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# Influence of polyunsaturated fatty acids on survival of skin allografts and tumor incidence in mice

**(immunoregulation/linoleic acid/deficient diet/autoclaving)**

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**ABSTRACT** **Subcutaneous or oral administration of the polyunsaturated fatty acid linoleic acid prolongs survival of skin allografts in mice. Mice fed on a diet deficient in polyunsaturated fatty acids show a relative immunopotentiation, as indicated by accelerated skin allograft rejection and decreased incidence and rate of development of methylcholanthrene-induced tumors. These observations support the hypothesis that polyunsaturated fatty acids take part in immunoregulatory mechanisms.**

Certain polyunsaturated fatty acids (PUFA) such as linoleic (C18:2) and arachidonic acid (C20:4) are essential constituents of mammalian diet and are important structural components of the phospholipids of plasma membranes. Earlier results of *in vitro* and *in vivo* investigations led to the proposal of the hypothesis that such PUFA may take part in immunoregulatory mechanisms (1–3). Preliminary studies of the effects of subcutaneous (s.c.) injections of C18:2 and C20:4 have here been extended. Oral administration of C18:2 was studied too. A variety of strain combinations representing different strengths of the histocompatibility barrier—H-2 and H-3 differences and the H-Y system—has been examined.

Our conception of an immunoregulatory action of PUFA implies that decreased PUFA serum levels should potentiate cell-mediated immune (CMI) responses. To test this assumption we have also examined allograft survival in mice on PUFA-deficient diets, and the effect of such a diet on the development of methylcholanthrene-induced tumors.

## MATERIALS AND METHODS

**Animals.** Three- to 8-week-old CBA/CaCRC, A, C3H/He/mg/CRC, Balb/c, and C57BL/10 mice were obtained from the Clinical Research Centre, Animal Division.

**Linoleic Acid.** C18:2 (*cis*-9-*cis*-12-octadecadienoic acid), approximately 99% pure, was obtained from Sigma Chemical Co., St. Louis, Mo. It was injected subcutaneously (s.c.) under aseptic precautions into the hind leg region three times a week according to a schedule already known to prolong skin allograft survival (4, 1). Injections were carried out using a Hamilton repeating syringe and the mean dose was 10 μl/day for 11–16 days. Oral administration was carried out by feeding the animals (under light ether anesthesia) with the help of a soft plastic esophagus tube, the daily dose being 50 μl of C18:2. Treatment was started on the day of grafting.

Abbreviations: PUFA, polyunsaturated fatty acids; C18:1, oleic acid (having an 18-carbon chain and one double bond); C18:2, linoleic acid (PUFA, having an 18-carbon chain and two double bonds); C20:4, arachidonic acid (PUFA, having a 20-carbon chain and four double bonds); CMI, cell-mediated immunity; s.c., subcutaneous(ly); i.p., intraperitoneal(ly).

In a control experiment C18:2 effect on graft survival was compared with the effect of oleic acid (C18:1; *cis*-9-octadecenoic acid), approximately 99% pure (Sigma).

**Allografts.** A modification of the technique of Billingham and Medawar (5) was used for tail skin grafting. In some experiments a second graft was applied (second set) at a time when most if not all of the first grafts had been rejected (first set). First and second rejection were scored daily. Tumor cells were allografted by intraperitoneal (i.p.) injection of $3 \times 10^7$ washed EL-4 ascites tumor cells.

**Diets.** The animals in which the effects of s.c. or oral C18:2 administration were studied received the diet normally used in our Animal Division (Spratts Laboratory Diet 1, Spillers Ltd.) and water ad lib. A diet containing levels of essential PUFA normally required for rodents (control diet) and a diet of the same base but PUFA-deficient were supplied by Cooper Nutrition Products Ltd. (Stepfield, Witham, Essex, England). The fatty acid contents are shown in Table 1. Mice at weaning (about 3 weeks old) were given these diets for 4–6 weeks before the start of the experiments. PUFA double bonds can be destroyed easily by physical means. Both diets were autoclaved and their effects on skin allograft survival were compared with those of untreated diets. Autoclaving was carried out using a Drayton Castle pulsing high vacuum autoclave for 20 min at 15 pounds/inch$^2$ (43 kPa) above atmospheric pressures and 121°. To assure even treatment the pellets were spread over trays to a depth of 4 cm and the trays were racked 1 inch (2.5 cm) apart.

**Theta-Positive Spleen Cells.** The number of theta-posi-

Table 1. Fat analysis of control and PUFA-deficient diets*

| | Control, % | Deficient, % |
|---|---|---|
| Total oil (ether extract) | 4.0 (4.2) | 1.5 (1.7) |
| Fatty acids as % of total oil (measured by gas-liquid chromatography) | | |
| Myristic acid | 4.7 (4.7) | 3.5 (3.5) |
| Palmitric acid | 9.0 (8.7) | 20.0 (18.2) |
| Stearic acid | 4.3 (3.4) | 10.4 (8.8) |
| Myristoleic acid | Trace (0.7) | Trace (1.8) |
| Palmitoleic acid | Trace (1.7) | Trace (4.7) |
| Oleic acid | 30.4 (27.4) | 40.3 (34.1) |
| Linoleic acid | 30.7 (30.7) | 5.6 (7.1) |
| Linolenic acid | 4.0 (4.5) | Trace (0.6) |
| Arachidonic acid | 1.0 (1.5) | 1.3 (4.2) |
| Glupanodenic acid | Trace (0.6) | Trace (1.8) |

* Both diets are of the same base containing, by weight, 22% protein and 2% fiber and fully supplemented with all necessary vitamins and trace minerals. Calculated values (in parentheses) are based on the ingredients.

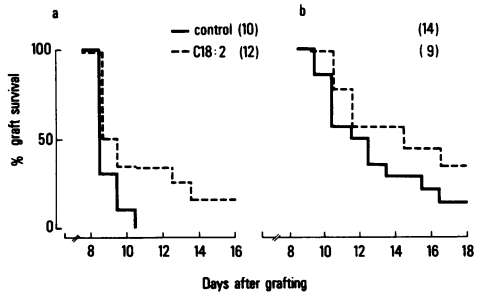


FIG. 1. Life tables of second set skin allograft survival times in female (a) and male (b) CBA mice grafted with C3H tail skin. The animals have been C18:2-treated for 16 days only during the first set. The number of animals per group is shown in parentheses.

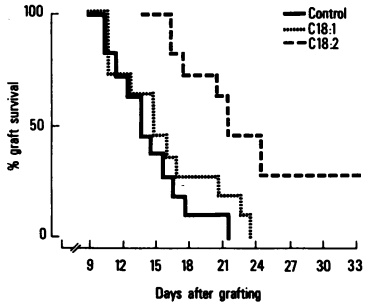


FIG. 2. Life table of skin graft survival in male CBA mice grafted with C3H tail skin. Untreated control and oleic acid (C18:1) or linoleic acid (C18:2) injected (s.c.) animals (11 per group).

tive cells in the spleens of both C18:2-treated mice and mice on PUFA-deficient diet was estimated using anti-theta (C3H) serum in the $^{51}Cr$ release assay already described (4). For these experiments Balb/c mice were allografted by i.p. injection of $3 \times 10^7$ washed C57BL/10 ascites tumor cells (EL-4) 14–15 days before the assay. Differences between controls and C18:2-treated animals in the number of theta-positive spleen cells in allografted mice have been shown previously (4).

**Tumor Induction.** Tumors were induced by s.c. injection in the dorsal region of 0.1 ml of 20-methylcholanthrene (5 mg/ml) dissolved in olive oil. Tumor appearance was assessed weekly by palpation.

## RESULTS

*C18:2 treatment* caused significant prolongation of allograft survival in CBA mice grafted with A strain (H-2 incompatible) and C3H tail skin (H-2 compatible, non-H-2 incompatible) and a marked difference in female C57BL/10 mice grafted with male skin of the same strain (H-Y system). In second set experiments done, differences in the survival times between controls and treated animals were significantly different despite a shorter period of treatment (11 days) (Table 2), suggesting C18:2 interference with the development of immunological memory as initiated by the first graft. This effect could also be observed in animals C18:2-treated only during the first set and without treatment during the second set period: they still showed slightly prolonged survival times (Fig. 1). This observation is consistent with recent results showing a decreased secondary cytotoxic response, as elicited *in vitro*, of mouse spleen cells when the animals were C18:2-treated during primary *in vivo* sensitization (1). Subcutaneous administration of C18:1 in the same dose regimen as used for C18:2 was virtually without effect on graft survival times (Fig. 2). Oral C18:2 administration also prolonged graft survival times (Table 2). This result confirms the finding of Ring *et al.* (6) in skin allografted rats.

*PUFA-deficient diet* caused a relative immunopotentiation, i.e., first and second skin allografts were rejected markedly faster than in the control diet groups (Fig. 3). There was also a smaller incidence of methylcholanthrene-induced tumors in PUFA-deficient animals (Fig. 4). Autoclaving the control diet likewise resulted in an acceleration of first and second set skin graft rejection similar to that found in PUFA-deficient animals, whereas autoclaving the deficient diet had no significant additional effect (Fig. 5). C18:2 injection could abrogate the effect of PUFA deficiency (Fig. 5).

Neither the administration of C18:2 nor PUFA deficiency caused an obvious difference between the death rates of treated and control groups.

*Theta-positive cells* in the spleens of allografted Balb/c mice were decreased in number when the animals were treated with C18:2 s.c. for 14 days, whereas the total number of spleen cells was found to be increased. No difference was observed between the numbers of theta-positive cells in animals on PUFA-deficient diet and their controls. However, the total numbers of spleen cells and their increase after allogeneic stimulation was significantly lower in the animals on the deficient diet (Table 3).

## DISCUSSION

Little is known yet about the extent to which nutritional factors may influence immune mechanisms. Hunt and Medawar (personal communication) have observed accelerated

Table 2. First and second set skin graft survival times of C18:2-treated mice given as mean expectation of life (MEL) in days

| Animals | Treatment | First set: Control No. | First set: Control MEL | First set: C18:2 (16 days) No. | First set: C18:2 (16 days) MEL | First set: Level of significance, %* | Second set: Control No. | Second set: Control MEL | Second set: C18:2 (11 days) No. | Second set: C18:2 (11 days) MEL | Second set: Level of significance, %* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A → CBA† | s.c. | 14 | 14.6 | 14 | 16.7 | 2.2 | | | Not done | | |
| C3H → CBA† | s.c. | 17 | 19.7 | 16 | 22.3 | 0.1 | 14 | 13.9 | 15 | 22.7 | 0.4 |
| C3H → CBA | Oral | 11 | 15.5 | 12 | 19.4 | 4.1 | | | Not done | | |
| ♂ → ♀ C57BL/10‡ | s.c. | 19 | 29.9 | 20 | 35.5 | 9.4‡ | 17 | 16.2 | 18 | 20.6 | 0.4 |

* The statistical difference from the controls as analyzed by a one-tailed test for nonparametric data (Wilcoxian test), significant differences being those <5%.

† Typical examples of at least three experiments.

‡ The marked difference in the MEL but lack of statistical significance of this experiment may reflect the wider range of variation inherent in this weak transplantation system.

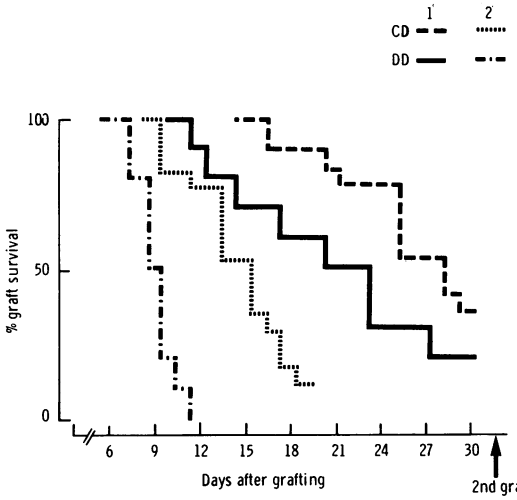


FIG. 3. Typical example of the effect of PUFA-deficient diet in first set (1°) and second set (2°) skin allograft rejection. In the first set it was noted that the median survival times of the grafts were greater than usual (compare Fig. 2). This may be due to the age of the animals, which were 9–10 weeks old at grafting (instead of 4–5 weeks) because of the diet pretreatment period. In our experience older animals tend to reject their grafts more slowly than younger ones. CD = Control diet; DD = PUFA-deficient diet.

skin allograft rejection and reduced tumor incidence in mice fed on autoclaved diets. Induction of tolerance also was affected, the animals getting autoclaved diet being more refractory to the induction.

Studying immune resistance in malnutrition, Jose and Good (7–9) have shown that CMI remained intact in mice with restricted protein intake, the protein content of the diet being as low as 5% protein calories. Beyond that limit, at 3% protein calories, CMI was depressed (8). This effect was irreversible when the mice were restricted in protein and calorie intake for 2 weeks at the time of weaning and then returned

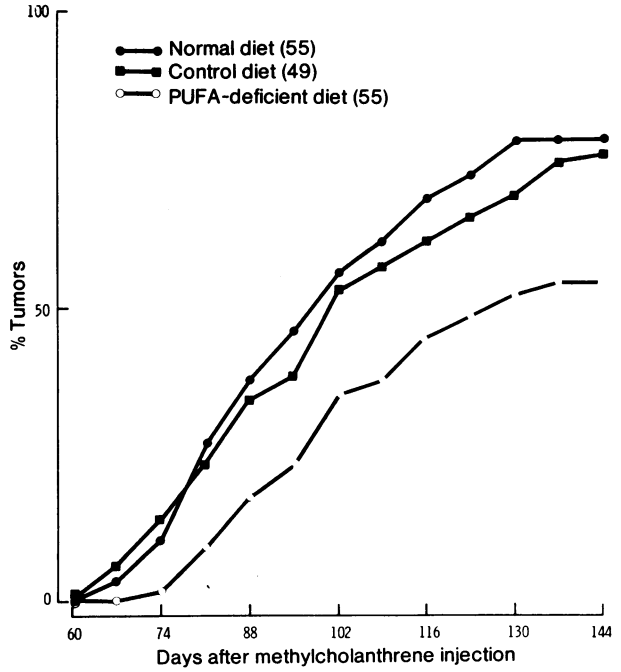


FIG. 4. Incidence of methylcholanthrene-induced tumors in CBA mice fed a PUFA-deficient diet. The number of animals in each group is given in parentheses.

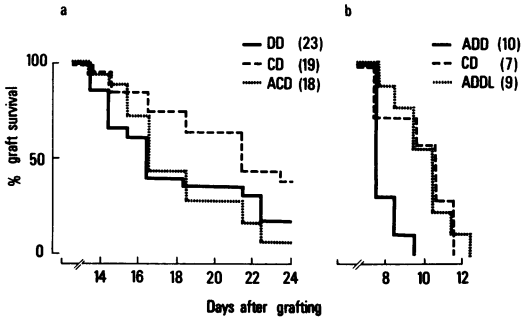


FIG. 5. Life tables of first (a) and second (b) set skin allograft survival times in PUFA-deficient female CBA mice grafted with C3H tail skin. Number of animals per group is shown in parentheses. DD = PUFA-deficient diet; CD = control diet; ACD = autoclaved control diet; ADD = autoclaved PUFA-deficient diet; ADDL = autoclaved PUFA-deficient diet + C18:2 s.c.

to a normal diet (9). However, in animals fed with a moderately restricted diet CMI was found more effective, this observation being interpreted by the authors as failure of a blocking antibody to develop (7).

Tumor-potentiating effects of PUFA-enriched diets have been interpreted by Tannenbaum and Silverstone (10) as resulting from increased efficiency of energy utilization by high fatty acid intake augmenting tumor growth. In our opinion the exercise of control by PUFA through an immunological surveillance system offers an alternative explanation for the long-known relationship of fatty acids and tumors (10) and our collected evidence supports this view.

Hopkins and West from the Australian National University have studied the influence of PUFA on experimental tu-

Table 3. Cytotoxicity of anti-theta serum on unseparated spleen cells from normal and allografted Balb/c mice treated with linoleic acid (C18:2) or fed on PUFA-deficient diet

| Treatment | Animal no. | Total spleen cells ($\times 10^{-7}$) | Lysed spleen cells ($\times 10^{-7}$) | % Lysis (± SEM)* |
|---|---|---|---|---|
| Control | 1 | 17.7 | 4.3 | 24.2 ± 0.60 |
| | 2 | 14.1 | 2.8 | 19.7 ± 0.45 |
| | 3 | 14.5 | 3.2 | 22.1 ± 0.19 |
| EL-4, saline | 1 | 21.1 | 4.3 | 20.6 ± 0.51 |
| | 2 | 26.2 | 4.6 | 17.5 ± 0.33 |
| | 3 | 21.0 | 3.9 | 18.8 ± 0.62 |
| EL-4, C18:2 | 1 | 38.4 | 3.2 | 8.3 ± 0.44 |
| | 2 | 29.5 | 2.7 | 9.1 ± 0.54 |
| | 3 | 43.6 | 3.2 | 7.4 ± 0.31 |
| Control diet | 1 | 15.3 | 3.0 | 19.4 ± 0.47 |
| | 2 | 14.7 | 3.2 | 21.5 ± 0.16 |
| Control diet + EL-4 | 1 | 21.0 | 4.7 | 22.5 ± 0.24 |
| | 2 | 23.6 | 4.9 | 20.8 ± 0.49 |
| PUFA-deficient diet | 1 | 11.9 | 2.4 | 19.8 ± 0.89 |
| | 2 | 12.3 | 2.4 | 19.9 ± 0.36 |
| PUFA-deficient diet + EL-4 | 1 | 14.3 | 3.3 | 23.2 ± 0.73 |
| | 2 | 16.2 | 3.0 | 18.7 ± 0.31 |

$$*\%\ \text{Lysis} = \frac{\text{mean counts released with anti-theta + complement} - \text{mean counts released with complement alone}}{\text{mean counts released with Triton (max. release)} - \text{machine background}} \times 100.$$

The figures represent replicates of three.

mors in animals (11). Rats given a carcinogenic polycyclic hydrocarbon showed increased tumor incidence when fed a diet containing polyunsaturated rather than saturated fatty acids. The increased incidence was observed when PUFA was fed after and not before carcinogen administration, suggesting PUFA affects survival and proliferation of tumor cells rather than the initial event of neoplastic transformation.

The results of our study strongly support the hypothesis of an immunoregulatory PUFA effect taking the form of immunoinhibition by PUFA treatment and immunopotentiation in the state of PUFA deficiency (1–3). The immunoregulatory aspect is stressed by the finding that the immunopotentiating effect of PUFA- deficient diet can be abrogated by C18:2 administration. This is in contrast to the observation mentioned above of irreversible changes in CMI induced by short-term calorie and protein deficiency (9).

The pathways through which PUFA may exert their immunoregulatory action are not yet known. Studies of the mechanisms of immunoinhibition by C18:2 and C20:4 have revealed a variety of effects of these substances on lymphoid and reticuloendothelial system. For example, spleen cells of mice receiving C18:2 s.c. have been shown to have less cytotoxic activity in an *in vitro* $^{51}Cr$ release assay (1). [$^{125}I$]Iododeoxyuridine uptake by spleen lymph nodes and bone marrow of mice was found to be affected by PUFA treatment and reticuloendothelial system function as measured by a carbon uptake assay could be increased (4).

Because various hormones take part in the regulation of PUFA serum and tissue levels (12), these fatty acids may represent mediators by which the hormonal system exerts influence on the regulation of immune mechanisms. Fatty acid levels have been found to be considerably higher in lymphoid cells than in cells of other organs (13). These cells thus may be in an outstanding position for being influenced in their metabolism and function by changes in PUFA levels. Site of action and mechanisms whereby such changes are effective are not known yet. However, two points may have to be taken into account: (*a*) the precursor relationship between PUFA and prostaglandins, some of the latter having been found to be immunoinhibitory (14); and (*b*) the effects that changing PUFA levels in the phospholipids of plasma membranes may have on specific reactions of immunologically competent cells. Further investigations have to be carried out to clarify PUFA action in immunoregulation and an extended insight into underlying processes may provide a possibility for manipulation of CMI in relevant clinical disorders.

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