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GANGLIOSIDES IMPROVE THE OUTCOME OF EXPERIMENTAL ALLERGIC NEURITIS (EAN)

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Abstract. The effect of gangliosides on the clinical expression of experimental allergic neuritis (EAN), a PNS counterpart of experimental allergic encephalomyelitis (EAE), was tested in Lewis rats sensitized with bovine intradural root myelin in complete Freund's adjuvant (CFA). A mixture of bovine brain gangliosides (GM_1, GD_{1a}, GD_{1b}, GT_{1b}) was injected intramuscularly at a daily dose of 20 mg per kg beginning 6 days post inoculation. The results show that GA treatment considerably reduces the incidence of mortality, delays its occurrence, and reduces the severity of clinical scores when either the whole population or survivors only are considered. Benefit from GA treatment appeared roughly proportional to disease severity. The neuronotrophic and anti-inflammatory effects possibly play a role in GA protecting effect.

Gangliosides (GA) are known to assert neuronotrophic effects both *in vitro* and *in vivo* (4, 9, 21, 22, 24, 28, 38, 39). They have been shown to facilitate protection and/or repair of the peripheral nervous system (PNS) as well as the central nervous system (CNS). This has been demonstrated in a large number of animal models, involving a variety of insults to PNS and CNS, such as mechanical, electrolytic and neurotoxic lesions (7, 8, 11, 16-18, 26, 27, 34, 35).

We have recently shown (23, 32) that an autoimmune affliction of the PNS, experimental allergic neuritis (EAN), also responded to GA treatment. This disease is characterized by invasion of inflammatory cells into the peripheral target tissues such as peripheral nerves, roots, and dorsal root ganglia. Neurological manifestations include paraparesis, tetraparesis and/or paraplegia (41). It is induced in susceptible species by sensitization with peripheral nerve homogenate, purified PNS myelin or the P_2 protein of myelin; all these antigens are administered with complete Freund's adjuvant (2). The underlying mechanisms are not well understood: although cell-mediated immune reactions appear to play a major role, humoral mechanisms have also been implicated (3, 41). EAN shares many features with the human polyneuropathy, Guillain-Barré Syndrome, and is therefore considered to be an experimental model of this disease (1, 37).

Because of many similarities in pathology (see 3), EAN is considered a PNS counterpart of EAE — experimental allergic encephalomyelitis, an immune-mediated, inflammatory disease of the CNS and an experimental analog of multiple sclerosis. It is this aspect of the studies of EAN that is pertinent to general discussion (see other articles, this volume) on various CNS insults and their repair.

In our studies we have used male inbred Lewis rats, sensitized with PNS myelin, isolated by a modification of the method of Norton and Poduslo (30). Coded purified ganglioside mixture (GA) from bovine containing GM_1 (21%), GD_{1a} (40%), GD_{1b} (16%) and GT_{1b} (19%) (Fidia Res. Lab.), in a daily dose of 20 mg/kg body weight or saline, was injected intramuscularly to two groups of animals, beginning six days post inoculation (dpi). This experiment has been described in a previous communication (23).

EAN-GA-injected animals showed marked reduction in mortality compared to saline-injected controls in most trials (23). It should be stressed that the percentage of mortality differed considerably from one experiment to another. The reason for these differences is not clear, but it is possibly related to the actual dosage of myelin used in sensitization (12, 23). Although we attempted to administer equivalent doses in each trial, the *de facto* level of antigen could have varied, according to the time and conditions of storage of the isolated myelin. In five of seven trials (when mortality was between 40-80%) mortality in the EAN-GA group was one half or less that of the EAN-saline group. In the remaining two trials there was little or no mortality in either group. We have applied various statistical treatments to all trials *in toto*: the Cochran — Mantel — Haenszel test for chi square distribution (20), the test based on the binomial distribution (10) and the mini-max Rubin

test, giving a reliable estimation of the expected value of mortality independently of the distribution (13). These all indicated a highly significant difference between EAN-saline and EAN-GA groups, pointing to a protective effect of GA against mortality in EAN.

GA-treatment not only reduced the incidence of mortality but also delayed it: while losses in the EAN-saline group began as early as 12 dpi and continued to 23 dpi, reaching a peak around 17-21 dpi, mortality in the EAN-GA group occurred in a narrower time frame of 16-19 dpi (23). The effect of GA-treatment on disease index was also evaluated this being done in a blind manner according to the following scale: grade 1, ruffled fur, flaccid tail and loss of weight; grade 2, abnormal gait with concomitant loss of whisker movement; grade 3, moderate paraparesis; grade 4, paraplegia; grade 5, paraplegia with some involvement of the forelimbs and respiratory distress; grade 6, death. Following death an animal was given a score of 6 on all subsequent days (23). Comparison of disease indices in individual experiments (consisting of 10 to 15 rats in each saline and GA group) by analizing the area under the curves (AUC) for all rats revealed that in five of the seven experiments, GA-treated animals had significantly lower scores than saline-treated ones. Again, as with mortality, average disease indices varied from one experiment to another, and may similarly depend on the "state" and amount of antigen used. In most trials there was a correlation between GA benefit to mortality and to general clinical severity.

We have become further interested also in GA effects upon the severity of the clinical index when survivors only were considered: below a detailed analysis of such effects is described. Since in most trials, as reported before (23), the high mortality rate considerably diminished the initial number of rats, especially in the unprotected EAN-saline groups, special analyses were applied allowing us to evaluate all results *in toto*.

The time course of mean clinical scores (according to the same evaluation scale as before) of surviving rats is shown in Fig. 1. It can be seen that in some instances the "saline" points lie higher than the "GA" ones while in other cases the opposite is true. However, even a superficial inspection shows that the former situation, i.e. "saline" points lying higher, is much more frequent — than the latter, suggesting that GA treatment is more efficient than saline treatment, also in the population of survivors. The sign test (6) was used to confirm or disprove this suggestion. We combined the data gathered from all seven experiments, and after estimating the error of the difference between the two experimental means, based on grading accuracy and the average sample size and assigning proper signs (plus, minus or zero in case of tie) we were

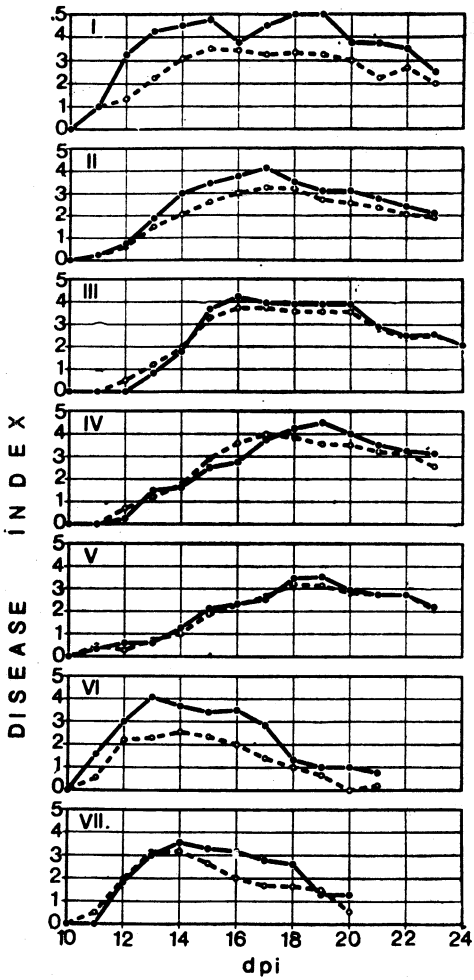

Fig. 1. Time course of mean clinical scores (disease index) in EAN-saline (solid line) and EAN-GA (broken line) surviving rats. Number of rats: EAN-saline: Exp. I — 2; II — 8; III — 9; IV — 4; V — 15; VI — 6; VII — 9; EAN-GA: Exp. I — 6; II — 10; III — 9; IV — 10; V — 15; VI — 10; VII — 11.

able to reject the hypothesis that both treatments were equally efficatious at $p < 0.05$.

A further proof of beneficial effect of GA treatment on the clinical scores in survivors was obtained by analysis of combined areas under curve (AUC) for all surviving treated rats in each group. Since the experiments in which the clinical scores were measured varied in length (see Fig. 1), for combined analysis we have standarized the time and considered only the measurements from dpi 10 to dpi 20.

Table I shows the results of individual experiments and combined data, together with the appropriate 95% confidence intervals. Table II shows the results obtained for all GA and saline-treated survivors as analyzed through two-way ANOVA. The two criteria of classification

TABLE I

Effect of ganglioside treatment on disease index in EAN surviving rats

Experiment	EAN-GA			EAN-saline			Mean	Pooled	95% confidence interval for Δ	
	n	Mean AUC	SD	n	Mean AUC	SD	Δ (SA-GA)	SD		
I	6	25.92	5.48	2	37.88	0.18	11.96	5.00	1.96	21.96
II	10	20.38	7.55	8	25.28	8.29	4.90	7.88	—3.02	12.83
III	9	23.28	3.02	9	24.17	2.09	0.89	2.60	—1.71	3.49
IV	10	23.30	3.15	4	23.12	1.64	—0.18	2.85	—3.85	3.50
V	15	17.00	4.53	15	18.27	5.82	1.27	5.21	—2.63	5.17
VI	10	15.00	5.12	6	24.92	4.82	9.92	5.01	4.37	15.47
VII	11	18.50	5.27	9	22.14	2.87	3.64	4.37	—0.49	7.76
Combined	71	19.86	5.92	53	22.84	6.26	2.98	6.07	0.80	5.16

AUC — Area under the curve estimated for the time interval from dpi 10 to dpi 20

TABLE II

Two-way analysis of variance on AUC in surviving rats

Source	*DF*	Sum of squares	*F* Value	*P*
Experiment	6	1311.55	8.57	0.0001
Treatment	1	508.16	19.92	0.0001
Exp × treatment	6	376.53	2.46	0.03
Error	110	2806.57		

AUC, see explanation in Table I.

were treatment and experiment plus interaction, termed experiment × treatment (Table II). The ANOVA analysis shows that both criteria proved significant. Although the effect varied through the experiments (Table I), the differences were in magnitude (except Exp. IV) and not in the direction, and thus the interaction experiment × treatment does not change the meaning of the results. Although, again, this inference does not give an insight into the characteristics of single experiments, it does show that generally GA treatment attenuates the severity of clinical expression in EAN surviving rats.

A special adjusting method based on symmetric moving average (31) which allows smoothing the irregular shape of experimental curves was further applied for a comparison of the maxima of the curves in GA-treated and saline-treated surviving animals in individual series of experiments. Briefly, the crude mean scores in the region of highest scores for each group in each experimental series were adjusted by taking

into account the crude mean of the scores on a given day postinoculation, 3 crude means preceding that day and 3 crude means following that day. Having found the maximum value for each curve and after estimating its standard error, we checked the significance of the difference between GA- and saline-treated rats in individual series of experiments. It was found that in five of seven experiments (except Exp. III and V, see Fig. 1) GA treatment resulted in a significant attenuation of the clinical maximum of the disease.

Similarly, as described for the whole "saline" and "GA" populations (23), there is also a correlation between GA clinical benefit and disease severity for survivors. The same appears to be true for other pathological signs of the disease in survivors: an apparent effect of GA on histopathology was suggested in preliminary studies where mortality and clinical scores were strongly expressed. This assumption was further supported by the results of a preliminary series of biochemical experiments (32), indicating that GA treatment led to protection against the decline of neuronal markers in EAN target tissues only when the disease was severely expressed.

Surviving rats, similarly to those which eventually died as reported before (23), showed rapid weight loss, but GA administration had no beneficial effect in terms of weight loss in either group independently of the fate of animals. This was similar to weight loss in diabetic animals, even though these responded positively in other ways to GA treatment (5, 14, 42). It is believed that immunological reactions may also contribute to development of pathological changes in diabetes (42).

The mechanism by which gangliosides exert their beneficial effect on the outcome of EAN rats is not clear. It is possible that, at least partly, the protective GA effect in EAN can be ascribed to the well-known neuronotrophic effect of gangliosides (4, 9, 21, 22, 24, 28, 38, 39). It has to be noted here that, although EAN is generally considered a model of inflammatory demyelination, neuronal/axonal damage is now recognized as a concomitant feature (12, 19, 25). This is supported by our preliminary biochemical investigations, as stated above (32).

An anti-inflammatory effect of other therapeutic treatments, e.g. silica quartz dust (40), was indicated as playing an important role in protection against EAN pathology. It is of some interest too that protection by GA against brain edema has been reported (16, 18, 27). As suggested by our preliminary biochemical and histopathological data, protection by GA against inflammation and/or edema may also take place in our conditions.

Several approaches to the treatment of EAN involve immunosuppression of immunological responses (3, 15, 29). However, no immunosuppres-

sive effect of GA on humoral immunity was observed in this disease, at least at the stage of clinical recovery when biochemical and histological deficits were still present (33). This accords with the recent observations of Presti et al. (36), who reported the lack of both humoral and cellular immunosuppression by GA *in vivo*. Further experimentation is needed in order to clarify the mechanism of beneficial action of GA upon the pathological changes in EAN.

In conclusion, GA treatment proved beneficial in recovery from yet another nervous system insult, i.e. an autoimmune condition.

Whether GA treatment would prove similarly beneficial in other autoimmune conditions, such as EAE, remains to be determined.

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