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Biochimica et Biophysica Acta 1223 (1994) 185–194

BBA Biochimica et Biophysica Acta

Inhibition of cytokine-stimulated thymic lymphocyte proliferation by fatty acids: the role of eicosanoids

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Received 13 December 1993

Abstract

The effect of individual fatty acids on the proliferation of thymic lymphocytes in response to interleukin-1 (IL-1) was investigated. Proliferation was estimated by measuring [^{3}H]thymidine incorporation into the acid insoluble fraction of the thymocytes. A concentration-dependent inhibition (in the range 1–100 μM) in the IL-1-stimulated proliferation was observed with the C_{20} fatty acids dihomo-γ-linolenic acid (DGLA), arachidonic acid and eicosapentaenoic acid (EPA). A less pronounced concentration-dependent inhibitory response was observed with the C_{18} fatty acids linoleic acid, α-linolenic acid and γ-linolenic acid. Palmitic acid and oleic did not have any effect on either basal or IL-1-stimulated proliferation at concentrations up to 100 μM. The potencies of each fatty acid for this effect at a concentration of 100 μM were: arachidonic acid > EPA ≥ DGLA > linoleic acid. DGLA, arachidonic acid and EPA also attenuated IL-2-stimulated proliferation. The inhibitory action of these fatty acids was not mediated by conversion to prostaglandins or other eicosanoids as the cyclooxygenase inhibitor, ketoprofen and NDGA did not alter their action. Incubation of thymocytes with radiolabelled DGLA and EPA followed by reverse-phase HPLC analysis revealed that DGLA is predominantly converted to a more polar metabolite which is not PGE_1 whereas EPA does not appear to be converted to any other detectable metabolite. The data indicate that the inhibitory actions of fatty acids on cell proliferation do not occur as a result of conversion to other metabolites but may be direct effects. The inhibition of cytokine-stimulated lymphocyte proliferation by unsaturated fatty acids would imply that they may attenuate cell-mediated immune reactions.

Key words: Fatty acid; Interleukin-1; Lymphocyte; Icosanoid; Cytokine

Introduction

Interleukin-1 (IL-1) plays a major role in host defence mechanisms during infection, it is an important immunoregulatory signal and is thought to be necessary for the development and proliferation of T-cells in the thymus [1]. This is the most commonly described action of IL-1 [2] as it greatly augments the proliferation of thymic lymphocytes and many T-helper cell lines in response to the low level proliferation induced by mitogenic lectins [3]. The amplification of cell proliferation by IL-1 is thought to occur via the induction of IL-2 [3]. This amplification step is thought to be an extremely important step in the maintenance and propagation of a cell-mediated immune response. The activation of target cells by IL-1 also often results in the biosynthesis of eicosanoids especially PGE_2 from almost every cell type stimulated by IL-1. Many of the actions of IL-1 are mediated by PGE_2 such as fever and inflammation [1]. However, very little information is available regarding the effect of the precursor fatty acids for prostanoid biosynthesis and interactions with other fatty acids which are not precursors for eicosanoid production. DGLA, arachidonic acid and EPA are the immediate precursors for the formation of 1-, 2- and 3-series prostaglandins respectively [4] and would, therefore, be expected to have some effect on immune responsiveness. Dietary lipids and some individual fatty acids have been shown to have a variable effect on a

Abbreviations: DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; IL, interleukin; NDGA, nordihydroguaiaretic acid; PGE, prostaglandin E; PHA, phytohaemagglutinin; TCA, trichloroacetic acid.

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SSDI 0167-4889(94)00079-T

variety of immune parameters especially lymphocyte proliferation in response to mitogenic lectins such as concanavalin A and phytohaemagglutinin [5–9] but there are few data regarding a systematic study of the direct effects of individual fatty acids on cytokine-stimulated proliferation.

Here we report that unsaturated fatty acids, especially arachidonic acid and EPA, can suppress the cytokine-stimulated proliferation of thymic lymphocytes which are not mediated via a conversion to eicosanoids. This indicates that unsaturated fatty acids can directly suppress lymphocyte proliferation and may thus attenuate cell-mediated immune reactions.

Materials and methods

Materials

All fatty acids, lipopolysaccharide (from *Salmonella abortus equi*), phytohaemagglutinin (from *Phaseolus vulgaris*), ketoprofen, ketoconazole, NDGA and endotoxin assay kits (E-Toxate) were obtained from Sigma. Recombinant (human) interleukins were obtained from Boehringer Mannheim (Sussex, UK). Analar reagents were used throughout. [^{3}H]Thymidine and radiolabelled fatty acids were obtained from Amersham International (England). RPMI 1640 culture medium and Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) were obtained from Gibco (Scotland).

Cell preparation

Thymocytes were prepared from male Aston strain mice, 3–6 weeks old. The thymus was aseptically removed, transferred to a sterile petri dish and washed with HBSS. Each thymus was teased apart using sterile forceps and its contents passed through a sterile 60 gauge nylon mesh, to obtain a single cell suspension, into a sterile tissue culture petri dish containing HBSS. The isolated thymocytes were left to equilibrate for 2 h at 37°C, 5% CO_2 in air, 100% humidity. The suspension was then decanted into a sterile universal container, centrifuged at $400 \times g$ for 10 min at room temperature, the supernatant was discarded and the cell pellet was resuspended in 3 ml of RPMI 1640 in the following incubation medium components: 10% heat inactivated foetal calf serum, 50 μM 2-mercaptoethanol, 200 IU/ml penicillin, 200 μg/ml streptomycin and 1.0 μg/ml PHA. Cells were counted and the volume of cell suspension adjusted to give 6×10^7 thymocytes/ml. Cell viability was determined using trypan blue exclusion and routinely found to be greater than 90%.

Incubation of cells with fatty acids

Thymocytes were incubated in the absence or presence of IL-1 or IL-2 without or with various concentrations of fatty acids. Fatty acids were initially dissolved in ethanol and prepared immediately before being used (in order to minimise oxidation) by being diluted 1000-fold in RPMI 1640 containing 5% foetal calf serum as a carrier. In control incubations, without fatty acid, the same volume of ethanol was diluted in RPMI 1640 containing foetal calf serum and identical volumes to those containing fatty acids were used. The concentrations of fatty acids were adjusted in RPMI 1640/5% foetal calf serum to yield the appropriate final concentration. Incubations were prepared in quintiplicate by adding up to 100 μl of the relevant substance (i.e. fatty acid, inhibitor, cytokine etc.) to 100 μl of thymocyte suspension (6×10^6 cells/well) in a final volume of 200 μl in 96-well, flat-bottomed tissue culture plates. The final incubation medium thus consisted of RPMI 1640 containing 5% heat inactivated foetal calf serum, 25 μM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/ml PHA. The 96-well plates were incubated at 37°C, 5% CO_2 in air and 100% humidity for 48 h. Cultures were then pulsed with 0.5 μCi [^{3}H]thymidine in 20 μl of RPMI 1640 per well and incubations continued for a further 24 h. Cells were aspirated from each well and transferred to Eppendorf tubes containing 600 μl of 10% TCA. Each well was then washed twice with 200 μl of HBSS and the washings transferred to the respective tubes. All tubes were then centrifuged at $13{,}000 \times g$ for 10 min, the supernatant was discarded and the pellet of acid insoluble material washed with 1 ml of 5% TCA. 0.5 ml solubilising solution (0.1 M NaOH; 2% w/v, sodium lauryl sulphate) was added to each tube and tubes were left for 24 h at room temperature. Solubilised samples were transferred to plastic scintillation vials and scintillation fluid was added to each vial. Samples were then counted for 5 min by liquid scintillation spectrometry and results were expressed as mean disintegrations per minute (dpm) after quench correction.

Incubation of radiolabelled fatty acids with thymocytes and analysis by reverse-phase HPLC

Materials. Solvents, supplied by BDH (Poole, UK) were HPLC grade and filtered at 0.2 μM using an all-glass vacuum filtration unit (Millipore, UK). Water was double distilled, deionised, reverse-phase filtered and passed through a 0.2 μm filter. A Waters 625 LC HPLC system containing a Nova-Pak C_{18} reverse-phase column (300 mm × 3.9 mm, 4 μm particle size) thermostatically controlled at 25°C, with a Wisp 712 autosampler (Millipore, UK), was used for HPLC chromatographic separation of free fatty acids and their metabolites. Detection of radiolabelled compounds was made by an on-line liquid scintillation radioactivity monitor (Reeve Analytical, Glasgow, UK), the scintillation fluid used was Flo-Scint A (Canberra Packard, Pangbourne, UK). Data recording and analysis of radiodetector

responses were performed by Dynamax HPLC Method Manager software (Rainin Instrument, MA, USA) running on a Macintosh IIfx computer (Apple, UK).

Preparation of samples for HPLC analysis. Samples for analysis by HPLC were pretreated using an ODS-silica cartridge method modified from Borgeat et al. [10]. Two fractions were obtained from each cell sample by the modified ODS-silica procedure. Fraction A was not retained by the ODS-silica cartridge (Sep Pak C_{18}, Millipore, USA), while fraction B was retained at pH 7.2. This modification was made in order to concentrate polar products and to reduce interference in the analysis by the precursor polyunsaturated fatty acids. Sep-Pak C_{18} cartridges were conditioned by methanol (2 ml) and water (10 ml).

Cells were aspirated from the multiwell plate and placed into an Eppendorf tube, the cells were then pelleted by centrifugation, $400 \times g$ 5 min at 20°C. The supernatant (400 μl) was transferred to a fresh Eppendorf tube containing methanol (150 μl) and water (450 μl). The resulting mixture, at pH 7.2, was applied directly to a conditioned C_{18} Sep-Pak cartridge, previously prepared as described above. The cartridge was then washed with methanol/water (15:85 v/v, 5 ml) then with water (5 ml), eluate and washings were collected in one test tube and termed fraction A. Methanol (2.0 ml) was passed through the cartridge, the first 0.5 ml eluate was discarded, the next 1.5 ml was collected and termed fraction B. Fraction A was acidified to pH 3.5 by the addition of HCl (1.0 M) and was reapplied to another conditioned C_{18} Sep-Pak cartridge. The initial methanol/water (15:85 v/v, 5 ml) and water (5 ml) eluates were discarded followed by passing methanol (2.0 ml) through the cartridge, the first 0.5 ml eluate was discarded and the subsequent 1.5 ml was collected and termed fraction C. Fractions B and C were separately processed by evaporation to dryness under N_2 (g), < 30°C, and were then reconstituted in methanol (225 μl) and transferred to vials for the autosampler for HPLC analysis.

Analysis of fractions by rp-HPLC. Reverse-phase HPLC analysis was conducted as described by Powell [11] using the following solvent compositions to generate the elution gradients used for the separation of fatty acids and polar metabolites: solvent I – acetonitrile/water/acetic acid (25:75:0.01 v/v/v), solvent II – methanol/acetonitrile/TFA (2%) (60:40:0.08 v/v/v). Methanol solutions (200 μl) of the separate fractions were applied to the rp-HPLC column in solvent I/solvent II (95:5 v/v) at a flow rate of 0.5 ml/min and the following gradient conditions were used for subsequent chromatographic separation and analysis. Initial conditions were maintained for 2 min then the flow rate was increased to 1 ml/min. A linear gradient of solvent I/solvent II (95:5 v/v) to solvent I/solvent II (20:80 v/v) was used between 5 min and 60 min. A second linear gradient of solvent I/solvent II (20:80 v/v) to solvent II (100%) was applied from 60 to 63 min, and maintained at this value from 80 to 100 min. A return to initial conditions was achieved by a linear gradient from 100 to 110 min.

Prostaglandin E_2 assay

Thymocytes were incubated exactly as described for the [^{3}H]thymidine incorporation (proliferation) assay and aliquots of the supernatant (5 μl) were collected for estimation of PGE_2. The sample of supernatant was diluted in HBSS to yield a PGE_2 concentration of 0.5–20 pg/100 μl which was within the range of the standard curve (0.25–25 pg/100 μl) for the PGE_2 assay.

PGE_2 was measured by radioimmunoassay using a kit supplied by New England Nuclear (Germany). The HBSS-diluted sample was used directly in the assay which was carried out exactly as described in the kit. Neither the HBSS, RPMI nor any of the agents used in this study was shown to alter the PGE_2 standard curve.

Results

The effect IL-1 and phytohaemagglutinin on thymocyte proliferation

Proliferation was estimated by measuring the incorporation of [^{3}H]thymidine into the acid insoluble fraction of non-adherent thymocytes. Initial experiments were carried out to ascertain the responsiveness of these thymocytes to PHA, IL-1β, and LPS. PHA alone increased the incorporation of [^{3}H]thymidine into thymocytes in a concentration-dependent manner with a threshold of 0.25 μg/ml and a maximal incorporation with 1.5 μg/ml. An approx. 35% maximal increase in proliferation was obtained with 0.5 μg/ml PHA and this concentration was included in all subsequent incubations to produce a suboptimal proliferation. IL-1α and IL-1β both increased the incorporation of [^{3}H]thymidine into thymocytes. The incorporation of radioactivity into cells from control incubations, with PHA alone, was 343 ± 103 dpm which increased to 3876 ± 672 dpm with IL-1α (10 ng/ml) and 4533 ± 332 dpm with 10 ng/ml IL-1β (all values are means of $n = 5$ $\pm$S.D.). The EC_{50} concentration for IL-1β-stimulated [^{3}H]thymidine incorporation was 25.22 ng/ml (1.45 nM).

In a separate series of experiments it was observed that the thymocytes were not responsive to LPS concentrations between 1 ng/ml and 10 μg/ml. The radioactivity incorporated into the acid insoluble fraction from thymocytes incubated with the highest concentration of LPS used (10 μg/ml) was 1576 ± 336 dpm compared with 1232 ± 249 dpm from control incubations (means $\pm$ S.D. for $n = 5$), indicating that these

cells would not be responsive to changes in endotoxin levels in any of the solutions used.

The effect of fatty acids on thymocyte proliferation ([^{3}H]thymidine incorporation)

A variety of fatty acids were incubated with thymocytes in the absence or presence of IL-1β (50 ng/ml) and subsequently measuring the [^{3}H]thymidine incorporated into the acid insoluble fraction. Each fatty acid was incubated with thymocytes at various concentrations between 1 μM and 100 μM. The fatty acid preparations at the concentrations used were tested for their endotoxin content using a commercial Limulus Amoebacyte lysate assay. All the fatty acids prepared in the final incubation medium at 100 μM contained less than 200 pg/ml endotoxin which was not greater than the level in medium alone ($< 200 > 100$ pg/ml).

Palmitic acid (16:0) did not have any significant effect on the IL-1-stimulated (50 ng/ml) incorporation of [^{3}H]thymidine into thymocytes at concentrations up to 10 μM palmitic acid. At 100 μM a slight decrease was observed (28.4 $\pm$ 15.4%, $P < 0.05$, means of $n = 5$ $\pm$S.D.). Higher concentrations of palmitic acid (200 μM) did not have any further effect. In the absence of IL-1 palmitic acid produced an apparent but not significant increase in proliferation at 100 μM. Oleic acid (18:1($n-9$)) did not significantly alter the incorporation of radioactivity into thymocytes either in the absence or presence of IL-1 (50 ng/ml) at concentrations up to 100 μM (Fig. 1). Higher concentrations were not used.

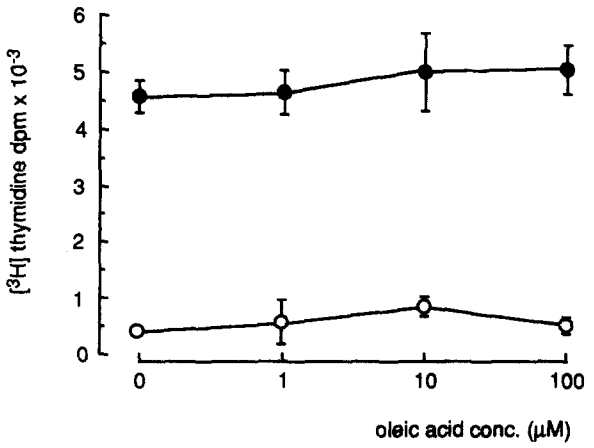

Fig. 1. The effect of oleic acid on the incorporation of [^{3}H]thymidine into thymocytes. Thymocytes were incubated with various concentrations of oleic acid in the absence (○) and presence of IL-1β (●, 50 ng/ml) for 72 h and the radioactivity (from [^{3}H]thymidine added for the last 24 h) incorporated into the acid insoluble fraction was measured. Values are the means of $n = 5$ $\pm$S.D. and are representative of three separate experiments.

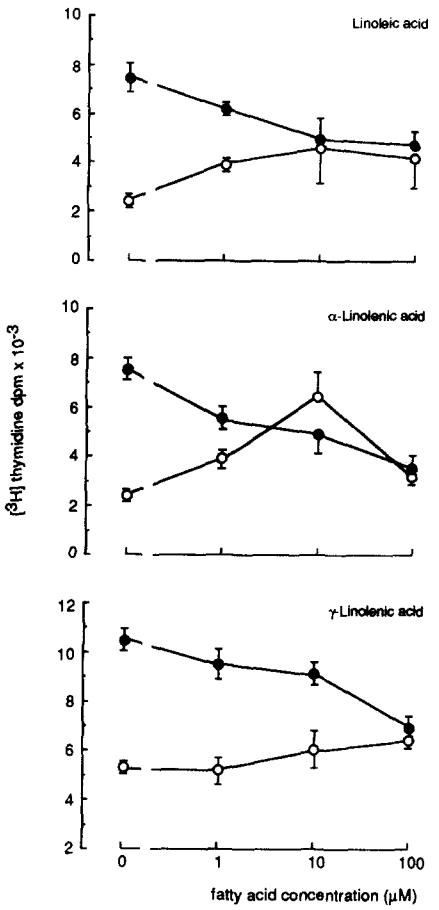

Fig. 2. The effect of the C_{18} fatty acids linoleic acid, α-linolenic and γ-linolenic acids on the incorporation of [^{3}H]thymidine into thymocytes. As for Fig. 1.

Linoleic acid (18:2($n-6$)), γ-linolenic acid (18:3($n-6$)) and α-linolenic acid (18:3($n-3$)) produced a concentration-dependent decrease in the IL-1-stimulated incorporation of [^{3}H]thymidine into thymocytes (Fig. 2). However, a small but significant increase in [^{3}H]thymidine incorporation was observed in thymocytes incubated with either of these three fatty acids in the absence of IL-1 (Fig. 2). The C_{20} fatty acids DGLA (20:3($n-6$)), arachidonic acid (20:4($n-6$)) and EPA (20:5($n-3$)) also produced a concentration-dependent decrease in the IL-1-stimulated incorporation of [^{3}H]thymidine into thymocytes which was qualitatively similar to the C_{18} fatty acids (Fig. 3). However, in incubations with fatty acid alone no effect on the [^{3}H]thymidine incorporation was observed.

In order to directly compare the potencies of the various fatty acids these were incubated at a concentration of 100 μM (which appeared to produce a maximal inhibition by those fatty acids which were inhibitory) in the absence and presence of IL-1β (50 ng/ml) and subsequently measuring the inhibition of [^{3}H]thymidine incorporation. The most profound inhibition was

observed with arachidonic acid which produced an almost complete inhibition of the IL-1-stimulated proliferation (Fig. 4). EPA was less inhibitory than arachidonic acid but more inhibitory than DGLA. The C_{18} fatty acids were less inhibitory than any of the C_{20} fatty acids and palmitic acid had very little effect with oleic acid having no effect (Fig. 4).

Prostaglandins of the E series were also inhibitory to the IL-1-stimulated thymocyte proliferation with a threshold above 10 nM, whereas basal proliferation was unaffected. All of the prostaglandins were inhibitory at 100 nM. Prostaglandin E_1 produced an inhibition of $51.1 \pm 6.6\%$, PGE_2 a $40.3 \pm 7.1\%$ and PGE_3 a $47.8 \pm 4.9\%$ decrease all at a concentration of 100 nM (means $\pm$ S.D., $n = 4$). Indicating that all of the E series prostaglandins were essentially equipotent in the suppression of IL-1-stimulated proliferation. The possibility that the effects of the fatty acids were mediated via their conversion to prostanoids was investigated by studying the effects of the cyclooxygenase inhibitor ketoprofen. We have previously shown that ketoprofen at a concentration of 20 μM can produce a

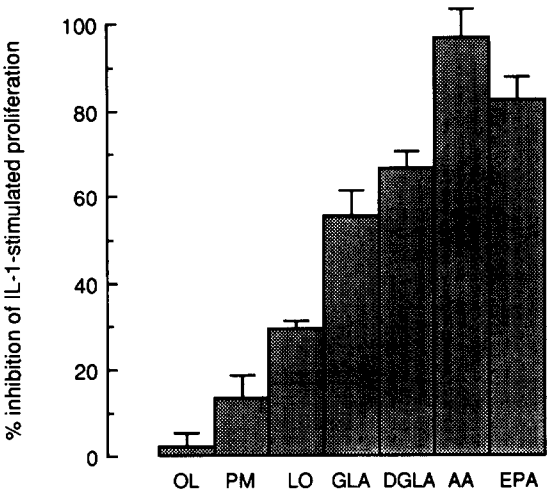

Fig. 4. Direct comparison of the effects of different fatty acids on the incorporation of [^{3}H]thymidine into thymocytes. Thymocytes were incubated with 100 μM of the appropriate fatty acid in the absence (open columns) and presence of IL-1β (shaded columns, 50 ng/ml) for 72 h and the radioactivity (from [^{3}H]thymidine added for the last 24 h) incorporated into the acid insoluble fraction was measured. Values are the means of $n = 5$ $\pm$S.D. and are representative of three separate experiments.

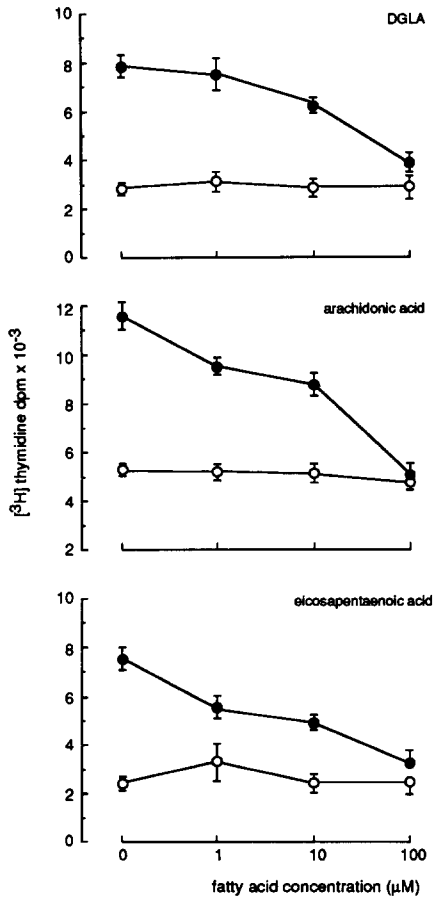

Fig. 3. The effect of the C_{20} fatty acids dihomo-γ-linolenic acid (DGLA), arachidonic acid and eicosapentaenoic acids on the incorporation of [^{3}H]thymidine into thymocytes. As for Fig. 1.

considerable inhibition of PGE_2 release from lymphocytes. In this study the effect of ketoprofen on PGE_2 production was confirmed by incubating thymocytes under exactly the same conditions as for the [^{3}H]thymidine uptake assays for the estimation of cell proliferation. The level of PGE_2 in supernatants from control cells was 856 ± 78 pg/well and in the presence of ketoprofen an inhibition in the order of almost 90% was observed with a decrease to 102 ± 48 pg PGE_2/well (means of $n = 4$ $\pm$S.D.). Thus, the effect of ketoprofen (20 μM) was studied on the inhibition of IL-1-stimulated thymocyte proliferation. Ketoprofen did not significantly alter the concentration-dependent inhibitory action of either arachidonic acid (Fig. 5) or γ-linolenic acid (not shown). A direct comparison was carried out where the effect of ketoprofen was studied on the inhibition of thymocyte proliferation in response to 10 μM of each of the fatty acids. The inhibitory response of all fatty acids used was unaltered by ketoprofen (Fig. 6). NDGA, which is known to suppress the synthesis of lipoxygenase products, was also used in several experiments with DGLA and arachidonic acid. NDGA did not significantly affect the inhibitory actions of either DGLA or arachidonic acid (both 10 μM) on IL-1-stimulated thymocyte proliferation. The control IL-1-stimulated incorporation was 7433 ± 632 dpm which was reduced to 2689 ± 431 dpm and 2178 ± 543 dpm with DGLA and arachidonic acid respectively. In the presence of NDGA (20 μM) the values were 2596 ± 344 dpm and 2022 ± 412 dpm for DGLA and arachidonic acid respectively (all values are the means of $n = 4$ $\pm$S.D.).

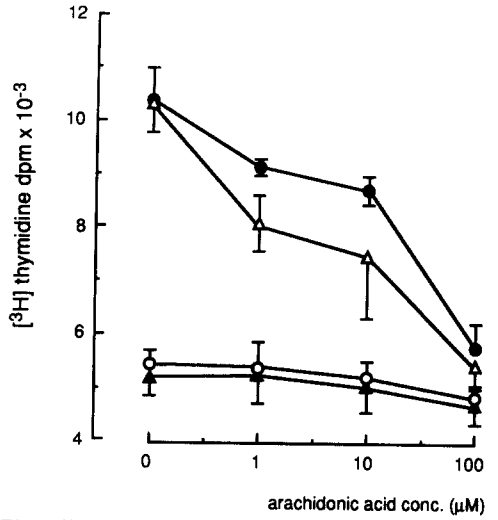

Fig. 5. The effect of ketoprofen on the arachidonic acid-induced suppression of thymocyte proliferation. Thymocytes were incubated either in the absence of any additions (○), or with IL-1β (50 ng/ml, ●), 20 μM ketoprofen alone (▲) or IL-1β + ketoprofen (△) for 72 h and the radioactivity (from [^{3}H]thymidine added for the last 24 h) incorporated into the acid insoluble fraction was measured. Values are the means of $n = 5$ ±S.D. and are representative of at least three separate experiments.

The effects of ketoconazole on the DGLA inhibition of IL-1-stimulated thymocyte proliferation was also estimated in order to indicate whether the epoxygenase pathway was involved in the inhibitory response. Ketoconazole at a low concentration (1 μM) did not significantly affect the DGLA attenuation of IL-1-stimulated proliferation with values of 3421 ± 323 dpm with DGLA alone and 3599 ± 341 dpm with DGLA in the presence of ketoconazole. The level of [^{3}H]thymidine incorporation in cells with IL-1 alone was 8312 ± 512 dpm and for IL-1 in the presence of ketoconazole the value was 8198 ± 433 dpm. Ketoconazole at 10 μM slightly augmented the inhibition by DGLA and reduced the level of [^{3}H]thymidine incorporation to 2766 ± 224 dpm ($P < 0.05$ compared to the value for the DGLA attenuation of IL-1 proliferation above). The value for IL-1 in the presence of 10 μM ketoconazole alone was 8223 ± 441 dpm.

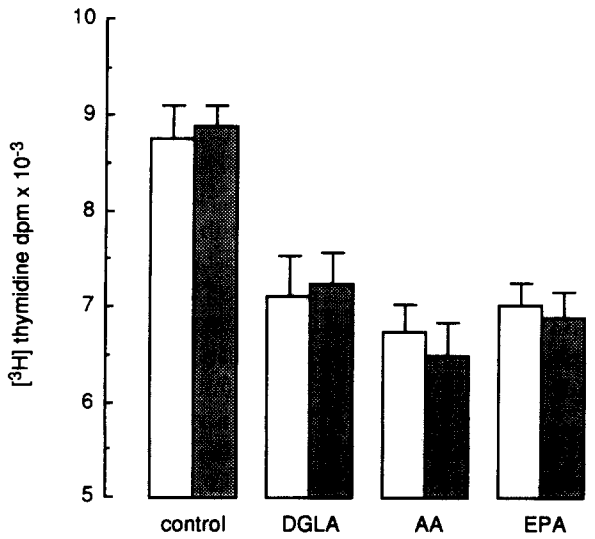

Fig. 6. The effect of ketoprofen on the fatty acid-induced suppression of thymocyte proliferation in response to IL-1. As for Fig. 5 except that thymocytes were incubated with either IL-1β alone (control) or with 100 μM of dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) or eicosapentaenoic acid (EPA) in the absence (open columns) or presence of 20 μM ketoprofen (shaded columns).

The effect of fatty acids on cell viability and number

It is well established that the detergent-like action of high concentrations of fatty acids may affect the viability of cells. The effect of the different fatty acid treatments on the viability of the non-adherent thymocytes used in this study was assessed by estimating the percentage of viable cells at the end of incubations compared to control cells (100%) incubated without fatty acid. The cells were incubated under identical conditions as those used in the experiments measuring the proliferation of thymocytes and a fixed concentration of 100 μM fatty acid was used. None of the fatty acids used (palmitic, oleic, DGLA, arachidonic and EPA) altered the percentage of viable cells. The viability in control incubations was 91.1 ± 2.2% and remained unchanged after incubation with any of the fatty acids; the range was 98.8 ± 2.3% for palmitic acid and 101.3 ± 2.2% for arachidonic acid compared to control values (all values are the means of $n = 4$ ±S.D.).

In order to check that the effects of the fatty acids actually affected cell numbers after incubation with IL-1 in the absence and presence of the fatty acid cells were incubated with 100 μM fatty acid under identical conditions as described above followed by cell counting. Control incubations were designated 100% and the effects of fatty acids were expressed as a change from the control value. γ-Linolenic acid decreased the cell numbers recovered at the end of incubations by 22.1 ± 9.5%, DGLA by 26.3 ± 10.3% and arachidonic acid reduced the cell number by 33.2 ± 9.2% (all values are the means of $n = 5$ ±S.D.).

The effect of fatty acids on IL-2-stimulated proliferation of thymocytes

It is well established that the proliferation of lymphocytes in response to IL-1 is mediated by the production and subsequent actions of IL-2. Thus, several experiments were carried out to ascertain whether the fatty acids could also affect the proliferation of thymocytes in response to IL-2, the final mitogenic signal for T-lymphocytes. IL-2 increased the incorporation of [^{3}H]thymidine into the acid insoluble fraction of thymocytes in a concentration-dependent manner. The threshold concentration for an increase in proliferation

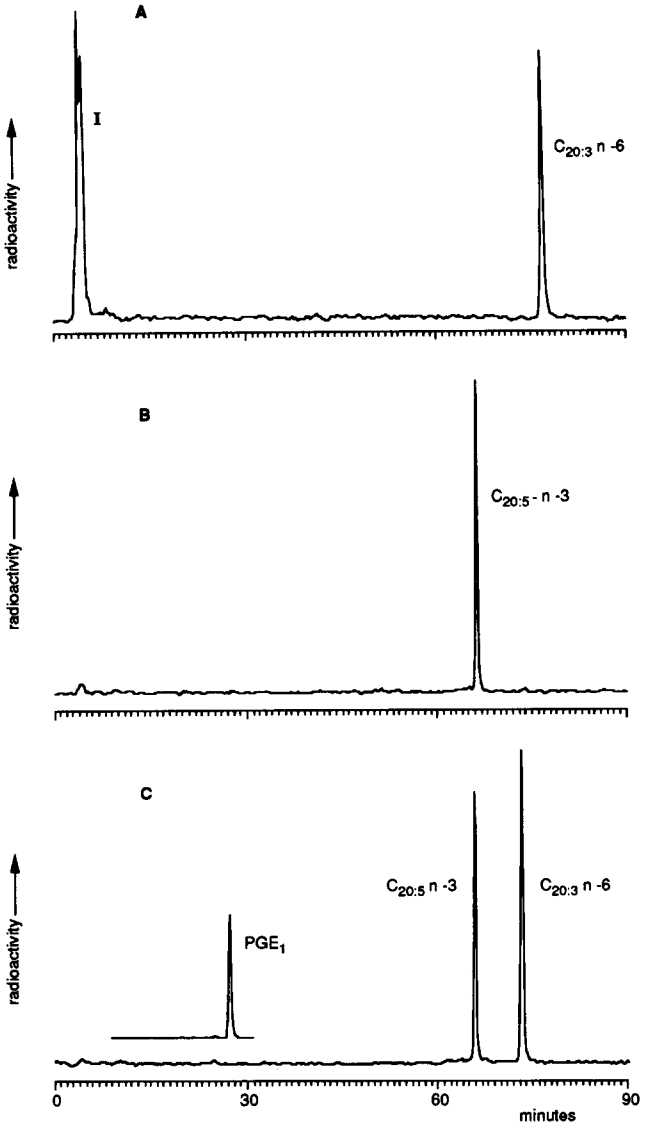

Fig. 7. HPLC analysis of radiolabelled fatty acids subsequent to incubation with thymocytes. Thymocytes were incubated in the presence of radiolabelled DGLA or EPA (1 μCi/well) for 72 h under identical conditions as those described for the [^{3}H]thymidine incorporation experiments (Figs. 1–6). The cell supernatants were collected and processed as described in Preparation of samples for HPLC analysis and the radioactivity analysed by reverse-phase HPLC (see Analysis of fractions by rp-HPLC). The upper trace (A) shows the profile of radioactivity from cells incubated with radiolabelled DGLA and indicates two peaks, the DGLA (20:3($n-6$)) and a major metabolite(s) at peak I. Trace B shows the radioactivity from cells incubated with eicosapentaenoic acid (20:5($n-3$)) and shows only one clear detectable peak which migrates with the same retention time as authentic EPA. Trace C shows the retention times of the respective radiolabelled standard DGLA and EPA, run together, which had been incubated under identical conditions as cell incubations except that cells were omitted. Trace C also shows for comparison the retention time of PGE_1 under these elution conditions It is not shown on the same axis as the DGLA and EPA as it was analysed in a separate run but in the same series of samples and the peak represents 7600 cpm.

was achieved between 0 and 50 units/ml of IL-2 and a maximal effect was obtained with 100 units/ml. The effects of DGLA, arachidonic acid and EPA on proliferation were studied with an IL-2 concentration of 100 units/ml. All three fatty acids at a concentration of 10 μM decreased the IL-2-dependent stimulation by 26.3 $\pm$ 6.3%, 38.9 $\pm$ 7.2% and 33.7 $\pm$ 4.6% for DGLA, arachidonic acid and EPA respectively (all values are the means of $n = 4$ $\pm$S.D.).

The metabolism of radiolabelled DGLA and EPA by thymocytes

Radiolabelled DGLA and EPA were incubated with thymocytes in the absence and presence of IL-1, under identical conditions to those used in the proliferation assays, in order to ascertain whether the thymocytes were able to convert the fatty acids to other mediators. Arachidonic acid, which in most cells is quickly converted to numerous other products, was not used in order to avoid a complex pattern of metabolites. The radiolabelled fatty acids were incubated with the thymocytes and the incubation medium was removed at the end of incubations for the HPLC analysis of released products. The cell-associated products were also analysed. DGLA was converted to a metabolite, possibly two, which were more polar than DGLA and eluted close to the solvent front in the predominantly aqueous conditions at the start of the gradient. Unchanged DGLA was also present in the incubation medium (Fig. 7). This pattern was also observed in the cell-associated radioactivity. This profile or the amount of the products was not changed in incubations carried out in the presence of IL-1. PGE_1 was not detected in any of the fractions. A standard PGE_1 profile was included in the analysis (Fig. 7). In contrast, EPA was not converted to any other detectable metabolite either in the incubation medium or the cell-associated free fatty acid fraction and only unconverted EPA was recovered (Fig. 7).

Discussion

The data presented clearly indicate that the IL-1-stimulated proliferation of thymic lymphocytes is suppressed in the presence of unsaturated fatty acids. The C_{20} fatty acids were more potent than the C_{18} fatty acids. The E series prostaglandins were also capable of inhibiting the IL-1-stimulated proliferation, however, the effect of the fatty acids, which are potential precursors of these prostaglandins, is unlikely to have occurred through conversion to prostanoids as the cyclooxygenase inhibitor, ketoprofen, did not alter their actions.

Previous studies have been conducted where unsaturated fatty acids have been given in the diet and the

lymphoproliferative response assessed ex vivo. The results of these studies, using mainly the $n-3$ family of fatty acids, are variable. Some indicate that dietary supplementation with polyunsaturated fatty acids reduces the proliferative response, while others have found either no detectable change [12] or that the proliferative response was greater in lymphocytes prepared from animals fed high levels of polyunsaturates [13]. A disadvantage of these studies is the use of oils of heterogeneous composition with respect to the spectrum of fatty acids present, as some fatty acids may have opposing actions and in vivo the integrated response may be manifested as zero change in activity. The composition of oils from natural sources can also vary widely from batch to batch and is occasionally prepared with other components such as antioxidants which could complicate the pattern of effect on bioactivity. In the present study only individual pure fatty acids were used providing an unambiguous indication of the effect of specific fatty acids without the potential complications of additives.

Several studies have investigated the direct actions of fatty acids in vitro on immune function especially lymphocyte proliferation [5–9]. These studies have documented the actions of fatty acids on mitogenic lectin-stimulated proliferation to simulate a non-specific antigenic stimulus. However, few data exist regarding the effect of fatty acids on cytokine-stimulated proliferation. This appears to be the normal pathway in cell-mediated immune responses where the low level proliferative response to antigen is greatly amplified via IL-1-induced IL-2 production [3]. The cytokine-augmented proliferation is very important in the generation of a cell-mediated response and inhibition of this pathway results in potent immunosuppression. The most successful immunosuppressive therapy, in order to prevent tissue rejection in organ transplantation, to date has been directed toward the inhibition of cytokine production by lymphocytes, specifically suppression of IL-2 production [14]. This is thought to be the mechanism by which therapeutic agents such as cyclosporin A exert their immunosuppressive actions.

The mechanism by which fatty acids used in this study can suppress cytokine-stimulated proliferation is not certain. As the cytokine pathway in lymphocyte proliferation involves the release of IL-2, in response to antigen or lectin in conjunction with IL-1, the inhibition of IL-2 release by fatty acids would thus be reflected as a decrease in the number of proliferating cells. A decrease in IL-2 release from human peripheral lymphocytes incubated with unsaturated fatty acids especially EPA and arachidonic acid has been reported by Calder and Newsholme [15] which correlates with a decrease in the proliferative response. However, in the present study it was shown that the proliferative response to IL-2 can also be attenuated by the C_{20} fatty acids EPA and arachidonic acid. This would indicate that the responsiveness of the cell to external stimuli, whether it is (a) the release of IL-2 or (b) the response to IL-2, is altered subsequent to incubation in the presence of the unsaturated fatty acids. The most common non-specific cellular action of lipid soluble agents is to penetrate the plasma membrane and alter its lipid dynamics (fluidity). Fatty acids have been shown to differentially alter membrane fluidity in a variety of different cells. In the study of Rand et al. [16] the provision of high levels of palm oil containing as much as 50% palmitic and other saturated fatty acids did not affect rat platelet membrane fluidity whereas oils containing high levels of linoleic acid (66%) and mainly polyunsaturated fatty acids significantly increased fluidity. This correlated with changes in platelet function in that an increased aggregability occurred with the polyunsaturates-rich oils and little or no effect on aggregation tendency was observed with the palmitic acid-rich oils [16]. The study of MacIntyre et al. [17] on the direct anti-aggregatory actions of unsaturated fatty acids also intimates a membrane site of action and a mechanism related to the physical properties of the fatty acids. The authors suggest that the biological behaviour of the different fatty acids correlates with differences in their melting point and hence their sequestration into separate domains in the membrane. This also correlated with changes in the membrane fluidity as measured by changes in the fluorescence polarisation of diphenylhexatriene [17]. A similar effect has also been reported in lymphocyte membranes where fatty acids affect receptor activity [18]. In the present study only the polyunsaturated fatty acids attenuated IL-1-stimulated proliferation with palmitic acid and oleic acid having little or no effect; a similar effect on membrane fluidity, analogous to those observed in the studies discussed above, could have occurred.

An increase in cytokine release in response to individual fatty acids has recently been reported in the lymphocyte murine T-helper cell line EL-4. The release of IL-2 from EL-4 cells is increased in response to both linoleic acid and arachidonic acid in the presence of phorbol myristate acetate where 10-fold and 8-fold increases respectively were reported [19]. However, no IL-2 release was detected from EL-4 cells in the absence of phorbol ester with fatty acid concentrations up to 200 μM [19]. It is possible that certain fatty acids may be able to regulate thymocyte responsiveness in a similar manner as not all of the fatty acids used in this study were effective. In the study of Bomalaski et al. [19] it was shown that IL-1 induces the release of IL-2 from EL-4 cells and that this can be mimicked by linoleic acid in the presence of phorbol ester. It was also shown that IL-1 stimulates phospholipase A_2 activity which specifically releases linoleic acid [19]. This suggests that linoleic acid may be a normal intra-

cellular regulator in EL-4 cells for IL-2 release and is released intracellularly in response to specific extracellular signals. Fatty acids such as arachidonic acid and DGLA may also be intracellular regulators and could be released under appropriate conditions and may subsequently be involved in the physiological downregulation of IL-1 responsiveness. These effects would be mimicked by the provision of extracellular fatty acids, which downregulated IL-1-stimulated proliferation of thymic lymphocytes in the present study, as they would be able to readily penetrate cells. It is also possible that these fatty acids can directly regulate gene expression and thus may regulate cell function at the gene level in a manner analogous to the direct regulation of genes by steroid hormones.

In the process of cellular differentiation the onset of differentiated function is associated with a downregulation of cell proliferation where mitogenic signals are suppressed and the cell upregulates mechanisms which are involved in differentiated function. However, very little is known of the intracellular or extracellular signals which control the onset of differentiation. In the present study the fatty acid-induced attenuation of IL-1-induced proliferation was not associated with a decrease in cell viability clearly indicating that although less cells were dividing in response to IL-1 in the presence of the fatty acids this did not occur as a result of cell death. It is possible that the fatty acids were exerting a differentiating influence on the cells with its associated switch-off of cell division. Arachidonic acid has been reported to be associated with the expression of differentiation markers in developing adipocytes [20,21], however, the mechanism by which this occurs is not known.

The inhibitory effects of the fatty acids used in this study raise the question of whether these are direct actions or are mediated via conversion to other metabolites. The three most effective fatty acids in the suppression of IL-1-stimulated activity were DGLA, arachidonic acid and EPA. These fatty acids are the immediate fatty acid precursors of the 1-, 2- and 3-series prostaglandins respectively. It is possible that the inhibitory actions of these fatty acids occur via the conversion to prostaglandins as prostanoids are generally inhibitory to many immune responses. In the present study prostaglandins E_1, E_2 and E_3 inhibited the proliferation of thymocytes in response to IL-1, therefore, conversion of the fatty acids to prostanoids would also have resulted in an inhibition. However, the study with ketoprofen, which shows that incubation with this inhibitor of prostaglandin synthesis does not reverse the actions of fatty acids, clearly indicates that prostaglandins are not involved in this action. This is further supported by the data obtained in experiments with radiolabelled DGLA and EPA where conversion to prostaglandins E_1 and E_3 by the thymocytes was not detected. It was, however, clearly demonstrated that the cells could produce PGE_2 indicating that there was a normal conversion of arachidonic acid to prostanoids. It is also unlikely that the inhibitory actions of the fatty acids are mediated by conversion to other pathways such as leukotrienes which appear to exert the opposite effect namely increase proliferation. Leukotriene B_4 has been shown to increase the proliferation of T-cells in response to low levels of IL-2 [22]. The failure of NDGA and ketoconazole to reverse the inhibitory actions of DGLA in the present study clearly indicates that the leukotriene and epoxygenase pathways are not involved in the inhibitory response. It is also unlikely that other oxidised metabolites are mediating this action as the most effective fatty acid was arachidonic acid whereas it would be expected that EPA with five double bonds would be oxidised to a greater extent and thus have a greater effect than either arachidonic acid with four double bonds or DGLA with three double bonds.

The data obtained clearly indicate that the activation of thymic lymphocytes by cytokines can be suppressed by unsaturated fatty acids. This would be an important aspect of immune activation as thymic lymphocytes play an important role in coordinating the cell-mediated immune response. It also indicates that it may be possible to modulate lymphocyte responses by modifying the spectrum of fatty acids in the diet. In addition, there are also implications for the modulation and possible impairment of immune responsiveness under circumstances in which the blood levels of free fatty acids are greatly elevated such as under intense exercise and in diabetes in which a chronic increase in blood levels of fatty acids is observed. Both intense exercise (as practised by athletes) and diabetes are associated with an increased susceptibility to infection and may be related to the sustained increases in free fatty acid levels.

Acknowledgements

This work was supported in part by the Scottish Office, Agriculture and Fisheries Department.

References

[1] Dinarello, C.A. (1991) Interleukin-1 and interleukin-1 antagonism. Blood 77, 1627–1652.
[2] Gery, I., Gerson, R.K. and Waksman, B.H. (1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. J. Exp. Med. 136, 128–142.
[3] Mizel, S.B. (1987) Interleukin-1 and T-cell activation. Immunol. Today 8, 330–332.
[4] Wahle, K.W.J. (1990) Dietary regulation of essential fatty acid metabolism and membrane phospholipid composition. Biochem. Soc. Trans. 18, 775–778.

[5] Johnston, P.V. (1983) Dietary fat, prostaglandins and immunity. Adv. Lipid Res. 21, 103–141.
[6] Calder, P.C., Bond, J.A., Bevan, S.J., Hunt, S.V. and Newsholme, E.A. (1991) Effect of fatty acids on the proliferation of concanavalin-A-stimulated rat lymph node lymphocytes. Int. J. Biochem. 23, 579–588.
[7] Valette, L., Croset, M., Prigent, A.F., Meskini, N. and Lagarde, M. (1991) Dietary polyunsaturated fatty acids modulate fatty acid composition and early activation steps of concanavalin A-stimulated rat thymocytes. J. Nutr. 121, 1844–1859.
[8] Calder, P.C., Bevan, S.J. and Newsholm, E.A. (1992) The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid independent mechanism. Immunology 75, 108–115.
[9] Chandra, R.K. and Amorin-Deganus, A.S. (1992) Lipids and immunoregulation. Nutr. Res. 12, S137–S145.
[10] Borgeat, P., Picard, S., Vallerand, P. and Sirois, P. (1981) Prostaglandins Med. 6, 557–563.
[11] Powell, W.S. (1985) Anal. Biochem. 148, 59–65.
[12] De Deckere, E.A.M., Jansen-Verplanke, C., Blonk, C.G. and Van Nielen, W.G.L. (1988) Effects of type and amount of dietary fat on rabbit and rat lymphocyte proliferation in vitro. J. Nutr. 118, 11–18.
[13] Carlomagno, M.A., O'Brian, B.C. and McMurray, D.N. (1983) Influence of early weaning and dietary fat on immune responses in adult rats. J. Nutr. 113, 610–617.
[14] Kasiaian, M.T. and Biron, C.A. (1990) Cyclosporine-A inhibition of interleukin-2 gene-expression, but not natural-killer-cell proliferation, after interferon induction in vivo. J. Exp. Med. 171, 745–762.
[15] Calder, P.C. and Newsholme, E.A. (1992) Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. Clin. Sci. 82, 695–700.
[16] Rand, M.L., Hennissen, A.A.H.M. and Hornstra, G. (1988) Effects of dietary palm oil on arterial thrombosis, platelet responses and platelet membrane fluidity in rats. Lipids 23, 1019–1023.
[17] MacIntyre, D.E., Hoover, R.L., Smith, M., Steer, M., Lynch, C., Karnovsky, M.J. and Salzman, E.W. (1984) Inhibition of platelet function by *cis*-unsaturated fatty acids. Blood 63, 848–857.
[18] Klausner, R.D., Bhalla, D.K., Dragsten, P., Hoover, R.L. and Karnovsky, M.J. (1980) Model for capping derived from inhibition of surface receptor capping by free fatty acids. Proc. Natl. Acad. Sci. USA 77, 437–441.
[19] Bomalaski, J.S., Steiner, M.R., Simon, P.L. and Clark, M.A. (1992) IL-1 increases phospholipase A_2-activity, expression of phospholipase A_2-activating protein and release of linoleic acid from the murine T helper cell line EL-4. J. Immunol. 148, 155–160.
[20] Amri, E.Z., Ailhaud, G. and Grimaldi, P. (1991) Regulation of adipose cell-differentiation. 2. Kinetics of induction of the ap2 gene by fatty-acids and modulation by dexamethasone. J. Lipid Res. 32, 1457–1463.
[21] Amri, E.Z., Bertrand, B., Ailhaud, G. and Grimaldi, P. (1991) Regulation of adipose cell-differentiation. 1. Fatty-acids are inducers of the ap2 gene-expression. J. Lipid Res. 32, 1449–1456.
[22] Atluru, D. and Goodwin, J.S. (1986) Leukotriene B_4 causes proliferation of interleukin 2-dependent T-cells in the presence of suboptimal levels of interleukin 2. Cell Immunol. 99, 444–452.