0014-4754/94/050489-04$1.50 + 0.20

Antiproliferative effect of polyunsaturated fatty acids and interleukin-2 on normal and abnormal human lymphocytes

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Received 2 June 1993; accepted 15 December 1993

Abstract. The polyunsaturated fatty acids (PUFAs), linoleic acid (LA), alpha linolenic acid (ALA), gamma linolenic acid (GLA), arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), showed inhibition of growth of both normal and abnormal (Molt-4) human lymphocytes, and inhibition was concentration-dependent. Interestingly, the production of the lymphokine Interleukin-2 (IL-2) was elevated in Molt-4 cells, but it was reduced in the normal human lymphocytes. Addition of GLA or IL-2 or a combination of both showed enhancement of SO_2^- and of lipid peroxidation levels, which were significantly higher in Molt-4 cells than in the normal lymphocytes. Reduction of protein concentration was also observed in both types of cells during this treatment. The data showed that the antiproliferative effects of GLA and IL-2 may partly be exerted through the elevated production of superoxide free radicals and peroxidation products. This is a novel finding and therefore, further exploitation of combinations of PUFAs and IL-2 may be a possible way of combating cancer cell growth.
Key words. Interleukin-2; polyunsaturated fatty acids; superoxide radical.

Interleukin-2 (IL-2) is a 15 kDa glycosylated protein produced by T cells[1]. It stimulates the proliferation of cytotoxic T lymphocytes and natural killer cells[2]. Recent studies reported that[3,4] the cytotoxicity was highly dependent upon the IL-2 dosage[5]. High levels of IL-2 also exerted powerful antimetastatic effects on several types of animal tumours[2].

Tumour cells are known to have low amounts of polyunsaturated fatty acids (PUFAs), owing to a deficiency or low levels of delta-6-desaturase activity[6,7]. In vitro studies have shown that some PUFAs and their metabolites exhibit cytotoxic effects on both tumour and normal cell growth[8–11]. Santoli et al.[12] recently reported that the inhibitory effects of dihomogamma linolenic acid (DGLA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) on normal human lymphocytes were independent of PGE (Prostaglandin E) involvement. PUFAs were shown to be able to augment free radical generation in neutrophils[13,14]. We have also shown that PUFAs inhibited the proliferation of normal human lymphocytes and the production of IL-2 and TNF (Tumour Necrosis Factor) through a free radical-dependent mechanism[15]. However, no reports or information are available on the effects of PUFAs on IL-2 production in tumour cells.

Some tumour cell lines have been reported to have a reduced level of superoxide dismutase (SOD) activity[10,16]. It would be useful to determine whether production of free radicals in malignant cells can result in inhibition of cell proliferation. Therefore PUFAs and IL-2 were used with the aim of determining the extent to which changes in free radical and lipid peroxide levels influence the proliferation of normal and abnormal human lymphocytes.

Materials and methods

Chemicals. RPMI 1640 and PUFAs were purchased from Sigma. Human IL-2 was obtained from Genzyme Co, USA. Dimethyl sulphoxide (DMSO) was supplied by E. Merck, Darmstadt (Germany). Test compounds were dissolved in DMSO and appropriate dilutions were made with the culture medium before use. In each cell suspension, the final DMSO concentration in each well was kept at $<1\%$ and at this concentration the growth of the cells was not affected. The fetal calf serum and antibiotic-antimycotic were purchased from Gibco, Grand Island (New York, USA).

Separation of fresh lymphocytes. Fresh blood was collected in heparinized tubes from healthy donors and the lymphocytes were isolated on a Ficoll-Hypaque Gradient[17]. The cells were washed twice with saline and cultured in RPMI medium 1640 (pH 7.2). The culture medium was supplemented with 5% heat inactivated fetal calf serum, 1% antibiotic-antimycotic and phytohemagglutinin (PHA) 5 µg/ml (Wellcome Lab, England). The cells were incubated at 37 °C, in an atmosphere containing 5% CO_2 as previously reported[18].

Cell culture. The leukemic cell line (Molt-4) was a gift from Dr. L. Edward, University of Florida, and was grown at 37 °C in a humidified atmosphere and subcultured every 4 days as previously reported[19]. The RPMI

medium (pH 7.2) was supplemented with 10 mM Hepes, 8 mM Mops, 10% (v/v) fetal calf serum, 1% antibiotic-antimycotic and 1% (w/v) glutamine.

Growth measurements. The normal and abnormal lymphocytes were plated at a density of 3×10^4 cells/ml/well and 3×10^5 cells/ml/well respectively in 24-well tissue culture plates. The PUFAs (1, 5, 10 or 20 µg/ml) and IL-2 (100 U/ml) were added singly to each of the cell cultures after 24 h incubation. After a further 48 h incubation period, the cell density was counted with a hemocytometer. All incubations were carried out in duplicate and repeated twice.

IL-2 measurement. The supernatant from each well was removed and assayed for IL-2 level using a commercially available ELISA kit (Genzyme Corporation, USA). The absorbance values were read by an ELISA reader, Ceres 900 (Bio Tech Inc., USA).

Estimation of free radicals; NBT reduction. Superoxide anion is able to reduce nitroblue tetrazolium (NBT) ion to form insoluble blue formazon. The reduction of NBT has been reported as a simple and reliable method for the assay for superoxide anion[10].

The cells (1×10^6 cells/ml) were seeded into 24-well plates and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then GLA (50 µg/ml), IL-2 (100 U/ml) or GLA + IL-2 (50 µg/ml + 100 U/ml respectively) were added into separate wells. After a further 48 h incubation, the cells were centrifuged, resuspended with saline and incubated with 0.4 ml NBT (0.1%) at 37 °C for 2 h. The reaction was terminated by the addition of 0.8 ml of glacial acetic acid and the solutions mixed well. Absorbance was determined at 560 nm.

Estimation of lipid peroxidation. The cells were seeded at 1×10^6 cells/ml in 24-well tissue culture plates and incubated at 37 °C in a humidified 5% CO_2 atmosphere. 24 h later, the cells were supplemented with either GLA (50 µg/ml) or IL-2 (100 U/ml). After a further 48 h incubation, the cells were assayed for thiobarbituric acid (TBA) reaction as described earlier[20]. Briefly, the culture medium was removed and the cells were washed with saline and solubilised in 1.4 ml of NaOH (0.1 N) followed by the addition of 0.2 ml of 10% trichloroacetic acid (TCA) and 0.4 ml of TBA (0.6 M). The reaction mixture was heated at 100 °C for 10 min. Upon cooling, the supernatant was taken and the absorbance was read at 532 mm using a Shimadzu dual beam UV-Spectrometer (model 160-A). The extent of lipid peroxidation was expressed as nmol. malondialdehyde (MDA) equivalence/µg protein calculated using the molar extinction co-efficient for MDA, $\Sigma = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (ref. 21).

Statistical analysis. In vitro results were reported as the means of ±S.D. of at least two separate experiments carried out in duplicates ($n = 4$). The level of significance was determined using Student's t-test[22].

Results

The PUFAs, linoleic acid (LA), alpha linolenic acid (ALA), gamma linolenic acid (ALA), docosahexaenoic acid (DHA), arachidonic acid (AA) and eicosapentaenoic acid (EPA) were able to inhibit proliferation of both normal lymphocytes and Molt-4 cells, in a concentration-dependent manner. For both types of cells, GLA was the most potent inhibitor, showing about 85% inhibition at a concentration of 20 µg/ml (figs 1 and 2). The order of potency for the PUFAs tested was GLA > ALA > EPA > AA > DHA > LA.

Interestingly, all the test compounds (LA, ALA, GLA, AA, DHA, EPA) signifiantly reduced ($p < 0.05$) the amount of IL-2 in the normal lymphocytes, but led to an elevation of the level of IL-2 in Molt-4 cells. The effect of PUFAs on the IL-2 level for both cell lines was also dose-dependent in the concentration range 1–20 µg (figs 3 and 4).

The levels of MDA equivalence in normal lymphocytes and Molt-4 cells are shown in table 1. Supplementation with the test compounds (GLA, IL-2, or GLA + IL-2) cells caused a significant increase in the MDA equivalence in Molt-4 cells but not in normal lymphocytes.

Although both GLA and IL-2 led to increases in $SO_2^{\cdot-}$ radicals in the two types of cells, higher levels of $SO_2^{\cdot-}$ free radicals were observed for the Molt-4 cells. The

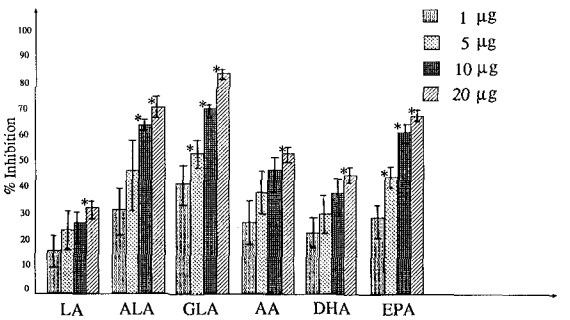

Figure 1. Cytotoxic effects of different concentrations of PUFAs on human lymphocyte cell proliferation. Data presented are average of at least 2 separate experiments determined in duplicates. *$p < 0.05$.

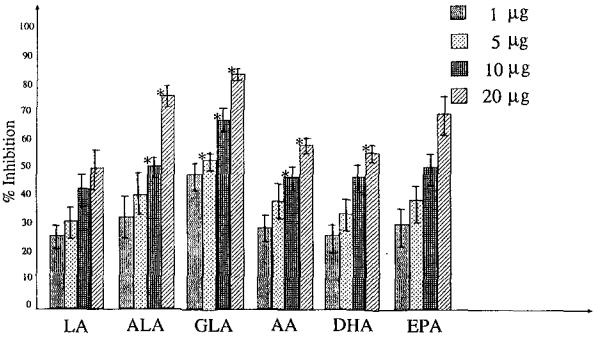

Figure 2. Cytotoxic effects of different concentrations of PUFAs on Molt-4 cell proliferation. Data presented are average of at least 2 separate experiments with duplicate determinations. *$p < 0.05$.

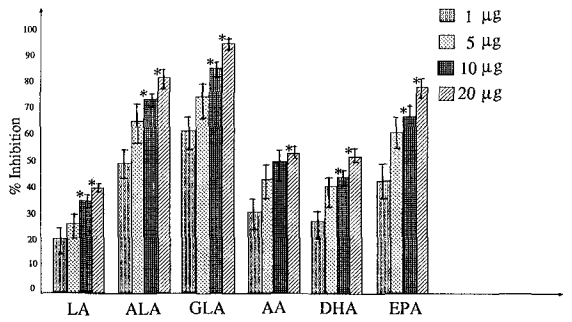

Figure 3. Effects of different concentrations of PUFAs on human IL-2 production. The IL-2 levels of human lymphocytes were measured as indicated in the 'Materials and methods' section. Each value represents (n = 4) the mean of two separate experiments with duplicate determinations. (Vertical lines indicate the range of values). The level of significance *p < 0.05 was determined according to Student's t-test.

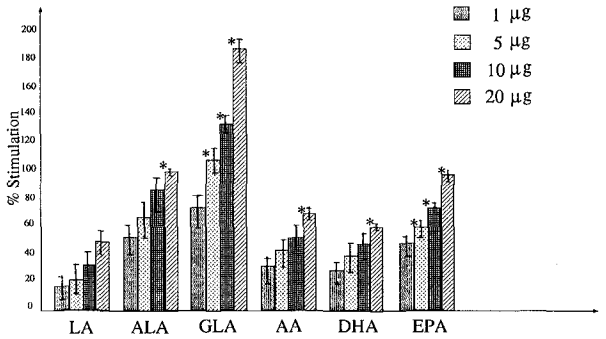

Figure 4. Effects of different concentrations of PUFAs on IL-2 production in Molt-4 cells. The IL-2 levels of Molt-4 cells were measured as indicated under 'Materials and methods' section. The control was taken as 0% stimulation. Each value represents (n = 4) the mean of two separate experiments with duplicate determinations. (Vertical lines indicate the range of values). The level of significance *p < 0.05 was determined using Student's t-test.

Table 2. In vitro effect of GLA and IL-2 on NBT reduction in normal and tumour cells.

Treatment	Normal human lymphocytes	Molt-4 Cells
Control	0.002 ± 0.002	0.002 ± 0.001
IL-2	0.008 ± 0.002	0.018 ± 0.004
GLA	0.007 ± 0.001	0.020 ± 0.002
IL-2 + GLA	0.010 ± 0.003	0.031 ± 0.003

simultaneous addition of IL-2 and GLA resulted in an additive stimulation of the $SO_2^{\cdot-}$ in Molt-4 cells (table 2).

Discussion

Currently it is known that lymphokines, interferon (IFN) and tumour necrosis factor (TNF) augment free radical generation in tumour cells and human neutrophils[23,24]. For the first time we have been able to demonstrate that in vitro IL-2 (a lymphokine) is able to enhance $SO_2^{\cdot-}$ radical generation as well as stimulate lipid peroxidation, in both Molt-4 and normal lymphocytes. However, the increases in $SO_2^{\cdot-}$ and MDA production in Molt-4 cells were significantly higher than in normal human lymphocytes. The results showed that there were associations between the $SO_2^{\cdot-}$ radical generation and lipid peroxidation (MDA equivalence) for both cells.

Additionally, the presence of both GLA and IL-2 caused greater inhibition of proliferation of Molt-4 cells than of normal human lymphocytes, as shown by the protein concentrations. Schlager et al.[25] showed that the PUFA linoleic acid was able to activate mouse peritoneal macrophages dose-dependently, and caused enhancement of formation of free radicals and oxidative products, which resulted in cytotoxicity. Thus, it is pertinent to suggest that the inhibition of cell prolifera-

Table 1. Effect of GLA and IL-2 on MDA formation in normal and tumour cells

Cell line	Treatment	MDA nmol	Protein µg	MDA nmol/protein µg
Normal human lymphocytes	Control	0.61 ± 0.3	126 ± 1.1 [a](0%)	0.48×10^{-2} [b](0%)
	IL-2	1.10 ± 0.4	41 ± 2.7 [a](67.5%)	2.60×10^{-2} [b](441%)
	GLA	2.30 ± 1.1	37 ± 0.9 [a](70.6%)	6.20×10^{-2} [b](1192%)
	IL-2 + GLA	2.50 ± 0.27	35 ± 0.8 [a](72.2%)	7.10×10^{-2} [b](1379%)
Molt-4	Control	0.48 ± 0.2	178 ± 1.1 [a](100%)	0.26×10^{-2} [b](0%)
	IL-2	2.80 ± 1.8	48 ± 2.7 [a](73.0%)	5.83×10^{-2} [b](2142%)
	GLA	3.20 ± 1.2	26 ± 0.9 [a](85.4%)	12.3×10^{-2} [b](4630%)
	IL-2 + GLA	3.20 ± 1.7	18 ± 0.8 [a](90%)	17.7×10^{-2} [b](6707%)

[a] % Inhibition of cell growth.
[b] % Increase in MDA equivalence.

tion by PUFAs and IL-2 may involve the participation of free radical generation and lipid peroxidation. This involvement has not been reported before. Activated T lymphocytes are known to play a major role in inflammatory diseases. There have been reports to show that GLA or IL-2 are able to reduce inflammatory diseases at least in part through the inhibition of lymphocyte proliferation[26,27]. Thus our present data indicates the possibility that the combination of PUFAs with IL-2 may be an approach which might be exploited in order to cause selective enhancement of free radicals in lymphocytes as a strategy for cytotoxicity.

In this study, we have also reported that PUFAs cause an increase in IL-2 production in Molt-4 cells but a decrease in normal lymphocytes. We therefore postulate that PUFAs act differently on normal and transformed lymphocytes. However, more work needs to be done before we can explain this difference in IL-2 level observed in the two different cell lines.

Acknowledgments. The authors are grateful to the National University of Singapore for the Research Scholarship awarded to M. Ambika Devi and also to the Shaw Foundation of Singapore for a generous research grant.

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