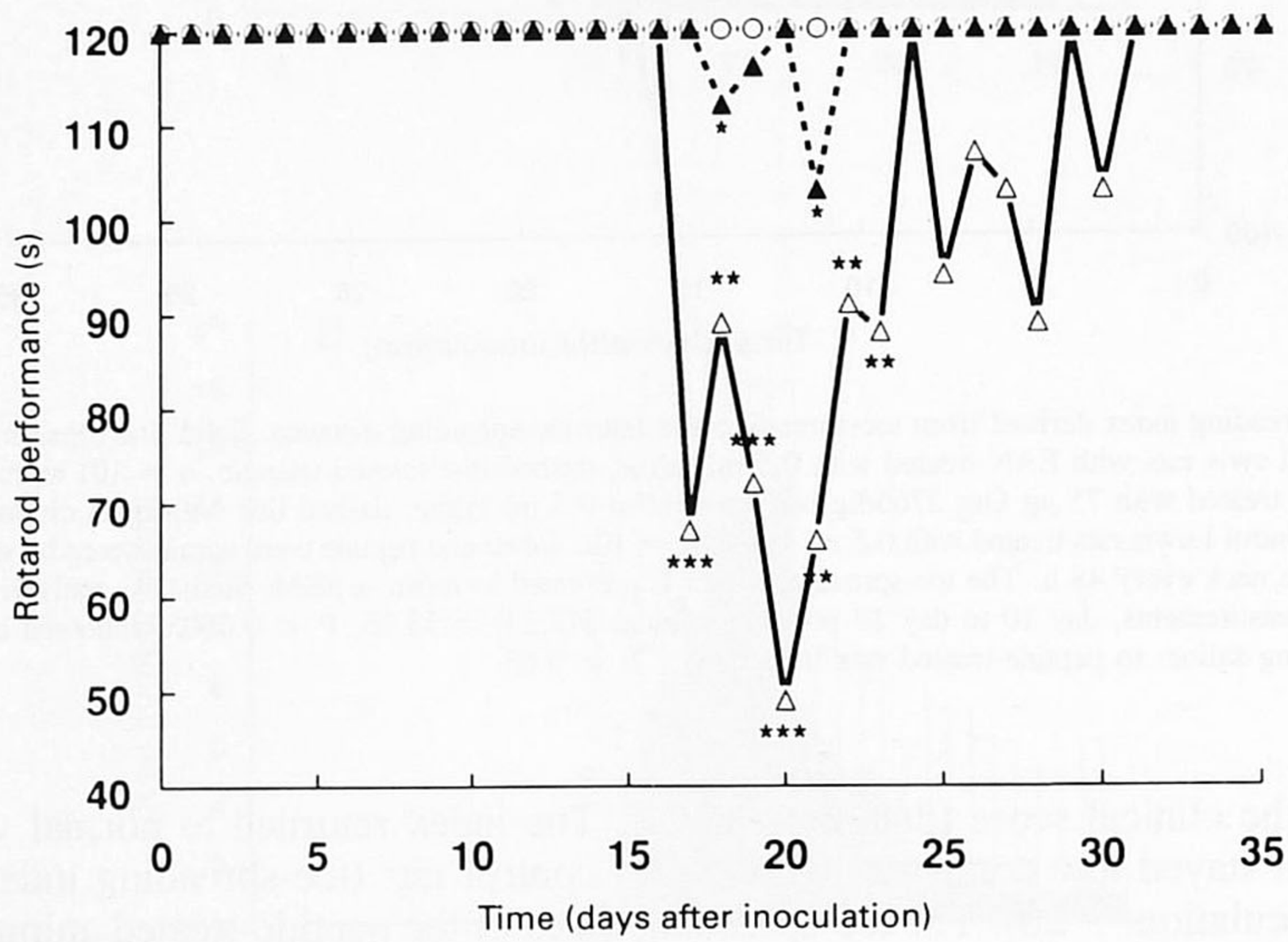

FIG. 4. Rotarod performance. Solid line (open triangle, $n = 19$) represents Lewis rats with EAN treated with 0.5 ml saline; dashed line (closed triangle, $n = 19$) represents Lewis rats with EAN treated with 75 μg Org 2766/kg body weight in 0.5 ml saline; dashed line with open circles represents age-matched, control Lewis rats treated with 0.5 ml saline ($n = 14$). Saline and peptide were administered by subcutaneous injections in the neck every 48 h. Rotarod performance scores are presented as medians. Statistics: Kruskal−Wallis test followed by Mann−Whitney *U* tests: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the age-matched control rats.

significantly over days 17−23 post inoculation (7 days) compared with the scores of the age-matched control rats. On day 31 post inoculation (17 days after the first manifestation of clinical symptoms) the rats of the EAN saline-treated group were again able to stay on the rotating rod for the maximal period of 2 min. Peptide treatment significantly improved the rotarod performance of rats with EAN (Fig. 4; median time spent on rotarod, day 20 post inoculation: EAN saline-treated rats 49 s, EAN peptide-treated rats 120 s). These peptide-treated animals had a decreased rotarod performance for only 2 days (day 18 and day 21 post inoculation) compared with the age-matched control group. Thus, under conditions that require maximal motor function, treatment with the neurotrophic

ACTH(4–9) analogue, Org 2766, greatly improved rats suffering from EAN. The ACTH(4–9) analogue not only protected against loss of motor function but also reduced the period of impaired motor performance.

Histology

On day 24 post inoculation (10 days after exacerbation), 10 rats of each treatment group were chosen at random and killed for histological analysis. At the end of the experiment when treatment codes were broken, the animals chosen for histology appeared to be representative groups and can be considered as a representative image of the status of the animals at day 24 post inoculation. An example of a transverse myelin-stained tissue section of a sural nerve of an EAN Lewis rat is shown in Fig. 5. In most of the sections of the sural nerve we have observed two fascicles (19 nerve sections). The remaining nerve sections consisted of either one fascicle (two nerve sections), three fascicles (five nerve sections) or four fascicles (three nerve sections). Histological assessment confirmed the beneficial effect of the ACTH(4–9) analogue in the EAN model. Saline-treated EAN animals had significantly fewer myelinated fibres (798 ± 67) in cross-sections of the sural nerve (Fig. 6) than age-matched controls (1019 ± 10). Org 2766 treatment normalized the number of myelinated fibres in the sural nerve. The total number of fibres in sections from EAN peptide-treated rats (1000 ± 30) was significantly higher than in saline-treated EAN rats and did not differ from that of age-matched controls [analysis of variance (ANOVA) $F(2,27) = 7.750$, $P = 0.002$; followed by Student's t test (EAN+saline versus EAN+peptide): $P = 0.016$; (EAN+peptide versus control): $P = 0.537$]. Four areas per section were chosen at random and examined by computer-assisted image analysis. As seen from the perimeter distribution (Fig. 7), EAN resulted in a marked loss of small and intermediate size fibres, a loss that was reversed by treatment with the ACTH(4–9) analogue [perimeter distribution statistics: ANOVA area under the curve (0–12 μm), all treatment groups: $F(2,27) = 10.023$, $P = 0.001$ followed by a supplementary t test (EAN+saline versus EAN+peptide) $P = 0.015$, (EAN+peptide versus control) $P = 0.287$, (EAN+saline versus control) $P < 0.0005$].

These beneficial effects of peptide treatment on functional and histological aspects of EAN were observed in three independent experiments.

DISCUSSION

In the present experiments we have shown that Org 2766, an ACTH(4–9) analogue devoid of corticotrophic and melanotrophic activity, suppressed the neurological symptoms and improved functional and histological parameters of EAN, a peripheral demyelinating neuropathy. The overall condition of the animals was assessed by clinical scoring and measuring body weight. Motor performance was tested by means of rotarod performance and walking pattern analysis. The functional tests employed in this study provide reliable information about qualitative and quantitative aspects of the function of the affected peripheral nerve. Rotarod performance requires the coordinated use of muscle groups and the ability to retain balance. Muscle weakness, muscle atrophy and ataxia can contribute to an impaired rotarod performance. Rotarod performance improved significantly in the ACTH(4–9) analogue-treated animals during the initial stages of the disease. During the recovery period, the difference in rotarod performance between the groups did not

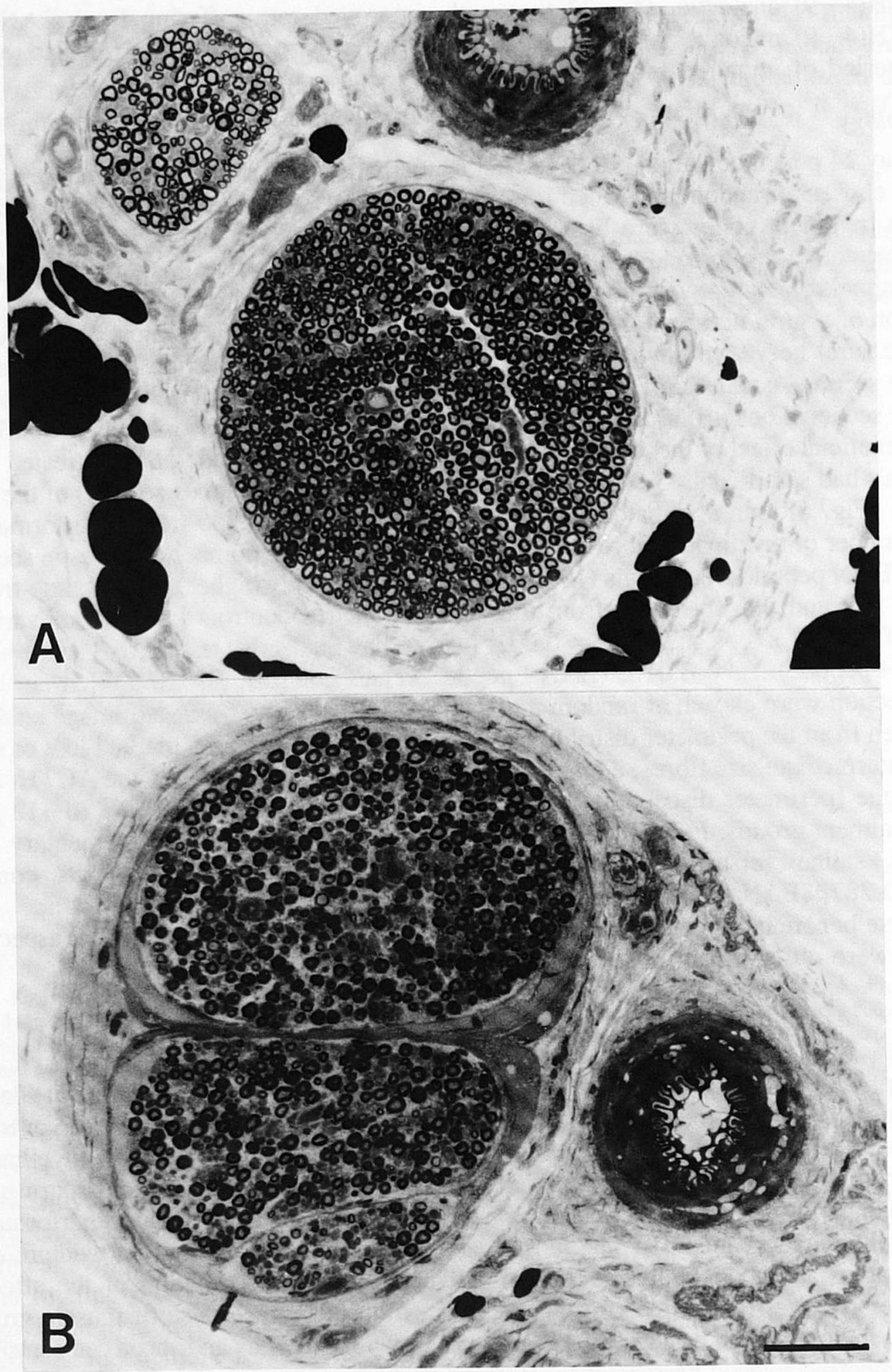

FIG. 5. Transverse sections of myelin-stained sural nerves. A, example of a transverse sural nerve section derived from an age-matched control rat at day 24 post inoculation. B, example of a transverse sural nerve section derived from a Lewis rat with severe EAN on day 24 post inoculation. The animal was treated with saline during the experiment. Bar represents 50 μm.

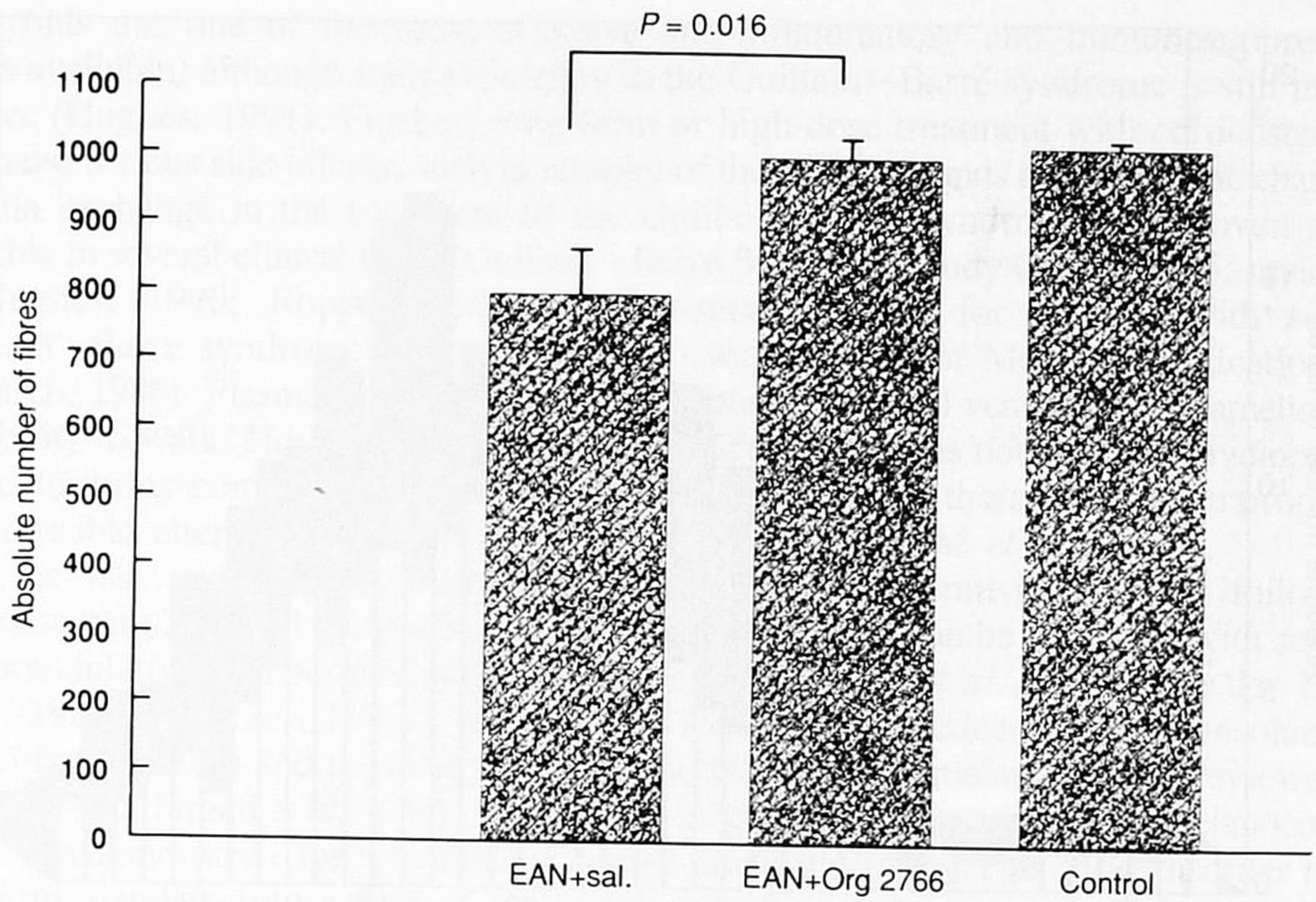

FIG. 6. Number of myelinated fibres in the sural nerve. On day 24 post inoculation (10 days after exacerbation), 10 rats from each treatment group were chosen at random and sacrificed for histological analysis. Cross–sections of the sural nerve were stained for myelin, photographed, and myelinated fibres were counted. The y-axis represent the absolute number of myelinated fibres; the x-axis represents the specific treatment groups. Statistics: analysis of variance (ANOVA) $F(2,27) = 7.750$, $P = 0.002$; followed by Student's t test (EAN+saline versus EAN+peptide): $P = 0.016$; (EAN+peptide versus control): $P = 0.537$.

change, suggesting that the peptide exerts its action during the first days of impaired rotarod performance, but has a long-lasting beneficial effect on nerve function. This corresponds to previous observations which have emphasized the importance of administering ACTH/α-melanocyte-stimulating hormone (MSH)-like peptides during the first 8 days of the recovery period following lesion of the sciatic nerve (Edwards *et al.*, 1984).

As second motor parameter in our experiments, we analysed the walking pattern, which provided detailed qualitative and quantitative information about the function of the afflicted nerve. Our results for the changes in walking pattern in rats suffering from EAN are similar to those reported by Wiethölter *et al.* (1990). The toe spreading of EAN rats was impaired, whereas print length, stride width and outward rotation were impaired only at a later stage. As found by Wiethölter *et al.*, the changes in walking pattern correlated closely with the progression of clinical impairment. The ACTH(4–9) analogue administration significantly enhanced the toe-spreading index during the development of EAN. Thus, for both aspects of motor function, i.e. performance on a rotating rod and analysis of toe spreading, peptide treatment provided a marked protection against EAN-related deficits in motor function. The beneficial effects of the ACTH(4–9) analogue on these parameters stresses the fact that enhanced recovery in this inflammatory demyelinating disorder is of functional significance. Furthermore, the peptide had a beneficial effect on weight loss during exacerbation of EAN. Whether this was the result

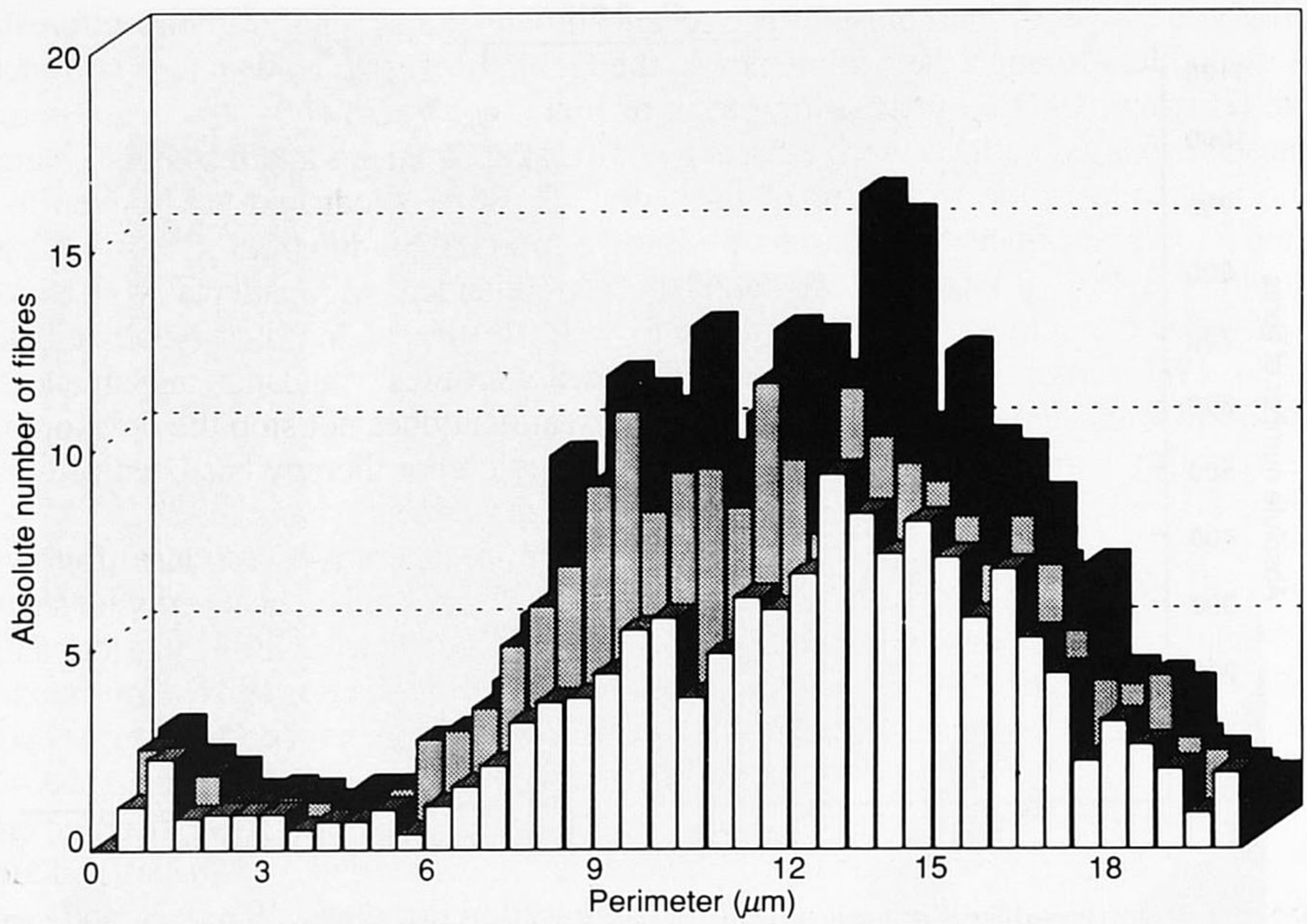

FIG. 7. Perimeter distributions of myelinated fibres in the sural nerve. The sural nerves were removed and fixed in glutaraldehyde. Examination of the sural nerve sections (1 μm, 3 cm distal to the sciatic nerve) was carried out by computer-assisted image analysis (TIM system, DIFA, Breda, NL) of four areas chosen at random (four fields of 35×50 μm) per section. The front histogram (white, $n = 10$) represents the group of rats with EAN treated with saline. The middle histogram (medium shading, $n = 10$) represents the group of rats with EAN treated with 75 μg ACTH(4−9) analogue/kg body weight in 0.5 ml saline. The back histogram (dark shading, $n = 9$) represents the group of age-matched control animals inoculated with 0.1 ml (PBS+CFA) emulsion and treated with saline. Statistics: ANOVA area under the curve (0−12 μm) all treatment groups: $F(2,27) = 10.023$, $P = 0.001$ followed by supplementary t test (EAN+saline versus EAN+peptide) $P = 0.015$, (EAN+peptide versus control) $P = 0.287$, (EAN+saline versus control) $P < 0.0005$.

of a direct effect of the peptide or an effect on the ability of the animal to obtain its daily intake of food and water remains unclear.

The allergic demyelination of EAN results in axonal degeneration as a concomitant effect of the fulminant reaction against myelin (Hartung *et al.*, 1988). The histomorphological assessment of the effect of the treatment with ACTH(4−9) analogue has provided a morphological basis for the improvement of functional parameters. The almost normal number of myelinated fibres in animals treated with the pepide may be due to either a protective action of the peptide or a facilitation of the formation of new nerve fibres. Since the myelinated fibres of the Org 2766-treated animals displayed a perimeter distribution comparable to the distribution of normal age-matched control rats and the peptide treatment resulted in a normal total number of myelinated fibres per sural nerve, it is feasible that the peptide protects the nerve against the immunological challenge.

Several therapeutic approaches have been used against autoimmune conditions like the Guillain−Barré syndrome or multiple sclerosis. Most of these treatments modulate the immune response by using immunosuppressive and anti-inflammatory drugs. Corti-

costeroids are one of the most effective anti-inflammatory and immunosuppressive drugs available, although their efficiency in the Guillain–Barré syndrome is still inconclusive (Hughes, 1991). Further, long-term or high-dose treatment with corticosteroids can cause serious side-effects, such as atrophy of the adrenal glands and metabolic changes. Plasma exchange in the treatment of the Guillain–Barré syndrome has proven to be valuable in several clinical trials (Guillain–Barré Syndrome Study Group, 1985, reviewed in Hughes, 1990; Ropper, 1992) and is recommended for patients with severe Guillain–Barré syndrome who are unable to walk (Office of Medical Applications of Research, 1986). Plasma exchange shortens the time of artificial ventilation and ameliorates the ability to walk. However, plasma exchange treatment does not stop the development of the disability completely. Most recently, immunoglobulin therapy has been proposed as a possible alternative to plasma exchange (van der Meché *et al.*, 1992).

In the last years it has become clear that the regenerative response following experimentally induced peripheral nerve damage in rodents can be enhanced with several neuromodulative compounds including nimodipine (Kater *et al.*, 1988; van der Hoop *et al.*, 1989; Bär *et al.*, 1990*a*), gangliosides (reviewed in Ledeen, 1984; Consolazione and Toffano, 1988) and peptides related to ACTH/α-MSH (melanocortins, reviewed in Gispen, 1990; Strand *et al.*, 1991). The beneficial effects of the neurotrophic melanocortins have been demonstrated in a number of animal models for peripheral nerve disorder ranging from a simple transection of the peripheral nerve to streptozocin-diabetic induced neuropathy and cisplatin-induced neuropathy (reviewed in Bär *et al.*, 1990*b*; Strand *et al.*, 1991). In these animal models, melanocortins either stimulated regenerative sprouting or prevented axonal loss. However, in experimentally induced allergic neuritis, the nerve is subjected to primary demyelination with concomitant axonal loss (Madrid and Wisniewski, 1977; Hahn *et al.*, 1988) rather than secondary demyelination as seen in the Wallerian degeneration occurring in the crush model and in the above-mentioned neuropathies. It is thus important to evaluate the neurotrophic action of these melanocortins in the experimental allergic neuritis model.

Given the observed beneficial effects in the present experiments, the question arises whether the ACTH(4–9) analogue, Org 2766, exerts its neurotrophic action by having a direct anti-inflammatory effect on the immune system, by affecting the damaged Schwann cell in the nerve or by exerting a direct effect on the neurons of the afflicted nerve. As the ACTH(4–9) analogue is devoid of corticotrophic action (de Wied and Jolles, 1982), the explanation of the present results by a possible anti-inflammatory mechanism seems unlikely. α-MSH has been referred to as a crucial mediator in thermoregulation and as an antipyretic peptide (Murphy *et al.*, 1983). Furthermore, α-MSH inhibits histamine-induced increases in vasopermeability. The amino acid sequence 11–13 (lysine-proline-valine) is regarded as being essential to the anti-pyretic properties of the α-MSH peptide (Richards and Lipton, 1984). However, in the nerve crush model, α-MSH (11–13) inhibited rather than enhanced the functional recovery of the rat (Van der Zee *et al.*, 1991). Structure activity studies demonstrated that the neurotrophic activity of the peptide resides in the 4–10 region of the peptide (Eberle, 1988; Van der Zee *et al.*, 1991). Furthermore, a direct neurotrophic effect of peptides related to ACTH and MSH has been shown on primary sensory and motor neurons in culture (van der Neut *et al.*, 1988; Bär *et al.*, 1992). A direct trophic effect of these peptides on neurons is also suggested by the recent observation that peptide administration stimulates the expression of the

transcription factor *c*-fos in primary neuronal cultures (Hol *et al.*, 1992). Thus, we favour the view that the ACTH(4–9) analogue exerts a direct trophic effect on neurons during the development of EAN or protects the Schwann cells against the autoimmune response. It has been postulated that melanocortins exert their beneficial effects on nerve recovery by mimicking or potentiating an endogenous peptide originating from the precursor of ACTH, pro-opiomelanocortin (Edwards *et al.*, 1984; Carr and Haynes, 1988; Hughes and Smith, 1988; Plantinga *et al.*, 1992).

The present study demonstrates that a peptide with neurotrophic properties can suppress neurological symptoms and improve functional and histological parameters in EAN. These beneficial effects underscore the potential application of this peptide in the treatment of peripheral nerve disorders (Strand *et al.*, 1975) and may provide an alternative or a supplementary treatment to immune-based therapies. The ACTH(4–9) analogue exerts neurotrophic and neuroprotective actions, probably at the site of the affected nerve, whereas most current therapies for demyelinating diseases modulate the (auto)-immune response. An advantage of Org 2766 therapy is that, until now, no toxic side-effects have been recorded, even after long-term treatment (de Wied and Jolles, 1982). The current results merit further investigation of the effectiveness of the ACTH(4–9) analogue in EAN and in the Guillain–Barré syndrome.

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