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Enhanced production of IgE and IgG antibodies associated with a diet enriched in eicosapentaenoic acid

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Summary. Eicosapentaenoic acid (EPA), a polyunsaturated fatty acid analog of arachidonic acid, alters certain platelet functions controlled by prostaglandins and thromboxanes, probably by inhibiting the synthesis of these molecules from arachidonic acid. This study reports the effects of a diet enriched in EPA (fish-fat diet, FFD) as compared with a diet lacking EPA (beef-fat diet, BFD) upon certain immunological and inflammatory responses in outbred Sprague Dawley rats. Induction of antibody formation to egg albumin (EA) produced four- to eight-fold greater titres of IgE ($P<0{\cdot}02$) and IgG ($P<0{\cdot}03$) anti-EA antibodies in FFD rats *v.* BFD rats. FFD rats had heightened active cutaneous anaphylaxis to EA, responding to a median [EA] of 10^{-4} mg/ml, *v.* 10^{-3} mg/ml in BFD rats ($P<0{\cdot}0032$). Similarly, active Arthus reaction to EA in FFD rats was elicited to a [EA] of 10^{-3} g/ml *v.* 10^{-1} g/ml in BFD rats ($P<0{\cdot}01$). Passive inflammatory reactions, as evaluated by passive cutaneous anaphylaxis elicited with IgE antibody, and by the intradermal injection of aggregated IgG, were not significantly different between the two groups. EPA constituted 7·3% of fatty acid in the livers of FFD rats, *v.* 0·3% in BFD rats ($P<0{\cdot}01$). These data demonstrate enhanced levels of IgE and IgG antibody in FFD rats, with subsequent increased active inflammatory reactivity in these animals. These alterations may be secondary to enrichment of tissue lipids with EPA, although effects due to changes in other fatty acids have not been excluded.

Abbreviations: PGs, prostaglandins; AA, arachidonic acid; EPA, eicosapentaenoic acid; FFD, fish-fat diet; BFD, beef-fat diet; PCA, passive cutaneous anaphylaxis; PBS, phosphate-buffered saline; ACA, active cutaneous anaphylaxis; EA, egg albumin; FIA, Freund's incomplete adjuvant; ELISA, enzyme-linked immunosorbent assay; EFA, essential fatty acid.

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INTRODUCTION

Prostaglandins (PGs) of the E series, derived from the fatty acid, arachidonic acid (AA), are mediators of the effector limb of acute inflammatory responses, producing vasodilation and edema both directly and through potentiation of other mediators such as bradykinin and histamine (Lewis, 1971; Horton, 1963; Williams & Morley, 1973). PGs also influence the proximal limb of immunological function, including phagocytosis of particles by macrophages (Razin, Zor & Globerson, 1979), regulation of B lymphocytes by T lymphocytes (Fulton & Levy, 1980), and production of antibody molecules by B lymphocytes (Zimecki & Webb, 1976).

Eicosapentaenoic acid (EPA), a twenty carbon analog of AA containing one additional double bond, is metabolized to a series of prostaglandins, thromboxanes, and leukotrienes structurally similar to the AA metabolites (Needleman, Minkes & Raz, 1976; Needleman, Raz, Minkes & Ferrendelli, 1979; Hammarstrom, 1980). The physiological effects of these EPA products differ from those produced by AA metabolites, and perhaps more importantly, EPA may act as an inhibitor of AA metabolism (Needleman *et al.*, 1979; Hammarstrom, 1980; Dyerberg, Bang, Stofferson, Moncada & Vane, 1978).

High dietary levels of EPA can produce alterations in platelet functions normally regulated by prostaglandins and thromboxanes (Bang, Dyerberg & Hjorne, 1976; Siess, Scherer, Böhlig, Roth, Kurzman & Weber, 1980). A diet enriched in EPA has also been shown to delay the development of proteinuria and to prolong survival in NZB × NZW F_1 mice (Prickett, Robinson & Steinberg, 1981). In view of the multiple effects of arachidonate metabolites in inflammatory responses, we have undertaken a series of experiments to study the effects of dietary EPA enrichment on certain models of inflammation, and on primary and secondary antibody responses in outbred rats.

METHODS

Diets

The basic diet consisted of fat-free powder (ICN Nutritional Biochemicals, Cleveland, Ohio), which contains by weight 21% casein, 15·6% cellulose, 58·5% sucrose, and 4% balanced salt mixture, plus essential vitamins. This ingredient was mixed three parts to one by weight with either refined whole menhaden oil (Zapata Haynie Corporation, Reedville, Va), a source rich in EPA, (Fish-Fat Diet, FFD) or melted beef tallow (ICN Nutritional; Beef-Fat Diet, BFD). Fatty acid analyses of these lipids are shown in Table 1.

Animals

Female Sprague-Dawley rats, 100–125 g in weight (Charles River Breeding Laboratory, Wilmington, Mass.) were housed four–six animals per cage under standard conditions of light, temperature, and water supply. Animals were placed on either BFD or FFD. Animals were weighed at 7–14 day intervals, and diets were adjusted to maintain weight gain within 10% of the mean of the two dietary groups. Animals were studied upon reaching body weights of 225–250 g, as follows:

Group 1 animals consisted of a pilot group of six animals on each diet. Studies performed on these animals were passive cutaneous anaphylaxis (PCA), and production of IgE anti-egg albumin antibodies (see Methods below).

Group 2 consisted of twenty animals on each diet. Studies performed on these animals, in sequence, included (i) reaction to intradermal injection of heat-aggregated human IgG; (ii) passive cutaneous anaphylaxis (PCA) with rat IgE antibodies; (iii) induction of IgE anti-egg albumin antibodies; (iv) active cutaneous anaphylaxis (ACA) to egg albumin; (v) induction of IgG anti-egg albumin antibodies; and (vi) active Arthus reaction. (See Methods below).

Elicitation of immunological reactions

Passive cutaneous anaphylaxis. Sera containing high titres of IgE antibodies to *Nippostrongylus brasiliensis* antigens and worm extract were prepared as previously described (Wilson & Bloch, 1968). A single pool of antiserum was used throughout. Samples were stored at −50°. PCA was performed as previously described (Wilson & Bloch, 1968). In brief, diluted antiserum was injected intradermally into test animals. After 24 hr, rats were injected intravenously with 50 μl of worm extract in 0·5 ml phosphate-buffered (0·01 M) saline, pH 7·4 (PBS) plus 0·5 ml of 2% Evans blue dye (Eastman Kodak Co., Rochester, N.Y.). Results were recorded at 40 min; the final dilution of serum yielding a blue visible lesion in the skin with a diameter of at least 5 mm was considered the end point of titration for the antiserum. Results were reported as the reciprocal of this titre.

Active cutaneous anaphylaxis. Animals immunized with egg albumin in alum (see below) were challenged with antigen injected intradermally on day 15. Nine sites were injected with 0·1 ml of solution containing 0·1 mg/ml, 0·01 mg/ml, 0·001 mg/ml ... 1·0 pg/ml. Each animal then received an intravenous injection of 1·0 ml of 1% Evans blue dye. Results were recorded 40 min later. The end point of titration was the lowest concentration of antigen producing a blue circular lesion in the skin with a diameter of at least 5 mm; results are recorded as the reciprocal of this concentration.

Active Arthus reaction. On day 60 after the first injection of egg albumin (EA), and 40 days after subsequent immunization with EA emulsified in Freund's incomplete adjuvant (FIA), rats were shaved

under light ether anaesthesia and injected intradermally with 0·1 ml of EA solution containing 0·1, 0·01, 0·0001, 0·00001 g/ml. At 2 and 4 hr later, test results were recorded. End point of the titration was the concentration of antigen producing a haemorrhagic lesion with a diameter of at least 5 mm; results are recorded as the reciprocal of this concentration.

Passive reaction to intradermal injection of aggregated human IgG. The procedure described by Christian (1960) was modified. Lyophilized human IgG (Miles Laboratories, Elkhart, Ind., number 64-145, lot 41) was reconstituted 10 mg/ml in PBS and incubated for 10 min at 63°. Serial two-fold dilutions in PBS were prepared. The rats were shaved on the dorsal surface and under light ether anaesthesia 0·1 ml of each dilution (ranging from undiluted to 1:256) of the IgG solution was injected intradermally. Results were recorded at 24 hr; the end point of titration was the lowest concentration of IgG solution producing palpable induration of greater than 3 mm. Results are reported as the reciprocal of this dilution. In one animal from each group, the site of a positive, negative, and PBS-control injection was biopsied. The tissue was fixed and sections stained with haematoxylin and eosin.

Induction of antibody formation

Induction of IgE anti-egg albumin antibodies. For all experiments with egg albumin a single lot of EA was used. Animals received an intraperitoneal injection of EA (five times crystalized, Miles Laboratories) 100 μg per animal together with 20 mg alum (Bloch, Ohman, Waltin & Cygan, 1973) in a total volume of 1·0 ml. Animals were bled from a cut in the tail on day 14. Blood was allowed to clot at 24° for 1 hr; serum was stored at −50°. The IgE antibody content of these sera was measured by PCA.

Induction of IgG anti-egg albumin antibodies. Twenty days after their initial i.p. injection with EA in alum, rats were injected with 2 mg EA in Freund's incomplete adjuvant. The emulsion was prepared in an Omni-Mixer (Sorvall, Newtown, Conn.); 1·0 ml of emulsion contained 2 mg EA, 0·5 ml PBS, 0·05 ml Aracel A (Sigma Chemical Company, St. Louis, Mo.) and 0·45 ml Drakeol 6VR (Pennsylvania Refining Co., Butler, Pa). Each animal was injected subcutaneously with 0·4 ml of emulsion in the right foot pad and intramuscularly with 0·6 ml in the left thigh. Animals were bled from the tail 15 days later; serum was stored at −20°. Animals were reinjected with 1·0 ml of emulsion on day 36 and day 49. Active Arthus reactions were elicited on day 60 as described above.

Enzyme-linked immunosorbent assay (ELISA) for IgG anti-EA antibodies. The ELISA assay (Voller, Bidwell & Bartlet, 1976) was performed with 96-well microtiter plates (Costar, number 3596, Cambridge, Mass.). EA (200 mg/ml) was dissolved in carbonate-bicarbonate coating buffer, pH 9·6. All other solutions were prepared with a PBS/Tween 20/NaN_3 buffer. Test sera were diluted 1:1000, 1:5000, 1:25,000 ... 1:3,125,000, and 50 μl of each dilution were assayed. Rabbit anti-rat IgG antiserum (Miles, number 65-128) and alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Miles, number 61-275) were used at a dilution of 1:500. The enzyme substrate consisted of 150 μl p-nitrophenyl phosphate (Sigma number 104 tablets), 1 mg/ml, in 10% diethanolamine. Incubation with substrate lasted 15 min, and was stopped by the addition of 50 μl 1·0 N NaOH. The end point of titration was the highest dilution of serum yielding a yellow solution; results are reported as the reciprocal of this titre.

Total haemolytic complement activity. Five animals on each diet in Group 2 were bled via the retro-orbital venous plexus. Blood was allowed to clot at 24° for 1 hr, then was centrifuged at 4° and serum stored frozen at −50°. One hundred microlitre aliquots of serum were assayed for CH_{50} using a standard procedure (Kabat & Mayer, 1961).

Tissue extractions. Three animals on each diet in Group 1 were killed at approximately 5 months of age, and the livers were frozen at −20°. 0·3–0·4 g (wet weight) of these tissues were homogenized and extracted in hot ethanol ether (3:1) mixture as described (Entenman, 1957). Methyl esters of fatty acids were prepared and analysed by gas-liquid chromatography (Prickett *et al.*, 1981). Fatty acid analysis of the beef tallow and menhaden oil has been reported previously (Prickett *et al.*, 1981) and is shown in Table 1.

Statistical analysis. Results of fatty acid analyses of liver were compared using a non-paired *t* test. All other data were analysed using the Mann-Whitney U test.

Table 1. Fatty acid analysis of dietary lipids and rat livers*

Fatty acid	Menhaden oil†	Beef tallow†	Liver FFD rats ($n=3$)	Liver BFD rats ($n=3$)
14:0	8·2	2·7	3·2±1·0	2·0±1·9
16:0	13·5	23·9	17·1±0·7	14·4±1·1‡
16:1	13·5	5·3	3·8±0·07	3·8±1·7
18:0	5·5	17·7	18·4±1·1	22·6±2·8
18:1 (oleic)	12·5	41·2	10·7±1·8	17·3±1·5§
18:2 (linoleic)	5·2	6·1	2·6±0·6	8·0±1·0§
20:3	1·4	<0·05	0·7±0·06	1·1±0·2¶
20:4 (arachidonic)	1·5	<0·05	11·7±0·2	17·0±5·6
20:5 (EPA)	14·4	<0·05	7·3±0·6	0·3±0·4§
22:6	8·0	<0·05	19·1±1·7	4·3±1·2§

* As percentage total fatty acid content, ±SD where indicated.
† Single determinations.
‡ Fish *v.* beef livers, $P<0·05$ statistical comparisons were made by non-paired *t* test.
§ Fish *v.* beef livers, $P<0·01$.
¶ Fish *v.* beef livers, $P<0·02$.

RESULTS

Elicitation of passive cutaneous anaphylaxis

The ability of rats fed either a fish- or beef-fat-enriched diet to undergo a PCA reaction was tested. Results of both Group 1 and Group 2 animals are shown in Fig. 1. For Group 1, the reciprocal of the median titres in rats on the FFD and BFD were 500 and 900, respectively (not significant, $P=0·11$). For Group 2, these values were 1000 and 1200 (not significant, $P=0·12$).

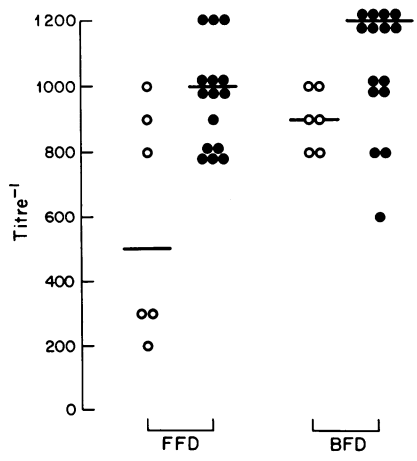

Figure 1. Passive cutaneous anaphylaxis to *N. brasiliensis*. Bars indicate median values. For Group 1 (○), $P=0·11$; for Group 2 (●), $P=0·12$.

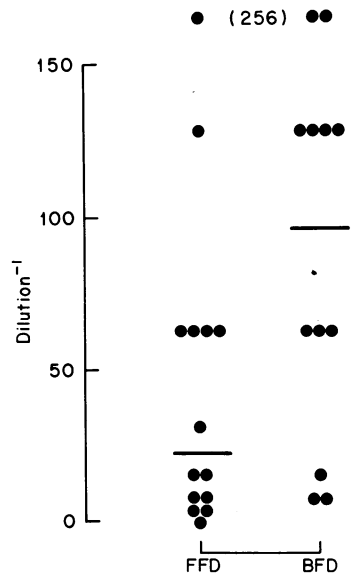

Figure 2. Response at 24 hr to intradermal heat-aggregated human IgG. Bars indicate median values. $P=0·057$.

Elicitation of intradermal reaction to heat-aggregated IgG

The ability of rats on the FFD and BFD to respond to intradermal injections of heat-aggregated human IgG was tested (Fig. 2). Rats on the BFD reacted to higher dilutions of IgG solution than did rats on the FFD; the reciprocals of the median dilutions were 96 and 24 respectively (not significant, $P=0{\cdot}057$).

Histological sections of both nodular and clinically non-reactive injection sites of heat-aggregated IgG from animals of both diets revealed infiltration by large numbers of a mixed population of lymphocytes and monocytes; occasional polymorphonuclear leucocytes were observed. Examination of PBS control injections revealed small infiltrates of lymphocytes and monocytes.

Induction of IgE antibodies to EA

Two weeks after injection of EA and alum, animals were bled and the IgE anti-EA antibody content of their sera was determined by PCA. These PCA tests were performed on Sprague-Dawley rats maintained on standard animal chow. For Group 1, the reciprocal of the median titre in rats on the FFD and BFD were 40 and 5, respectively ($P=0{\cdot}02$; Fig. 3). For Group 2 animals, the respective values were 20 and 5 ($P<0{\cdot}0012$).

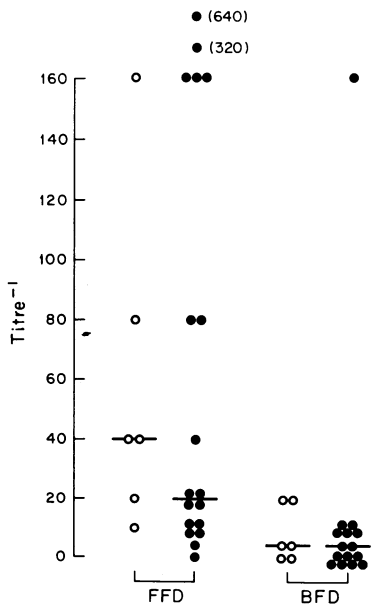

Figure 3. Induction of IgE antibodies to egg albumin, as assayed by passive cutaneous anaphylaxis in normal rats. Bars indicate median values. For Group 1 (○), $P=0{\cdot}02$; for Group 2 (●), $P<0{\cdot}0012$.

Elicitation of active cutaneous anaphylaxis with EA

Animals of both dietary groups were immunized so as to produce endogenous IgE anti-EA antibodies and were challenged by intradermal injection of EA (Fig. 4). FFD animals responded to a median [EA] of 10^{-4}mg/ml; BFD animals responded to a median [EA] of 10^{-3}mg/ml ($P<0{\cdot}0032$).

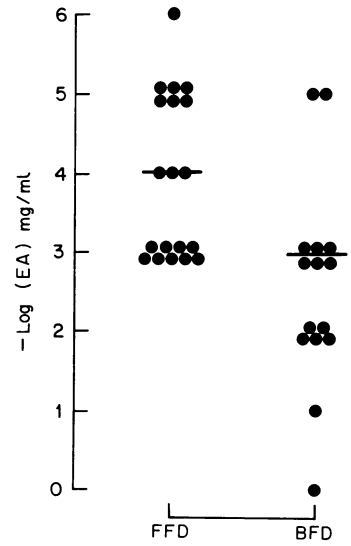

Figure 4. Active cutaneous anaphylaxis to egg albumin. Bars indicate median values. $P<0{\cdot}0032$.

Induction of IgG antibodies to EA

Rats initially primed so as to induce an IgE antibody response (see above) were subsequently injected with EA in FIA. The IgG antibody response was measured by the ELISA method. Two weeks after the first injection of EA emulsified in FIA, the median reciprocal titre of the FFD and BFD animals were 625×10^3 and 125×10^3, respectively ($P<0{\cdot}03$; Fig. 5).

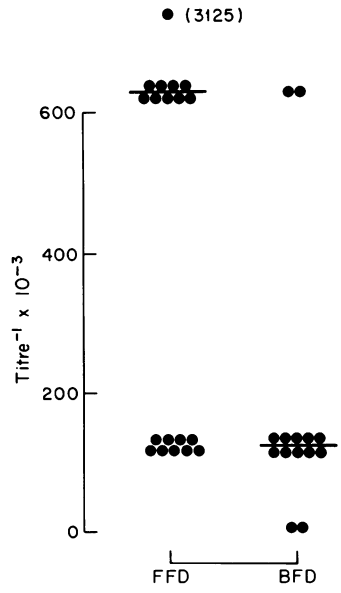

Figure 5. Induction of IgG antibodies to egg albumin, as assayed by ELISA. Bars indicate median values. $P < 0{\cdot}03$.

Elicitation of active Arthus reaction with EA

After the third injection of EA in FIA above, rats were tested for their ability to undergo an Arthus reaction to intradermal injection of EA. Results were identical at 2 and 4 hr, and are shown in Fig. 6. FFD animals reacted to a median antigen challenge of 10^{-3} g/ml, while BFD animals responded to a median antigen concentration of 10^{-1} g/ml ($P < 0{\cdot}01$).

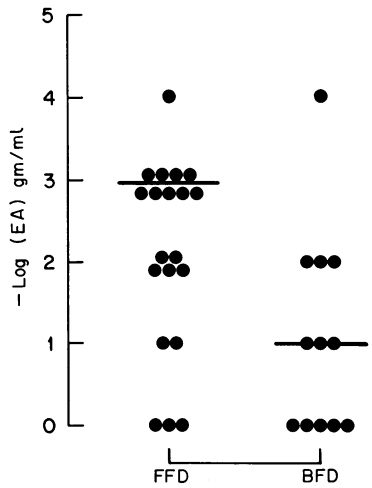

Figure 6. Active Arthus reaction to egg albumin. Bars indicate mean values. $P < 0{\cdot}01$.

Total haemolytic complement activity

The influence of the FFD and BFD on the serum total haemolytic complement activity, CH_{50}, was investigated. The median CH_{50} values for five FFD and five BFD animals were 145 and 127 CH_{50} units per ml of serum, respectively (not significant, $P > 0{\cdot}76$).

Fatty acid analysis. Fatty acid analyses of dietary lipids, and livers of test animals, are presented in Table 1. Each dietary lipid contained comparable amounts of linoleic acid. EPA constituted 14·4% of the menhaden oil, but was undetectable in the beef tallow.

Although analyses of fatty acids in the livers of the two groups of animals show several statistically significant variations, the most striking of these involved C18:2 (linoleic), C20:5 (EPA), and C22:6, a metabolite of EPA (Tinoco, Babcock, Hincenbergs, Medwadowski, Miljanich & Williams, 1979). Linoleic acid constituted 2·6% of fatty acid in the FFD animals, *v.* 8·0% in the BFD. EPA and the C22:6 derivative constituted 7·3 and 19·1% of the fatty acid in the FFD livers, *v.* 0·3% and 4·3% in the BFD livers, respectively ($P < 0{\cdot}01$ for each).

DISCUSSION

These experiments were undertaken to determine the effects of a diet enriched in the polyunsaturated fatty acid EPA upon the *in vivo* induction of humoral immune responses and the elicitation of acute inflammatory responses. FFD rats showed markedly increased levels of both IgE and IgG antibodies relative to the BFD rats. Although kinetic analyses were not performed, the dose of antigen (EA) and alum, as well as the sampling interval, were those which have been shown previously to yield maximal titres of IgE anti-EA antibodies in Sprague-Dawley rats fed a normal laboratory diet (unpublished data). Similarly, the increased levels of IgG anti-EA antibodies observed 15 days after injection of EA emulsified in FIA and the heightened active Arthus reactions elicited 4 weeks later in FFD compared with BFD rats, suggest that the fish-fat-enriched diet promotes enhanced antibody production. There was no statistically significant difference in the passive inflammatory

responses between the two groups, although (in both Groups 1 and Group 2) animals on the FFD reacted less than those on the BFD. However, the increased levels of IgE and IgG antibodies in the FFD rats appear to have resulted in heightened active cutaneous anaphylaxis and active Arthus reactions in these rats, thereby more than compensating for the marginal decrease in inflammatory responsiveness in the FFD animals.

Prostaglandins have been demonstrated to affect multiple steps in the immunological processing of antigen and elaboration of antibody. PGE_2 in physiological concentrations enhances phagocytosis of foreign particles by macrophages (Razin *et al.*, 1979), and is also subsequently released by these cells, suppressing certain T-cells activities (Tracey & Adkinson, 1980). T cells themselves may exert immunological suppression at least in part via release of PGs (Fulton & Levy, 1980). *In vivo*, secondary antibody synthesis in humans can be enhanced by administration of indomethacin, an agent which inhibits PG synthesis (Goodwin, Selinger, Messner & Reed, 1978). Purified populations of B lymphocytes respond to PG synthesis inhibition by both increased or decreased antibody secretion, depending upon the timing of such inhibition after antigen exposure (Zimecki & Webb, 1976). Thus, it is evident that PGs exert effects upon the primary and secondary antibody response that have only been partially characterized. To the extent that EPA interferes with the generation of active metabolites from arachidonic acid, or provides substrate for the formation of less active compounds (Needleman *et al.*, 1979; Hammarstrom, 1980; Dyerberg *et al.*, 1978), the enhanced antibody synthesis of the FFD rats may be attributable to a reduction in suppressive effects mediated by metabolites of arachidonic acid.

An hypothesis of this study was that the primary difference between the two dietary groups was the enrichment of the FFD with EPA. Although beef fat and fish fat vary in the proportion of several fatty acids, the most profound differences involved EPA and its metabolite, C22:6. Animals maintained on the FFD and BFD showed several statistically significant variations in fatty acid composition of their livers. These variations ranged from 20% to 60% except for the differences in composition of linoleic acid, EPA, and C22:6. The livers of rats on the BFD had a higher content of linoleic acid than did those rats of the FFD; similarly the livers of rats on the BFD had a 45% greater content of AA, the major metabolite of linoleic acid, although this difference was not of statistical significance. Nevertheless, the differences observed are still far less dramatic than the 2400 and 440% elevations in EPA and C22:6 composition, respectively, observed in the FFD animals.

Animals in our study were fed so that the mean weight of animals in either group did not differ by more than 10%, eliminating starvation as an explanation for differences observed between the groups. Essential fatty acid (EFA) deficiency can also profoundly influence immunological and inflammatory responses, possibly by limiting the availability of precursors of PGs (Bonta, Bult, Vincent & Zijistra, 1977; Denko, 1976). EFA deficiency has been defined biochemically by a tissue ratio of C20:3 to C20:4 of $>0{\cdot}4$ (Holman, 1960). This ratio in both our FFD and BFD animals was 0·06, far below that of EFA deficiency.

Substitution of polyunsaturated fatty acids for saturated fatty acids increases fluidity of cell membranes (Seelig & Seelig, 1977; Burns, Luttenegger, Dudley, Buettner & Spector, 1979); alterations in membrane fluidity in turn influence membrane functions and cellular interactions (Burns *et al.*, 1979; Ishizaka, Hirata, Ishizaka & Axelrod, 1980). Thus, alterations in membrane fluidity of cells involved in the immune response of FFD rats may also account for the changes observed in the present study.

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