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REPORTS

Beneficial Effect of an Essential Fatty Acid Deficient Diet in NZB/NZW F_1 Mice

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New Zealand Black by White (B/W) hybrid mice spontaneously develop a disease similar to systemic lupus erythematosus (SLE). Subepidermal immunoglobulin deposits (Se-Ig) and antibodies to double-standed DNA (anti-dsDNA) develop in aging mice. Death from glomerulonephritis occurs at 8 to 12 mo. Previous findings suggest that epidermal DNA:anti-dsDNA complexes form *in situ* since Se-Ig correlates with anti-ds DNA and Se-Ig accumulation is augmented by increased epidermal proliferation (presumably due to enhanced epidermal DNA release). Since essential fatty acid (EFA) deficiency is known to increase epidermal proliferation we have studied the effect of an essential fatty acid deficient EFA-d diet on: (1) Se-Ig, (2) anti-dsDNA, and (3) survival. Ten-mo B/W mice on an EFA-d diet were compared with 14 controls on a calorically equivalent standard diet. Both groups were initiated on their diets at 2 mo of age. Only female mice were used. All were weighed weekly; tested for anti-ds DNA (*Crithidia luciliae* assay) each month; and biopsied for direct immunofluorescence (IF) staining of skin at 6, 7.5, 9, 10.5, and 12 mo. Tissue (skin and kidney) was also obtained for light and IF microscopy. Weights in the 2 study groups were essentially identical. All disease manifestations examined were strikingly altered in the EFA-d animals. Only 2 of 14 (14%) control animals survived to 9 mo and both had anti-dsDNA and Se-Ig. In contrast, 8 of 10 (80%) EFA-d mice were alive at 9 mo and none had anti-dsDNA or Se-Ig. The kidneys from EFA-d mice at 10 mo were normal; however, all kidneys from 7 to 9 mo control mice were abnormal by both light and IF microscopy. Eight of the 10 EFA-d mice were alive at 10 mo. None had Se-Ig but one had anti-dsDNA. At 16 mo (4 mo after controls had died) 7 of 10 EFA-d mice were living and 60% were anti-ds DNA positive. These findings strongly suggest that (1) SE-Ig is present in mice with anti-dsDNA and severe renal disease and (2) EFA-d produces a profoundly beneficial effect in the disease process.

The F_1 hybrid of the New Zealand Black and White mice spontaneously develops an autoimmune disease that is remarkably similar to human systemic lupus erythematosus (SLE) [1-3]. In 1975, we reported the finding of subepidermal immunoglobulin deposits in 5- to 12-mo-old female NZB/W mice [4]. The appearance of the cutaneous Ig deposits in these animals closely resembles the subepidermal Ig deposits in clinically normal skin of SLE patients.

The temporal relationship between subepidermal Ig deposition, autoantibody production, glomerular Ig deposition, and azotemia in female B/W mice from our colony were also studied [4]. Cutaneous immune deposits first appear at 5 to 6 mo—2 to 3 mo after the appearance of antinuclear antibodies and glomerular Ig deposits and 1 to 2 mo before the onset of azotemia. Most animals die of immune complex mediated renal disease by 8 to 10 mo of age. Fifty percent are dead by 8 mo and over 90% by 10 mo.

Findings from earlier studies [5] have suggested that immune complexes composed of DNA from the epidermis and anti-DNA from the circulation form in the skin. Subepidermal Ig correlates with circulating anti-dsDNA antibodies and subepidermal Ig accumulation is augmented by local stimulation of epidermal proliferation [6]. This has suggested that a persistent and generalized increase in epidermal turnover, with attendant increased DNA release from keratinizing cells, may lead to exacerbation of the disease by providing additional DNA for the formation of more immune complexes both in the skin and kidneys. Essential fatty acid deficiency is known to cause a generalized and persistent increase in epidermal proliferation [7]. To determine whether an essential fatty acid deficient (EFA-d) diet would worsen the disease and enhance subepidermal immunoglobulin accumulation we have given an EFA-d diet to NZB/W mice.

MATERIALS AND METHODS

Animals

Only femal mice were used in these studies. The NZB/NZW F_1 (B/W) animals were obtained from our own colony which was established with mice originally obtained from Dunedin, New Zealand, in 1967. Each group of animals was entered into the study when the mice were 6 to 11 weeks of age. The groups consisted of: 14 untreated colony control animals and 12 animals on an essential fatty acid deficient (EFA-d) diet containing 20% coconut oil. All animals were sacrificed when obviously moribund. However, tissues were not available for study in a number of animals that died. Each animal was weighed daily for the first 2 mo, and weekly thereafter.

Diets

Colony control animals were fed regular laboratory chow. The EFA-d diet consisted of casein (25%), dextrins (35%), sucrose (15%), a complete vitamin mixture (2%), a complete mineral mixture (3%) [8] and the coconut oil (20%). Diets were given *ad libitum*. The biochemical analyses of the fatty acid composition of the coconut oil employed in the present study are given in the Table. The methyl esters of the fatty acids were prepared according to the procedure of Morrison and Smith. [9]. The fatty acid methyl esters were dissolved in carbon disulfide and were separated by gas-liquid chromatography employing a Hewlett Packard Model 583A gas chromatograph with an integrator (utilizing columns of 10% S-2330 on 100/120 chromosorb W at 200°C). The amounts of eluted fatty acid methyl esters were computed using heptadecanoic acid as an internal standard.

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Abbreviations:
anti-dsDNA: antibodies to double-stranded DNA
EFA-d: essential fatty acid deficient
FITC: fluorescein isothiocyanate
IF: immunofluorescence
Se-Ig: subepidermal immunoglobulin deposits

Biochemical composition of coconut oil (EFA-d) diet

No. C: No. Double Bonds	Coconut Oil (%)
8:0	9.9
10:0	7.1
12:0 (Lauric)	53.0
14:0 (Myristic)	15.7
16:0 (Palmitic)	6.5
18:0 (Stearic)	1.8
18:1 (Oleic)	4.2
18:2 (Linoleic)	<1.0

Plasma

Blood was obtained monthly from the retrobulbar venous plexus of each mouse (approx. 200 μl per animal) and the harvested plasma was stored at −70°C until assayed.

Preparation of Tissues

Animals were examined at frequent intervals and when they became moribund (hunched posture, decreased activity, rapid shallow breathing, and pitting edema) they were sacrificed and tissues were removed and prepared for study. Portions of the renal tissue were fixed in formalin for light and electron microscopy and other portions were immersed in Tissue-Tek II embedding medium (Lab-Tek Products, Naperville, Illinois), frozen and stored at −70°C for examination by direct immunofluorescence.

Immunofluorescence

Kidney and skin tissues obtained at the time of sacrifice were examined for the presence of immunoglobulin deposits by the direct immunofluorescence technique. Skin biopsies were also obtained from living animals at 6-week intervals. Fresh tissue was frozen immediately and embedded in Tissue-Tek II (Lab-Tek Products, Naperville, Illinois). Cryostat sections (4 μm) were stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG and IgM (Meloy Laboratories, Inc., Springfield, VA), washed 3 times and mounted in buffered glycerol.

Tissue sections were examined with a Leitz Orthoplan fluorescence microscope equipped with epi-illumination (E. Leitz, Inc., Rockleigh, NJ). An HBO 100 ultra-high pressure mercury lamp and K490 and K510 filters were employed. Photomicrographs were taken with a Leitz Orthomat camera on Tri-X Pan black and white film, ASA 400 (Eastman Kodak Co., Rochester, NY). Kidney tissue sections were graded 0 through 4+ according to distribution and intensity of fluorescence. Separate scores were given for capillary loops, mesangium, and overall appearance of the glomerulus. All kidney and skin sections were coded and examined without knowledge of the source.

Light Microscopy

Formalin-fixed tissue was embedded in paraffin. Hematoxylin and eosin and PAS-stained kidney tissue sections were prepared from paraffin blocks for routine light microscopic examination. All tissue sections were coded and examined without knowledge of treatment category. Histologic abnormalities of the kidney were scored on the basis of 6 separate categories: (1) glomerular cellularity, (2) alterations in mesangial matrix, (3) presence of epithelial crescents, (4) fibrinoid changes and presence of wire loop-like alterations, and (5) amount of perivascular and interstitial mononuclear cell infiltration. An overall score was derived from an average of the above 5 categories.

Measurement of Antibodies

Antinuclear antibodies were measured by indirect immunofluorescence using K-B carcinoma cells on slides as the nuclear substrate (Electro-Nucleonics, Inc., Bethesda, Md.). Antibodies to native DNA (anti-nDNA) were assessed utilizing the *Crithidia luciliae* (ATCC 14765, Rockville, Md.) indirect immunofluorescence technique as previously described [10]. In this assay, the *C. luciliae* kinetoplast (a giant mitochondrion containing histone-free dsDNA) is used as the dsDNA substrate. Kinetoplast fluorescence after exposure to test sera followed by FITC-conjugated goat antisera to mouse Ig indicates the presence of anti-dsDNA. Serum antibody concentration was determined by serial dilutions to the point of disappearance of kinetoplast fluorescence. Kinetoplast fluorescence following the application of undiluted test serum was considered positive since undiluted mouse serum from a large number of nonautoimmune strains have been consistently negative. The class composition of anti-dsDNA antibodies in age-matched pooled sera from each study group was evaluated using monospecific antimouse IgG or IgM conjugates in the final step of the procedure.

RESULTS

Skin Histology

The photomicrograph shown in Fig 1(*a*) is of skin from an animal on the EFA-d diet. The animals on the EFA-d diet had the expected gross and histologic skin changes of EFA deficiency—acanthosis, hypergranulosis, and hyperkeratosis. A section of normal mouse skin for comparison is shown in Fig. 1(*b*). Normally mouse epidermis is only 2 to 3 cell layers thick.

Weights

The mean body weight in the age matched groups was essentially the same.

Survival

Figure 2 shows the percent survival over an observation period of 20 mo in the EFA deficient animals and in the untreated controls. The remarkable prolongation of survival of EFA deficient B/W mice was unexpected. At 12 mo, 8 of 10 (80%) of the EFA-d animals were living compared to only one of 14 (7%) of the control animals. Three EFA-d animals were sacrificed at 13 mo although they appeared healthy. Five of the EFA-d animals were still alive at the end of the study. These 5 animals were sacrificed at 20 mo to examine the renal pathology.

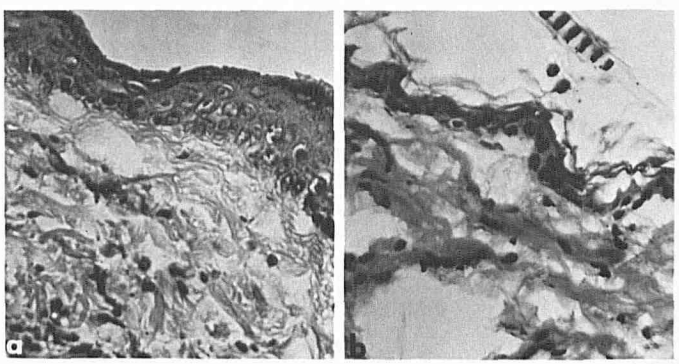

FIG 1. *a*, Skin biopsy from an animal on the EFA-d diet, demonstrating acanthosis, hypergranulosis and hyperkeratosis (H & E, reduced from × 400). *b*, Skin biopsy from NZB/NZW F_1 mouse on the normal diet. None of the changes noted in Fig 1(*a*) are present (H & E, reduced from × 400).

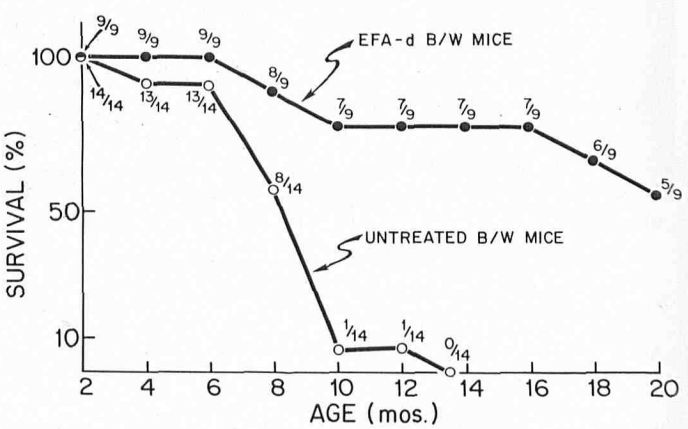

FIG 2. Percent survival in untreated and EFA-d animals. In this and the following figures, numbers in the numerator reflect the number of animals surviving in each age group; numbers in the denominator reflect the total number of animals in each group. EFA-d *vs* untreated ($p = .02$).

Autoantibodies

Results of the antinuclear antibodies (ANA) tests are shown in Fig 3. The control group had a sharp rise in ANA positivity at 6 mo with almost all becoming positive by 8 mo. In contrast, the increase in ANA positivity was delayed until 12 mo in the EFA-d animals.

Figure 4 shows a similar delay in onset of dsDNA antibody production in the EFA-d animals compared to the control group. Anti-dsDNA antibodies were undetectable until the EFA-d animals had reached 12 mo of age. The frequency of positivity then increased to a maximum of 80% by 18 mo.

Subepidermal Immunoglobulin Staining

One of the most striking differences between these 2 groups of animals was in the frequency of subepidermal immunoglobulin deposits, as shown in Fig 5. As expected, the control animals showed a sharp increase in percentage of subepidermal immune deposits between 6 and 9 mo of age. All except one control animal died during the same period. In contrast, none of the 10 EFA-d animals biopsied at 6, 7.5, 9, 10.5 and 12 mo showed the slightest trace of immunoglobulin deposited in the skin.

Figure 6(*a*) shows a typical example of the heavy subepidermal immunoglobulin deposits found in skin from the untreated control mice. Figure 6(*b*) shows tissue that was taken from a 20-mo-old female B/W mouse maintained on the EFA-d diet for 18 mo. Only an autofluorescent hair is present in the dermis. The subepidermal area is completely free of immunoglobulin.

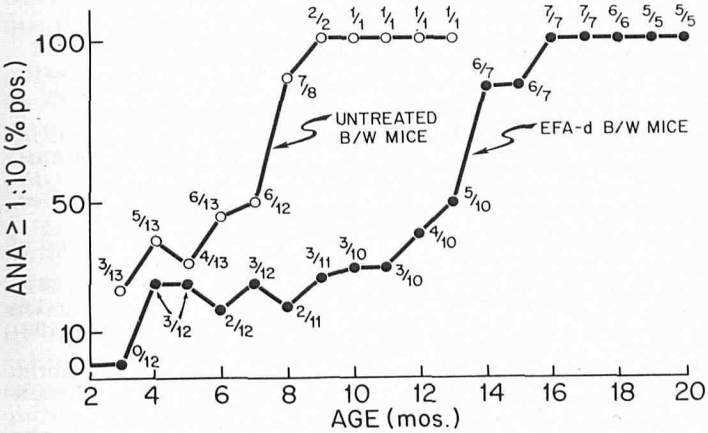

FIG 3. Percent positivity of antinuclear antibodies in untreated and EFA-d animals. EFA-d *vs* untreated ($p < .05$).

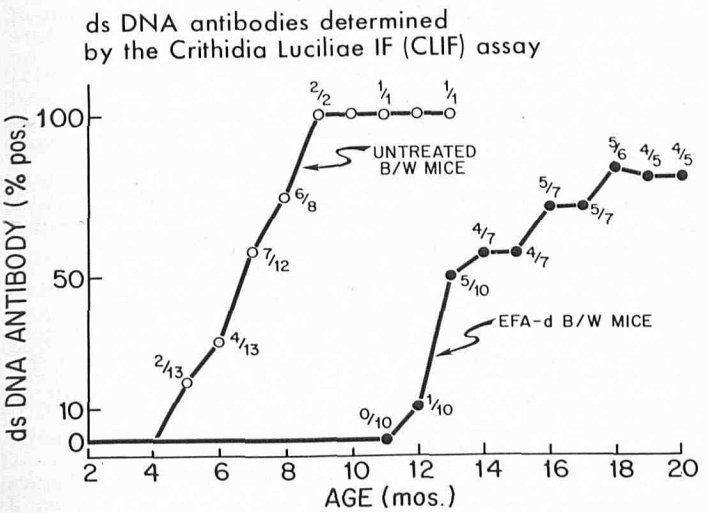

FIG 4. Anti-dsDNA antibodies in untreated and EFA-d animals. EFA-d *vs* untreated ($p = .02$).

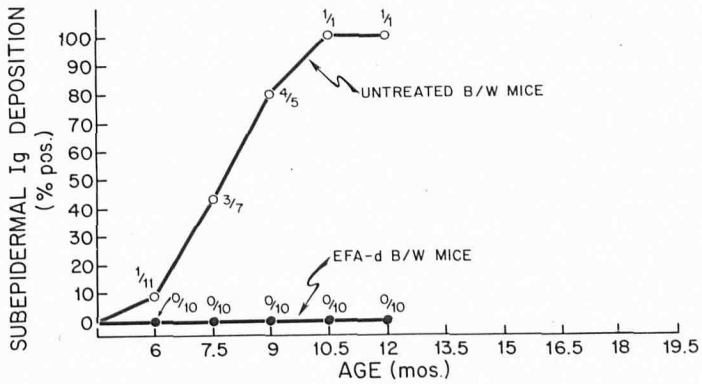

FIG 5. Subepidermal Ig deposition in untreated and EFA-d animals. EFA-d *vs* untreated ($p < 0.001$).

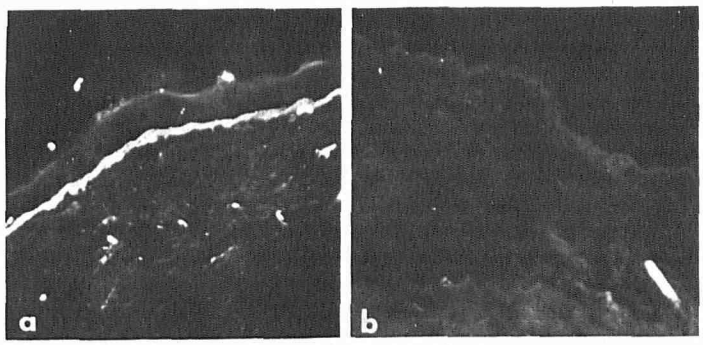

FIG 6. *a*, Example of heavy subepidermal IgG deposits in an 8-mo-old untreated female B/W mouse; *b*, Skin biopsy from a 20-mo-old female B/W maintained on an EFA-d diet. No subepidermal staining is present.

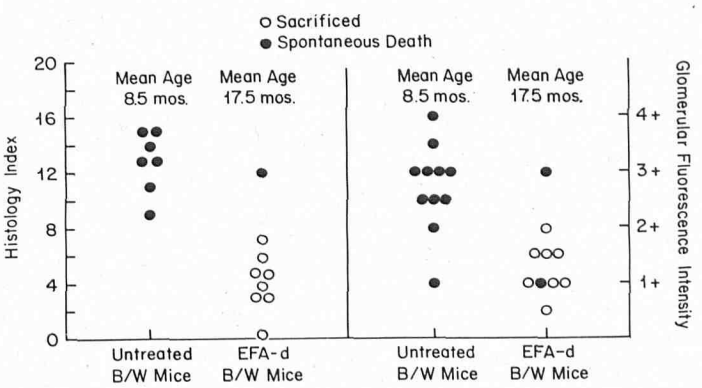

FIG 7. Kidney histology and glomerular Ig fluorescence staining in untreated and EFA-d animals. The *open circles* represent animals sacrificed at the termination of the study whereas *closed circles* are animals sacrificed when moribund. The mean age of the animals at the time tissues were obtained is shown at the top of each group. For histology index, EFA-d *vs* untreated ($p = .02$). For Ig values, EFA-d *vs* untreated ($p = .02$).

Kidney Histology and Immunopathology

Figure 7 shows the renal pathology and renal immunopathology in both study groups. The histologic changes (histology index) were graded on a predefined zero (normal) to 20 scale. As shown in this Figure, the renal pathology was significantly more advanced in the untreated animals even though this group was considerably younger (mean age 8.5 mo) than the EFA-d group (mean age 17.5 mo). The only EFA-d animal that died spontaneously had histologic changes in the same range as seen

in the control group. Glomerular fluorescence intensity was graded on a 0 to 4+ scale. The differences in fluorescence intensity are less striking than the histology index but the differences between the control and EFA-d group were significant ($p = .02$).

DISCUSSION

In the present study, the following indices of disease severity were significantly altered in animals receiving the EFA-d diet: (1) a delay in autoantibody production; (2) less severe renal disease; (3) absence of subepidermal immunoglobulin deposits; and (4) a marked increase in survival.

These findings clearly indicate that essential fatty acid deficiency dramatically alters the course of the lupus-like disease in NZB/W mice. The mechanism of this beneficial effect is unclear. Fernandes and others have shown increased survival in B/W mice on restricted caloric intake [11,12]. However, we do not believe that simple under-feeding played a significant role in the beneficial effects observed in the EFA-d animals. Both mean weights and percentage weight gained were similar in both study groups. In recent studies, Zurier and co-workers [13,14] have demonstrated increased survival and lessening of renal disease in B/W mice given pharmacological amounts of PGE_1. In these studies PGE_1 minimally influenced autoantibody production suggesting that the improvement was due to an antiinflammatory effect.

A number of studies have examined the effect of EFA-deficiency on various animals models of immunologic disease and inflammation. In studies by Denko [15], adjuvant arthritis was suppressed in rats deficient in EFA. The reduction in the chronic phase of adjuvant inflammation was restored to usual levels by feeding a small supplement of corn oil as a dietary source of EFA. It was concluded that the EFA deficiency diminished the adjuvant-induced inflammation by reducing available PG in the mediation of inflammation. Using various animal models of chronic inflammation, Bonta and co-workers [16–18] found a reduction in inflammation in EFA-deficient rats. They showed that carrageenin induced hind paw inflammation is suppressed partially in EFA-deficient rats and that arachidonic acid given to these animals restored the suppressed carrageenin inflammation [17]. Using kaolin-induced pouch granulomas, these same investigators demonstrated a reduction of exudate production in EFA-deficient rats when compared with normal animals [18]. The exudates from normal rats contained large amounts of PGE but in the exudates from EFA-deficient rats the amount of PGE was reduced very markedly.

In studies by Mertin and Hunt [19], mice fed a diet deficient in polyunsaturated fatty acids showed a relative immunopotentiation, as indicated by accelerated skin allograft rejection and decreased incidence and rate of development of methycholanthrene induced tumors. Their cell-mediated immune responses appeared to be potentiated. Enhancement of delayed hypersentivity to PPD in rats fed an EFA-deficient diet has also been reported by Parnham and co-workers [20]. Thus, EFA-d is associated with a reduced inflammatory response and an enhanced cell-mediated immune response.

Since arachidonic acid is preferentially incorporated into the phospholipids of activated lymphocytes [21] and constitutes nearly 20% of the total phospholipid fatty acid content of macrophage membranes [22], EFA-d might conceivably impair function of these 2 cell types. Such an impairment of function might result in a beneficial effect in the pathogenesis of B/W mouse disease.

The present findings suggest that the overall physiological effect of arachidonate metabolites on the autoimmune process in B/W mice is more harmful than helpful. Indeed, some of the prostaglandins or related products of arachidonic acid metabolism, e.g., prostacyclin, thromboxane or products of the lipoxygenase pathway, may be necessary for the full expression of autoimmunity in these animals. Studies of the role of arachidonate metabolites in regulating the immune system and inflammatory responses in mice may lead to a more rational approach to the treatment of autoimmune diseases in man. Such studies are currently in progress in our laboratories.

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