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Linoleic acid therapy in severe experimental allergic encephalomyelitis in the guinea-pig: suppression by continuous treatment

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SUMMARY

The effect of oral linoleic acid (LA) treatment on experimental allergic encephalomyelitis (EAE) in guinea-pigs in three trials of differing disease intensity has been investigated. The efficacy of LA treatment was linked to the severity of the disease being suppressed. The trial with the greatest disease severity showed no beneficial effect. The other two trials with less severe disease showed a marked therapeutic response to LA, but only when treatment was started before immunization and given continuously. This was apparent in both clinical and histopathological responses. These results support an immunoregulatory mechanism for LA treatment in EAE and by analogy in multiple sclerosis.

INTRODUCTION

Hypotheses connecting the irregular geographical incidence of multiple sclerosis (MS) with varying patterns of fat intake, especially diets deficient in polyunsaturated fatty acids (PUFA), supported by evidence of altered linoleic acid (LA) content in brain, serum and blood cells in this disease (reviewed by Mertin & Meade, 1977) prompted Millar *et al.* (1973) to test the therapeutic value of oral LA. Their double-blind trial of dietary supplementation with sunflower-seed oil, rich in LA, resulted in fewer, shorter and less severe relapses, though the overall rate of clinical deterioration was not affected.

The pathological mechanisms responsible for the clinical symptoms of MS are not understood, but a state of autoimmunity to antigens within the central nervous system (CNS) is implicated (reviewed by Knight, 1977). Research into MS has leaned heavily on experimental allergic encephalomyelitis (EAE), an animal model: a T cell-dependent cell-mediated autoimmune disease of the CNS induced by immunization with myelin basic protein (MBP) containing CNS material usually in Freund's complete adjuvant (Caspary, 1978). Although not a true model of MS, the immune-mediated damage in EAE provides a useful parallel (Caspary, 1979). The influence of dietary LA on EAE has been investigated by Clausen & Møller (1969), who showed that a diet deficient from birth in essential fatty acids (EFA) increased the severity of EAE in rats. This result was confirmed by Selivonchick & Johnston (1975), who in addition found that this dietary effect was partially corrected by LA supplementation.

We have therefore tested under double-blind conditions the *in vivo* effect of oral LA medication on the clinical signs and histopathology of EAE in the guinea-pig. Several treatment schedules were

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used with LA being given at different times in relation to immunization and development of the disease. The immunization was arranged to produce disease with severe neurological signs in contrast to a separate study recently published by one of us (J.M.) in which LA modulation of EAE with minimal clinical disease was reported (Meade *et al.*, 1978). Our experiments showed that a clear reduction in both the clinical and histological course of EAE was produced by oral LA, but only when given continuously from well before the clinical episode. This is in accord with a study by Simon, Contag & Pöllinger (1979), published after the present work was completed, in which substantial inhibition of clinical and histological signs followed intravenous administration of polyunsaturated phospholipids with a disease incidence intermediate between that reported by Meade *et al.* (1978) and the present study.

MATERIALS AND METHODS

Three separate trials of the effect of oral linoleic acid on EAE were done. The first was 'open' while the second and third were 'blind', in that assessment of the disease was made without knowledge of the treatment and previous history.

First trial

Animals. Five groups of six random-bred Hartley guinea-pigs of equivalent weight and sex distribution were selected. The average weight within each group varied between 390–430 g (range 310–610 g). They were fed on sow pellets (Oxoid diet 18) supplemented with cabbage three times a week and water *ad libitum*.

Immunization. Animals were immunized in the dorsum of the right hind-foot by a single intracutaneous injection of 0·1 ml containing 40 μg of human myelin basic protein (MBP) (Caspary & Field, 1965) emulsified in Freund's complete adjuvant (FCA) (DIFCO—*M. tuberculosis* H37Ra). The emulsion contained 2 volumes of FCA to 1 volume of MBP in normal saline.

Treatment. Linoleic acid (cis-9-cis-12-octadecadienoic acid; Sigma Chemical Company; grade II—90–95% pure) was given orally with a tuberculin syringe, 0·3 ml per day according to the schedule (Fig. 1). Daily dose was equivalent to 0·25 g pure linoleic acid per day or 0·6 g/kg (based on average weight). Control animals were given the same volume of normal saline for the longest treatment period only, i.e. −7+21 (Fig. 1) to allow for the maximum stress effects of daily oral administration.

Disease assessment. All guinea-pigs were weighed and examined daily. Clinical signs of EAE were recorded and later converted to a 10-point scoring system for statistical analysis: score 0, no disease; score 1, quiet and sensitive to handling, weight static or slight loss; score 2, hypotonia, especially in hind limbs and abdomen, animal trembling or shaking, some ataxia, some hind limb paraparesis, weight loss may be appreciable; score 3, ataxia, paraparesis or pronounced hypotonia, animal has hind limb weakness but able to walk though tending to fall to one side, weight loss significant; score 4, paraparesis, slight dragging of hind limbs often to one side as animal moves on front limbs, hind limb movement possible following strong stimulation, weight loss marked; score 5, as score 4 with incontinence; score 6, pronounced dragging of hind limbs which show no movement even after strong stimulation, considerable weight loss; score 7, as score 6 but with incontinence and more rarely faecal impaction; score 8, complete paralysis of hind limbs and some involvement of fore-limbs, unable to eat or drink; score 9, as score 8 but with incontinence; score 10, death due to EAE.

The trial was terminated at 21 days and the animals killed and brain and spinal cord fixed in 10% formaldehyde. The extent and severity of perivascular infiltrates were assessed on haematoxylin and eosin sections and scored on an arbitrary 3-point scale. Results were expressed as total CNS score, i.e. sum of scores for all areas: frontal, parietal, temporal and mid-brain, pons, cerebellum and medulla as well as cervical, thoracic and lumbar spinal cord.

Second trial

Linoleic acid or control medication was administered in the form of coded emulsions, and weight and clinical assessments were entered on a daily pro forma without access to previous records.

Animals. Four groups of nine random-bred Hartley guinea-pigs were used: five males and four females in each. Average weight was 446 g (range 375–520 g).

Treatment. Two emulsions were prepared using the 'dry gum' method.

(a) *Emulsion A* (containing 50% linoleic acid). Primary emulsion: linoleic acid (Sigma grade II) 4 parts (230 ml); water 2 parts (115 ml); gum acacia 1 part (57·5 g); made up to a final volume of 460 ml.

(b) *Emulsion B* (containing 15% olive oil). Primary emulsion: olive oil 4 parts (75 ml); water 2 parts (37·5 ml); gum acacia 1 part (18·75 g); made up to a final volume of 500 ml.

An equal quantity (0·6 ml) of either emulsion A or B was given as previously (equivalent to 0·28 g pure linoleic acid or 0·08 g olive oil per animal). All guinea-pigs were given emulsion B (olive oil) every day with substitution of emulsion A (linoleic acid) according to the schedule (Fig. 1).

Disease assessment. Guinea-pigs surviving at 21 days were killed and examined as before.

Third trial

In the third trial while the clinical assessment was 'blind' treatment was 'open' as the previous trial had shown it was possible to identify the coded medication. Daily pro formas recording clinical scores were completed before treatment and without access to previous clinical history.

Animals. Four groups of six male random-bred Hartley guinea-pigs were used. Average weight 413 g (range 375–495 g).

Treatment. Guinea-pigs were treated as the first trial: experimental animals receiving 0·3 ml linoleic acid orally and control animals 0·3 ml normal saline, according to the schedule (Fig. 1).

Clinical and histological assessment. As for second trial.

Statistical analysis. All clinical and histopathological scores are ranks rather than measured values and are expressed as means for practical convenience only. However, as all the data are non-parametric, it has been analysed for statistical significance using the Mann–Whitney *U*-test and Fisher's exact probability test.

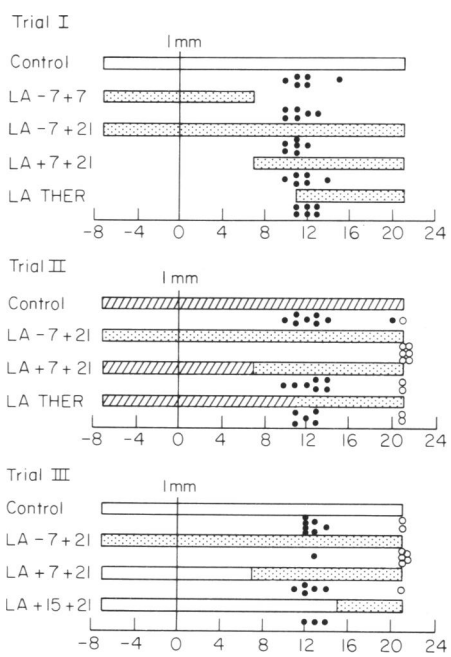

Fig. 1. Treatment schedules for linoleic acid (LA) therapy (THER) of EAE also showing day of onset (clinical score 1) for individual animals. Time given in days before and after immunization (Imm). (□) Saline, (▨) oleic acid, (▩) linoleic acid, (●) onset of EAE in individual guinea-pigs, (○) guinea-pigs that never developed EAE.

RESULTS

Clinical assessment

First trial

The results of the first trial are summarized in Fig. 2. Clinical EAE was seen in all animals with uniform time of onset of definite signs (Fig. 1) (range 10·8–12·0 days). The control group (Sal −7+21) had 2/6 spontaneous deaths and a mean peak clinical score of 6·5. The treatment with linoleic acid from 7 days before to 7 days after immunization (−7+7) gave no significant change. Continuous treatment until 21 days after immunization (−7+21) gave greater severity with a score of 8·3, but this was not statistically significant. The lowest death rate (1/6) and lowest mean peak clinical score (5·0) was seen in the group treated therapeutically, i.e. treatment started when the early signs of clinical EAE first became apparent (score 1, at 10–11 days). In marked contrast LA treatment started 3–4 days earlier (+7+21) not only produced the highest death rate (5/6), but also the highest clinical score of 9·8, significantly higher ($P=0·026$) than the group treated therapeutically. There was no difference between the groups in the extent of recovery from the acute phase of the disease.

Changes in weight tended to reflect the general health of the animals. Increases of 10–30 g a day were initially recorded falling to about 5 g or becoming static with the first clinical signs. Definite clinical signs (weakness or paralysis) were associated with weight losses of up to 60 g/day, but these did not correlate with the disability of individual animals. Clinical symptoms improved from 14–17 days after immunization usually with concomitant weight gains even though clinical scores were still high, e.g. gains of 25–35 g a day in animals with paralysis (score 5–7).

In summary, LA given therapeutically at first clinical signs gave some suppression, but enhanced the disease when administered from before immunization and continuously through the disease period (Fig. 1).

Second trial

The results of the second trial are summarized in Fig. 2. The severity of EAE in the control group (mean 5·7) was milder than in the first trial, with the mean time of onset being delayed slightly (13·0 days) (Fig. 1) though the mortality was the same. In contrast to the first trial the prolonged LA

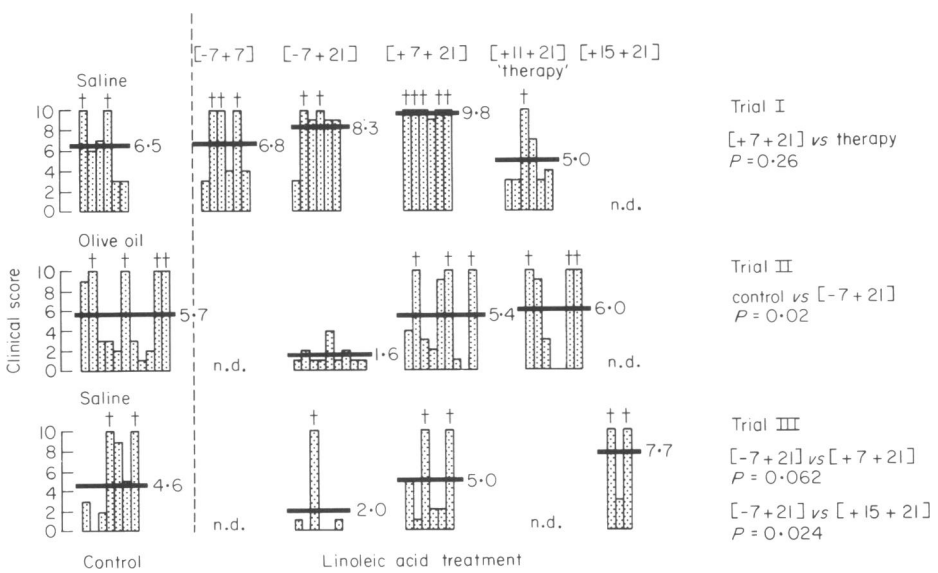

Fig. 2. Results of effect of linoleic acid treatment on clinical assessment of EAE. Statistical significance was calculated using the Mann-Whitney *U*-Test (two-tailed). (†) Indicates spontaneous death, n.d. = not done.

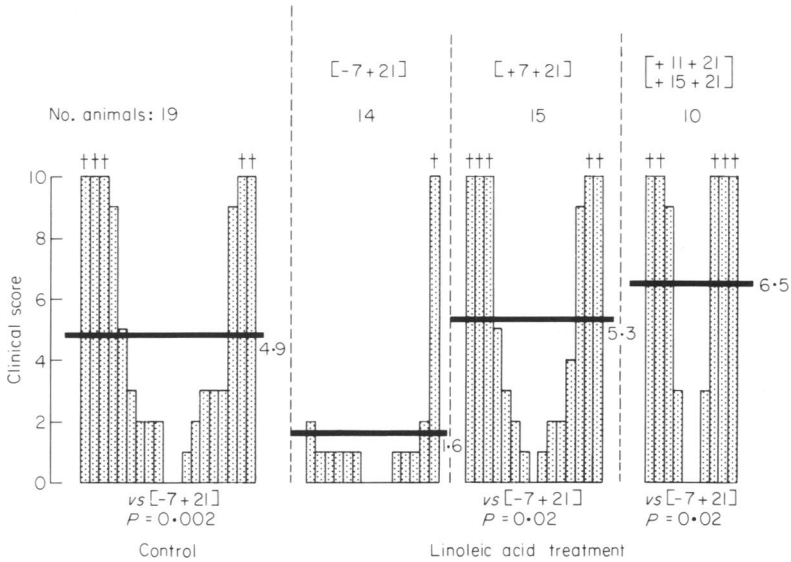

Fig. 3. Combined results of trials II and III. Effects of linoleic acid treatment on clinical assessment of EAE. Statistical significance was calculated using the Mann-Whitney *U*-test (two-tailed). (†) Indicates spontaneous death.

treatment (−7+21) gave a striking reduction in both mortality and clinical score. The death rate (0%) in this group is significantly lower than the 36% and 43% in the control and +11+21 groups respectively, while the mean peak clinical score of 1·6 is significantly reduced ($P=0{\cdot}02$) from the control value of 5·7. The other two LA treated groups (+7+21) and (+11+21) were essentially similar to the control group in mean peak scores and mortality, but both groups included one or two animals free of clinical disease, compared to a 100% incidence in the controls. Once again weight change, while a reliable indicator of imminent clinical EAE, was not proportional to the severity of the disease. The prolonged treatment group also showed the most complete recovery with seven out of nine guinea-pigs returning to zero score at 21 days after immunization. In addition all animals showed a net weight gain, with the LA-treated animals gaining more than the controls.

Third trial

The results of the third trial are summarized in Figs 1 and 2. The average intensity of clinical EAE in the control group (4·6) was lower than that produced in the first and second trials with one animal remaining free of clinical signs, though showing a brief period of weight loss comparable to the affected animals. No group differed significantly from the control; however, in accord with the second trial, the prolonged treatment (−7+21) group showed a greatly reduced mean peak score (2·0) and also the lowest mortality. All animals in this group, apart from one that died, were either disease-free or only minimally affected. All surviving animals had made a complete recovery from clinical EAE by 21 days after immunization with the exception of a single control scored 1. This may be attributed to less severe initial EAE.

Second and third trials combined

As the second and third trials were in close agreement, their results were combined to allow statistical analysis of larger groups (Fig. 3). The severity of the disease was significantly reduced ($P<0{\cdot}002$) only in the longest treatment group (−7+21 days): 4·9 to 1·6. The other two LA shorter treatments failed to show any significant effect, with however a tendency to increased severity.

Effect of linoleic acid on incidence of EAE

An alternative way of examining the effect of LA on EAE is to look at incidence (Fig. 1) under the

Table. 1. Distribution of clinical severity of EAE according to linoleic acid treatment: combined results of trials II and III

Treatment schedule	No. of animals in group	Percentage of animals with clinical scores above:									
		1	2	3	4	5	6	7	8	9	10
Control	19	90	84	63	42	42	37	37	37	32	32
−7+21	14	79	21*	7†	7‡	7‡	7	7	7	7	7
+7+21	15	93	80	60	53	47	40	40	40	33	33
{+11+21 / +15+21}	10	80	80	80	60	60	60	60	60	50	50

Statistical analysis (compared with control):
* $P=0{\cdot}001$. (χ^2 test with Yates' correction for continuity; two-tailed.)
† $P=0{\cdot}005$. (χ^2 test with Yates' correction as above.)
‡ $P=0{\cdot}06$. (Fisher's exact probability test; two-tailed.)

various treatment regimens. In Table 1 the proportions of guinea-pigs reaching a particular score in trials II and III are shown. A significant difference when compared against the control group emerges only with the continuous treatment (−7+21) group, where the incidence of the earliest neurological signs of ataxia (score 2) was reduced from 84 to 21% ($P<0{\cdot}001$) and score 3 from 63 to 7% ($P<0{\cdot}005$). The reduction of the incidence of paraparesis (scores 4 and 5) was just under statistical significance, being reduced from 42 to 7% ($P<0{\cdot}06$). The reduced incidence of the more severe disease (scores 6–10) was not statistically significant.

Histopathological assessment

If all the animals, irrespective of treatment, are considered together there is a reasonable relationship between clinical and histopathological scores. In the lower half of the clinical scale the degree of inflammation increases rapidly reaching its maximum at the mid-point of the clinical score.

Broadly speaking the histopathological changes associated with the various LA treatments are in agreement with the results already described for clinical signs. Trial I showed no statistically significant difference between the groups, although as with the clinical findings the group on continuous treatment (−7+21) gave the highest score. The results are distorted to some extent by the high mortality, which reduced the number of animals available for histological examination. Further, several of the animals exceeded the mid-point on the clinical scale above which there is only minimal increase in histological severity. In contrast the inflammatory responses in trials II and III were in good agreement with the clinical picture. In both, the longest LA treatment gave the least inflammatory changes with the difference being statistically significant from the control in the latter ($P=0{\cdot}03$). The groups in which LA treatment was delayed until onset of clinical disease showed the most severe changes.

DISCUSSION

Our results show a clear reduction in the severity of EAE by oral LA in two out of the three trials. Clinical and histological scores were depressed to one-third of the control values, but only when the animals were treated continuously and from well before the clinical episode. This supports the earlier work (Selivonchick & Johnston, 1975; Meade *et al.*, 1978) showing that dietary LA suppresses EAE and extends it to disease of greater clinical severity. Thus the present work may provide a better parallel to the acute clinical episode of multiple sclerosis.

The results of our second and third trials are in close agreement: both showing a marked beneficial effect. A statistical analysis of the combined results of these two trials shows a significant reduction of the disease ($P=0{\cdot}002$) when LA was given daily from 7 days before immunization until

21 days after, which includes the expected period of clinical symptoms. However, in contrast the shorter treatments slightly enhanced the disease. Pathological severity and neurological signs are in good agreement, with the prolonged treatment groups showing the least inflammatory activity ($P=0{\cdot}03$ in the third trial). In contrast the first trial showed no beneficial effect: the severity of the disease being the most intense of the three trials, and therefore perhaps less amenable to suppression.

Meade *et al.* (1978) recently reported a small protective effect of LA as shown by clinical score, weight loss and histological changes. However, this was in EAE of low severity: half of the animals remaining disease-free and the mean clinical score of the control group being approximately half that of the present study. The small beneficial effect of LA was on the borderline of significance, but oleic acid also appeared to be effective. Their LA treatment schedule ($+7+21$) was not effective in the present study. In a discussion of oleic acid as the usual control for LA in treatment of MS and suppression of EAE, they concluded that it was not inert and could be involved in LA metabolism. It has also been shown to depress lymphocyte function *in vitro* (Mertin & Hughes, 1975; Weyman *et al.*, 1975) and reticuloendothelial activity *in vivo* (Spratt & Kratzing, 1975). Therefore, oleic acid, if used as a control for LA, may reduce its apparent effect.

Three recent studies used more complex forms of PUFA treatment and are not strictly comparable to the present work. Mertin & Stackpoole (1978, 1979) showed that EAE severe enough to give 100% incidence in Lewis rats could be significantly reduced by daily doses ($+7+21$) of oil of evening primrose (Naudicelle), which contains gamma-linolenic acid in addition to LA. Gamma-linolenic acid is an intermediate product in the biosynthesis of arachidonic acid from LA and has been suggested to be more effective in the therapy of MS (Field & Joyce, 1978). Simon *et al.* (1979) produced a high degree of protection with EAE of moderate severity (75% incidence in controls) using intravenous polyunsaturated phospholipids (Lipostabil) composed of linolenic and oleic acids in addition to LA.

Earlier studies viewed the role of LA in EAE from the standpoint of dietary deficiency during myelin development. Clausen & Møller (1969) produced a very high incidence of EAE in Wistar rats deprived of essential fatty acids from birth, while animals of this susceptible strain fed a complete control diet were disease-free. However, the absence of EAE in this group may be related to the massive dose of encephalitogenic material (0·5 g of whole brain per rat being about 50 times that normally required) which could itself protect from the disease (unpublished observations) thus complicating any interpretation. A later study from Selivonchick & Johnston (1975) showed that Spraque–Dawley rats fed a fat-free diet gave a greater disease incidence and severity, and in addition that LA supplementation of this diet reduced the disease incidence, but not its severity to control levels. However, this strain of rats has relatively low EAE susceptibility, and additionally the animals were immunized when very young and consequently showed both low severity and incidence (about 50%) of disease.

EAE is generally considered to be the best available experimental model for multiple sclerosis while differing in many fundamentals (Maugh, 1977). Although the relapsing form of EAE (reviewed by Raine, 1976; Wisniewski & Keith, 1977) is a much closer parallel to the human disease, variability in occurrence and timing of relapses makes it very difficult to provide sufficient numbers of animals for a valid experimental design. The acute EAE adopted for this study is a monophasic condition and consequently the effect of LA on the relapse rate cannot be assessed. However, its effect on the severity of the single episode can be equated with any beneficial effect of LA on MS patients, where the exacerbation may be considered equivalent to the acute phase of EAE. Millar *et al.* (1973) found that over a 2-year period MS patients given LA tended to have less frequent relapses, which were significantly less severe and of shorter duration than patients given oleic acid as a control. Bates *et al.* (1977) showed that treatment with LA or evening primrose oil (Naudicelle) was without effect in patients with chronic progressive MS. However in relapsing-remitting disease treated with LA (Bates *et al.*, 1978) exacerbations were shorter and less severe, but the rate of clinical deterioration and frequency of attacks were unchanged. Oil of evening primrose had no effect, though the low dose used is in dispute (Hassam & Crawford, 1979; Bates *et al.*, 1979). Another trial with LA (Paty *et al.*, 1978) failed to demonstrate any therapeutic benefit, though 'control' patients given oleic acid showed a reduced relapse rate. The efficacy of PUFA treatment may be dose-depen-

dent. While the successful EAE studies have all employed daily doses of between 500–1,500 mg/kg the marginal effects in MS trials were obtained with much lower daily doses (245–330 mg/kg). Intravenous treatment of EAE with polyunsaturated phospholipids (Simon *et al.*, 1979) was effective at the very low dose of 50 mg/kg, suggesting that systemic is more efficient than oral administration. The more complex chemical nature of this preparation may also enable it to persist longer in the circulation and its therapeutic value should be assessed in MS.

These studies using PUFA in treating CNS disease have shown beneficial effects that are at best marginal in MS, but more definite in EAE. Three main theories have been advanced to explain the mechanism of LA therapy: two metabolic and the other immunological. The association of abnormal fat intake with MS led Swank (1950) to propose that the high prevalence of this disease in some geographical areas was due to high fat diet, an idea that was later extended by Sinclair (1956) to a more specific dietary deficiency in PUFA. An alternative metabolic explanation was advanced by Thompson (1966) who attributed the LA deficiency in MS tissues reported by some to an inborn error or metabolism. However, this LA deficit has been disputed and also reported to be not specific to MS (reviewed by Mertin & Meade, 1977). Mertin has suggested (Mertin, 1976) an alternative hypothesis of LA acting as an immunoregulator, probably as a precursor of prostaglandins which are known to interfere with immune responsiveness (Pelus & Strausser, 1977). LA has been shown to suppress *in vitro* lymphocyte activation to both mitogens and specific antigens using lymphocyte transformation, inositol incorporation and macrophage electrophoretic mobility (reviewed by Mertin & Meade, 1977). Prolongation by LA of skin allograft survival in rodents (reviewed by Hughes, Caspary & Wisniewski, 1975) and enhancement of short-term kidney graft survival in humans (McHugh *et al.*, 1977) have been reported. Inhibitors of prostaglandin synthesis, such as indomethacin (Vane, 1971) not only increase the severity of EAE (McIlhenny *et al.*, 1978; Davison, 1978) andMS (Niewodniczy & Pozniak-Patewicz, 1973; P. J. Rudge, personal communication) but also abrogate the beneficial effect of LA in EAE (Mertin & Stackpoole, 1978). The alternative explanation of LA exerting a beneficial therapeutic effect in MS by altering the composition of abnormal myelin (Field, 1979) or perhaps by stabilizing membranes previously susceptible to immune or other forms of damage (Field, Joyce & Smith, 1977) lacks direct evidence. The suggestion of prolonged prophylactic PUFA treatment of children deemed to be at risk of developing multiple sclerosis (Field, 1979) is based on speculation and, until supported by direct evidence, should be treated with reserve. On balance the facts favour an immunoregulatory mechanism for LA therapy.

The experimental findings in EAE, principally the high dose of LA required, selection of placebo and possible non-responsiveness of very severe disease should be borne in mind in the design of any further clinical trials of PUFA therapy in MS.

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