Triacylglycerol metabolism by lymphocytes and the effect of triacylglycerols on lymphocyte proliferation

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This study investigates the ability of lymphocytes to utilize fatty acids originating from triacylglycerols and the effect of triacylglycerols upon mitogen-stimulated lymphocyte proliferation. Lymphocytes isolated from rat lymph nodes, spleen, thymus and lymphatic duct had a lipoprotein lipase activity of approx. 10 units/mg of protein, indicating that the fatty acids of circulating triacylglycerols are accessible to these cells. In culture, lymph node lymphocytes hydrolysed triacylglycerols added to the medium as emulsions. Both non-esterified fatty acids and free glycerol appeared in the cell culture medium, but their concentrations indicated that a high proportion of each (65–90% of fatty acids and 60–80% of glycerol) was taken up by the cells. The incorporation and fate of triacylglycerol-fatty acids was studied by culturing the cells in the presence of tri[^{3}H]oleoylglycerol or tri[^{14}C]linoleoylglycerol. Both fatty acids were incorporated into lymphocyte lipids in a time-dependent manner; linoleic acid was incorporated at a significantly greater rate than oleic acid. The majority of oleic acid (greater than 70%) was incorporated into cellular triacylglycerol, while less than 10% was incorporated into phospholipids. In contrast, linoleic acid incorporation into cellular triacylglycerol never exceeded 25%, while up to 45% was incorporated into phospholipids. Triacylglycerols containing polyunsaturated fatty acids inhibited concanavalin A-stimulated lymphocyte proliferation in a concentration- and time-dependent manner; triacylglycerols containing saturated fatty acids or oleic acid were not inhibitory. Such direct effects of certain triacylglycerols on lymphocyte function may explain why some clinical trials of polyunsaturated fatty acid-rich diets have been successful in improving the condition of patients suffering from inflammatory diseases.

INTRODUCTION

Inflammatory and autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis and multiple sclerosis are characterized by an overactive immune system which is directed against host tissues [1,2]. Epidemiological studies indicate that the incidence of these disorders is low in regions where the dietary intake of vegetable or fish oils is high [3–5]. This has led to the suggestion that increasing the proportion of vegetable or fish oils in the diet could be of benefit to patients suffering from inflammatory diseases [3–5]. Indeed, such dietary manipulation has been reported to improve the clinical condition of patients suffering from rheumatoid arthritis [6,7], psoriasis [8] and multiple sclerosis [9,10]. These oils are rich in the $n-6$ polyunsaturated fatty acids (PUFAs) linoleic acid (e.g. corn and sunflower oils) or γ-linolenic acid (e.g. evening primrose oil), or in the $n-3$ PUFAs eicosapentaenoic and docosahexaenoic acids (fish oils). Despite their use in the clinical situation, the mechanism(s) by which PUFAs might affect the immune system is still not clear.

In vitro experiments show immunosuppressive effects of non-esterified unsaturated fatty acids, which may explain the apparent benefits of diets enriched in such fatty acids in inflammatory disorders. For example, a number of unsaturated fatty acids, including oleic ($C_{18:1,n-9}$), linoleic ($C_{18:2,n-6}$), α-linolenic ($C_{18:3,n-3}$), arachidonic ($C_{20:4,n-6}$), eicosapentaenoic ($C_{20:5,n-3}$) and docosahexaenoic ($C_{22:6,n-3}$) acids, inhibit mitogen-stimulated proliferation of rat and human lymphocytes [11–13] and suppress production of interleukin-2, an immunoregulatory cytokine, by such cells [13,14]. In addition, oleic, γ-linolenic ($C_{18:3,n-6}$) and eicosapentaenoic acids inhibit the natural killer cell activity of human lymphocytes [15,16]. Although these findings indicate the potential mechanisms by which dietary lipids could suppress immune functions, caution must be exercised in extending the findings of *in vitro* studies to the *in vivo* situation. This is because fatty acids of dietary origin are transported in the bloodstream as triacylglycerols, rather than as non-esterified fatty acids, and little is known about the ability of lymphocytes to utilize triacylglycerol-fatty acids or about the effects of triacylglycerols on lymphocyte functions. Fatty acids are released from circulating triacylglycerols (present in chylomicrons and very-low-density lipoproteins) by the action of lipoprotein lipase (EC 3.1.1.34); however, the activity of this enzyme in lymphocytes is not known.

The present study investigated the ability of lymphocytes to utilize fatty acids present in triacylglycerols by (a) measuring the lipoprotein lipase activity of lymphocytes; (b) measuring the breakdown of various triacylglycerols by lymphocytes in culture; and (c) measuring the incorporation of radioactively labelled fatty acid from triacylglycerol into lymphocytes in culture. The potential ability of circulating triacylglycerols to cause immunosuppression was also studied by investigating the effect of a variety of triacylglycerols upon *in vitro* mitogen-stimulated lymphocyte proliferation. The triacylglycerols used in this study were trimyristroyl-, tripalmitoyl-, tristearoyl-, trioleoyl-, trilinoleoyl-, tri(α)linolenoyl- and triarachidonoyl-glycerol. To our knowledge, the metabolism of triacylglycerols by lymphocytes and their effects on lymphocyte proliferation have not been previously reported.

MATERIALS AND METHODS

Animals and chemicals

Male Wistar rats were purchased from Harlan-Olac, Bicester, Oxon., U.K. They were housed in the Department of Bio-

Abbreviations used: ConA, concanavalin A; PC, dioleyl-phosphatidylcholine; PUFA, polyunsaturated fatty acid.
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chemistry animal house and, unless otherwise indicated, were allowed *ad libitum* access to food and water.

The sources of chemicals, culture medium and medium supplements were as described previously [11,17]. In addition, [1-^{14}C]linoleic acid, [U-^{14}C]glycerol and tri[9,10-^{3}H]oleoylglycerol were obtained from Amersham International, Amersham, Bucks., U.K.; non-radioactively labelled trioleylglycerol and all other triacylglycerols, dioleyl-phosphatidylcholine (PC), creatine phosphate, lipid standards and oxalyl chloride were obtained from Sigma Chemical Co., Poole, Dorset, U.K.; silica gel 60 t.l.c. plates, iodine, glycerol and all solvents were obtained from BDH, Poole, Dorset, U.K.; creatine kinase was obtained from Boehringer Corp., Lewes, E. Sussex, U.K.; DEAE-cellulose filter paper discs were obtained from Whatman International, Maidstone, Kent, U.K.; and Lymphoprep (a solution of sodium metrizoate/Ficoll with a density of 1.077 g/ml) was obtained from Nycomed Pharma AS, Oslo, Norway.

Synthesis of tri[1-^{14}C]linoleoylglycerol

Linoleoyl chloride was formed by refluxing 1.4 mmol of [1-^{14}C]linoleic acid (specific radioactivity 36.2 μCi/mmol) with 3.5 mmol of oxalyl chloride in 10 ml of carbon tetrachloride for 30 min. Tri[1-^{14}C]linoleoylglycerol was formed by mixing the [1-^{14}C]linoleoyl chloride with an excess (0.5 mmol) of glycerol in the presence of 150 μl of pyridine. The reaction was allowed to proceed for 3 days at room temperature and the formation of triacylglycerol was monitored by t.l.c. The triacylglycerol formed was isolated from the reaction mixture by filtration through a fine scinter funnel and hexane extraction of the resulting filtrate. The hexane extract was purified by passage through a silica column; after washing the column with hexane and then hexane/ether (40:1, v/v), the triacylglycerol was eluted with hexane/ether (20:1, v/v). The product was dried to a constant weight. The yield of triacylglycerol was approx. 20% and the product had a specific radioactivity of 0.12 μCi/μmol.

Lymphocyte preparation

Cervical lymph nodes, thymus and spleen were dissected free of fatty tissue and were gently ground. Thoracic duct lymphocytes were obtained by cannulation of the thoracic duct as described in detail by Hunt [18]. The cells were washed once and lymphocytes were collected by centrifugation on Lymphoprep (1500 *g*, 20 min). The lymphocytes were washed once more and resuspended. For enzyme assays the lymphocytes were resuspended in the appropriate homogenizing buffer and for cell culture they were resuspended in culture medium.

Measurement of lipoprotein lipase activity

Lymphocytes were prepared for the assay of lipoprotein lipase by the method of Baltzell et al. [19]. The cells were resuspended in ice-cold 50 mM Tris, pH 8.0, containing 15 units/ml heparin and were homogenized for 15 s with a Polytron homogenizer at speed 7. The homogenate was kept on ice for 30 min and then centrifuged (1000 *g*, 10 min). The supernatant was used to determine the activity of lipoprotein lipase by the method of Nilsson-Ehle et al. [20].

The substrate for the lipoprotein lipase assay was formed by dissolving 8 μmol of trioleoylglycerol, 16.6 μCi of tri[9,10-^{3}H]oleoylglycerol and 0.4 mg of PC in 0.5 ml of benzene. After evaporation to dryness, 2.5 ml of 0.2 M Tris, pH 8.1, was added and the mixture was sonicated (4 × 1 min at an amplitude of 12 μm) in an MSE sonicator. Then 0.3 ml of 4% defatted BSA and 0.2 ml of serum prepared from a rat which had been fasted overnight were added. Blank tubes contained NaCl (final concentration 1 M) instead of serum.

Supernatant (0.1 ml) was incubated for 30 min at 37 °C with 0.1 ml of substrate. The reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (28:25:20, by vol.) followed by 1.05 ml of 0.1 M potassium carbonate, pH 10.0. After mixing, the tubes were centrifuged briefly to separate the layers and an aliquot of the upper layer (which contains the non-esterified fatty acid released from triacylglycerol) was removed and added to scintillant prior to liquid scintillation counting. One unit of lipoprotein lipase activity is defined as the amount of enzyme which releases 1 nmol of fatty acid per 30 min. Preliminary experiments showed that this reaction is linear for at least 30 min.

Formation of triacylglycerol emulsions for use in cell culture

Triacylglycerol (7.5 μmol) in a small volume of toluene was mixed with 0.4 mg of PC. After evaporation to dryness, 7.5 ml of Mg^{2+}- and Ca^{2+}-free PBS, pH 7.2, was added and the mixture was sonicated as described above. This gave a 1 mM stock solution of triacylglycerol which was further diluted with a sonicated mixture of PC in PBS to give solutions of lower concentration. These solutions were added to the cell culture medium to give the required triacylglycerol concentration (see the Results section for concentrations used).

Utilization of triacylglycerols by lymphocytes *in vitro*

Lymph node lymphocytes were cultured at 37 °C in an air/CO_2 (19:1) atmosphere at a density of 5×10^6 cells per well (final volume 2 ml) in 24-well plates in Hepes-buffered RPMI supplemented with 10 mM glucose, 2 mM glutamine, 10% foetal calf serum, antibiotics (streptomycin and penicillin) and concanavalin A (ConA). The medium also contained emulsions of various triacylglycerols (final concentration 100 μM). After 48 h the medium was collected and the concentrations of triacylglycerol, and free glycerol and non-esterified fatty acid in the medium were determined by the assays of McGowan et al. [21] and Okabe et al. [22] respectively.

Incorporation of glycerol or of fatty acid from triacylglycerol by lymphocytes *in vitro*

Lymph node lymphocytes were cultured as described above in medium containing 1 mM [U-^{14}C]glycerol (specific radioactivity 0.039 μCi/μmol) or an emulsion of 100 μM tri[9,10-^{3}H]oleoylglycerol (specific radioactivity 5.85 μCi/μmol) or 100 μM tri[1-^{14}C]linoleoylglycerol (specific radioactivity 0.068 μCi/μmol). After various times the cells were collected and washed three times with PBS. Lipid was extracted and analysed as described below.

Lipid extraction and analysis

Cells were resuspended in a small volume of PBS and sonicated (3 × 15 s at an amplitude of 12 μm) in an MSE sonicator. Lipid was extracted using chloroform/methanol (2:1, v/v). Lipid classes were separated by t.l.c. on silica gel 60 plates using hexane/diethyl ether/acetic acid (70:30:2, by vol.). After t.l.c., lipids were detected by staining with iodine. The position of each

lipid class was determined by comparison with a commercially available standard lipid mixture which was separated on the same plate as the samples. Each identified spot was scraped from the t.l.c. plate and the lipid was extracted with chloroform/methanol (2:1, v/v). The lipid extract was transferred to scintillation counting vials, dried and the incorporation of radioactivity was determined by liquid scintillation counting.

Lymphocyte proliferation assay

Lymph node lymphocytes were cultured as described above except that 5×10^5 cells per well (final volume 200 μl) and 96-well micro-titre plates were used. The medium contained emulsions formed with various triacylglycerols (see the Results section for concentrations used). After 24 h or 48 h, [6-^{3}H]thymidine was added (0.2 μCi/well) and the cells were incubated for a further 18 h. The cells were then harvested on to glass fibre filters, washed and dried using a Skatron Cell Harvester. Incorporation of radioactivity was measured by liquid scintillation counting and was used to indicate lymphocyte proliferation [23].

Other procedures

Macrophages were removed from lymphocyte preparations by adherence to cell culture dishes, as described elsewhere [12]. Murine thioglycollate-elicited peritoneal macrophages were prepared and purified as described previously [17]. Glycerol kinase (EC 2.7.1.30) activity was determined by the method of Newsholme et al. [24]. Protein concentrations were measured by the method of Bradford [25].

Data presentation and statistical analysis

Unless otherwise indicated, all data are expressed as means $\pm$ S.E.M. for an indicated number of samples. Statistical significance was determined using the unpaired Student's t test.

RESULTS

Lipoprotein lipase activity of lymphocytes

Lymphocytes isolated from lymph nodes, spleen or thymus contained a similar lipoprotein lipase activity (Table 1). The lipoprotein lipase activity of lymphocytes (80–105 units/g of tissue and 7–11 units/mg of protein; Table 1) was much lower than that of adipose tissue (933 ± 15 units/g of tissue and 51.1 ± 8.7 units/mg of protein; $n = 6$) extracted and measured using the same techniques.

The lymphocyte preparations from lymph nodes, spleen and thymus contain a small proportion of macrophages (usually less than 5%), and the macrophage is known to synthesize and secrete lipoprotein lipase [26–28]. In this study, the lipoprotein lipase activity of murine peritoneal macrophages was found to be 97.9 ± 5.8 units/mg of protein ($n = 3$). Therefore it was possible that the lipoprotein lipase activity measured in the lymphocyte preparations could be due to the presence of contaminating macrophages. Lymphocytes collected by cannulation of the thoracic duct are completely devoid of phagocytic cells, including macrophages [18,29]; these lymphocytes contained a lipoprotein lipase activity similar to that of the other lymphocyte preparations (Table 1). It is possible to remove contaminating macrophages from lymphocyte preparations using a variety of procedures, including adherence to the surface of cell culture dishes (see [18]). We have previously shown that this technique depletes lymph node lymphocyte preparations of macrophages [12]. The resulting purified lymphocyte preparation (i.e. the non-adherent cells) had a lipoprotein lipase activity of 114 ± 13 units/g of tissue and 9.4 ± 2.4 units/mg of protein ($n = 3$). This accounted for approx. 90% of the lipoprotein lipase activity of the original cell preparation. The remaining adherent cells (mostly macrophages) had a lipoprotein lipase activity of 23 ± 4 units/g of tissue and 495 ± 58 units/mg of protein ($n = 3$). These findings suggest that a large proportion of the lipoprotein lipase activity measured in lymphocyte preparations (Table 1) is due to the presence of the enzyme within or bound to the surface of the lymphocytes themselves.

Utilization of triacylglycerols by lymphocytes *in vitro*

Lymphocytes were cultured with various triacylglycerols (initial concentration 100 μM) and the concentrations of triacylglycerol, free glycerol and non-esterified fatty acids in the medium were measured after 48 h. The rate of triacylglycerol breakdown varied from approx. 8 to approx. 12 nmol/48 h for the various triacylglycerols (Table 2). Interestingly, triacylglycerols containing PUFA were hydrolysed to a greater extent (approx. 30%) than those containing saturated fatty acids or oleic acid. Non-esterified fatty acids and free glycerol appeared in the culture medium (Table 2). The amount of non-esterified fatty acid which accumulated in the culture medium accounted for between 12

Table 1 Lipoprotein lipase activities of lymphocytes of different origins

Rat lymph node, spleen, thymus and lymphatic duct lymphocytes were prepared. The cells were homogenized and the lipoprotein lipase activity was determined as described in the Materials and methods section. Activity is expressed per g of original tissue and per mg of lymphocyte protein. One unit of lipoprotein lipase activity released 1 nmol of fatty acid per 30 min. Data are means $\pm$ S.E.M. of the indicated number of cell preparations. The values shown for thoracic duct lymphocytes are the activities measured for two separate cell preparations.

	Lipoprotein lipase activity (units)	
Lymphocyte source	per g of tissue	per mg of protein
Cervical lymph node ($n = 6$)	105 ± 12	7.3 ± 1.9
Spleen ($n = 3$)	83 ± 15	9.3 ± 1.7
Thymus ($n = 3$)	80 ± 15	10.8 ± 1.6
Thoracic duct	–	10.5, 6.8

Table 2 Breakdown of triacylglycerols by lymphocytes *in vitro*

Rat cervical lymph node lymphocytes were prepared and cultured in medium containing emulsions of various triacylglycerols at a concentration of 100 μM. After 48 h the medium was collected and assayed for triacylglycerol (TAG), free glycerol and non-esterified fatty acid (NEFA). Data are means $\pm$ S.E.M. of three cell preparations.

Triacylglycerol	TAG breakdown (nmol/48 h)	NEFA in medium (nmol)	Free glycerol in medium (nmol)
Trimyristoyl	9.0 ± 1.2	8.9 ± 3.3	3.3 ± 1.3
Tripalmitoyl	8.4 ± 0.6	6.7 ± 1.9	1.8 ± 0.4
Tristearoyl	7.8 ± 1.0	5.7 ± 2.1	2.6 ± 0.6
Trioleoyl	9.0 ± 1.1	8.3 ± 2.0	2.5 ± 0.9
Trilinoleoyl	11.8 ± 1.3	6.8 ± 1.1	4.1 ± 0.8
Tri(α)linolenoyl	11.2 ± 1.0	4.0 ± 1.7	2.2 ± 0.3
Triarachidonoyl	10.8 ± 1.2	5.7 ± 1.7	2.7 ± 0.4

Table 3 Incorporation of oleic acid from trioleoylglycerol by lymphocytes *in vitro*

Rat cervical lymph node lymphocytes were prepared and cultured in medium containing tri[9,10-^{3}H]oleoylglycerol. After 18, 40 and 64 h the cells were collected and washed and the lipid extracted. The fate of the incorporated oleic acid was determined by t.l.c. (see the Materials and methods section). Data are the means ± S.E.M. of five cell preparations. Statistical significance versus results at 18 h is indicated by $*P < 0.05$; $**P < 0.02$; $***P < 0.01$; and versus the results at 40 h by $\dagger P < 0.05$. TAG indicates triacylglycerol; DAG + MAG indicates diacylglycerol plus monoacylglycerol; NEFA indicates non-esterified fatty acid; PL indicates phospholipid.

Time (h)	Oleic acid incorporation (nmol/mg of protein)				
	Total lipid	TAG	DAG + MAG	NEFA	PL
18	34.2 ± 3.2	24.1 ± 1.9	3.0 ± 0.6	1.7 ± 0.2	0.7 ± 0.2
40	47.5 ± 3.1**	38.2 ± 3.9**	5.1 ± 0.2**	2.7 ± 0.3*	1.4 ± 0.1**
64	58.9 ± 4.0***	43.7 ± 3.7***	7.7 ± 0.9***†	3.4 ± 0.4***	4.2 ± 1.0***†

Table 4 Incorporation of linoleic acid from trilinoleoylglycerol by lymphocytes *in vitro*

Rat cervical lymph node lymphocytes were prepared and cultured in medium containing glycerol tri[1-^{14}C]linoleoylglycerol. After 18, 40 and 64 h the cells were collected and washed and the lipid extracted. The fate of the incorporated linoleic acid was determined by t.l.c. (see the Materials and methods section). Data are the means ± S.E.M. of five cell preparations. Statistical significance versus the results at 18 h is indicated by $*P < 0.01$; $**P < 0.001$ and versus the results at 40 h by $\dagger P < 0.05$; $\dagger\dagger P < 0.01$. Abbreviations are described in the legend to Table 3.

Time (h)	Linoleic acid incorporation (nmol/mg of protein)				
	Total lipid	TAG	DAG + MAG	NEFA	PL
18	77.0 ± 2.5	28.4 ± 3.6	18.6 ± 1.7	13.3 ± 0.6	19.9 ± 1.4
40	225.7 ± 23.2**	42.1 ± 6.0	62.8 ± 7.5**	30.1 ± 2.7**	77.6 ± 13.4*
64	327.4 ± 27.0**†	56.9 ± 2.2**†	91.3 ± 6.5**†	44.8 ± 1.6**††	143.7 ± 12.7**††

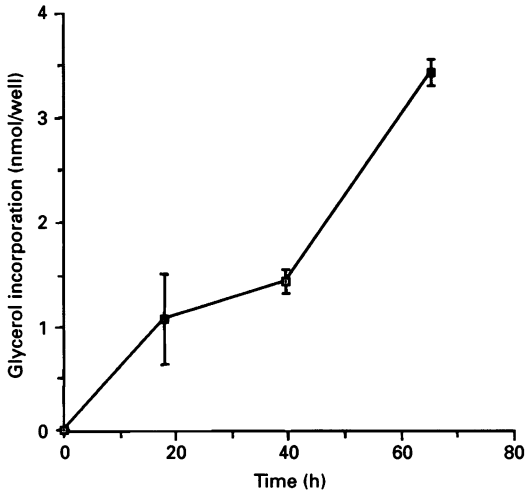

Figure 1 Incorporation of glycerol into lymphocyte lipids *in vitro*

Lymphocytes were cultured in medium containing 1 mM [U-^{14}C]glycerol. After various times the cells were collected and washed and the lipid was extracted. Data are means ± S.E.M. of three cell preparations.

and 33% of the amount of fatty acid which would have been released from the hydrolysed triacylglycerol. Similarly, the amount of free glycerol in the culture medium only accounted for 20–37% of the triacylglycerol that was hydrolysed. These findings indicate that the cells have taken up much of the fatty acid and glycerol released by triacylglycerol hydrolysis.

Incorporation of fatty acids from the triacylglycerols by lymphocytes *in vitro*

Triacylglycerols added to the culture medium are hydrolysed by lymphocytes, with only a small increase in the concentration of non-esterified fatty acids in the medium (Table 2), indicating that lymphocytes may be capable of utilizing triacylglycerol-derived fatty acids. This was studied by incubating lymphocytes with either trioleoylglycerol or trilinoleoylglycerol containing radioactivity labelled fatty acid.

Oleic acid from triacylglycerol was incorporated into lymphocyte lipids (Table 3). Incorporation of oleic acid into all lipid fractions increased with time in culture (Table 3). At all three time points more than 70% of the oleic acid incorporation was into cellular triacylglycerols, while incorporation into the phospholipid fraction never exceeded 10% of the total incorporation.

Linoleic acid from trilinoleoylglycerol was incorporated into lymphocyte lipids (Table 4). The rate of linoleic acid incorporation was much greater than that of oleic acid (see Table 3). This is consistent with the higher rate of hydrolysis of trilinoleoylglycerol reported in Table 2, and the apparently greater uptake of released fatty acid (Table 2). The incorporation of linoleic acid into lymphocyte lipids and into all lipid fractions increased with increasing time of culture (Table 4). Incorporation into cellular triacylglycerols never exceeded 40% of the total linoleic acid incorporation; incorporation of linoleic acid into the phospholipid fraction accounted for 25% of the total incorporation at 18 h and almost 45% at 64 h (Table 4).

Incorporation of glycerol by lymphocytes *in vitro*

The increase in the glycerol concentration in the medium when lymphocytes were cultured with emulsions containing triacyl-

Table 5 Effect of various triacylglycerols on lymphocyte proliferation *in vitro*

Rat cervical lymph node lymphocytes were cultured in the presence of emulsions of various triacylglycerols. Lymphocyte proliferation was measured as [^{3}H]thymidine incorporation over the final 18 h of a 66 h culture period. Data are the means ± S.E.M. of six determinations. Statistical significance versus control: $^*P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.001$.

	[^{3}H]Thymidine incorporation (d.p.m./well)			
Triacylglycerol	10 μM	30 μM	50 μM	100 μM
Trimyristoyl	104400 ± 4575	98819 ± 6340	95776 ± 6864	87956 ± 5941*
Tripalmitoyl	100160 ± 4499	99206 ± 9253	92082 ± 8541	84386 ± 5743**
Tristearoyl	100489 ± 4163	94471 ± 5650	93420 ± 7574	94323 ± 8133
Trioleoyl	99109 ± 4185	98113 ± 7357	94477 ± 9342	97707 ± 5280
Trilinoleoyl	91509 ± 7626	67602 ± 4320***	59242 ± 9983***	31859 ± 4341***
Tri(α)linolenoyl	72306 ± 5139***	51149 ± 6042***	35733 ± 4215***	24392 ± 4042***
Triarachidonoyl	61861 ± 5758***	41140 ± 5049***	26591 ± 6137***	14411 ± 3267***

Table 6 Time course of the effect of various triacylglycerols on lymphocyte proliferation *in vitro*

Rat cervical lymph node lymphocytes were cultured in the presence of emulsions of various triacylglycerols (final concentration 100 μM). Lymphocyte proliferation was measured as [^{3}H]thymidine incorporation over the final 18 h of a 42 h or a 66 h culture period. Data are the means ± S.E.M. of six determinations. Statistical significance versus control: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

	[^{3}H]Thymidine incorporation (d.p.m./well)	
Triacylglycerol	42 h	66 h
None	63088 ± 4243	117495 ± 5330
Trimyristoyl	69497 ± 7194	98819 ± 6340*
Tripalmitoyl	62255 ± 4108	84386 ± 5745**
Tristearoyl	66743 ± 3598	94323 ± 8133*
Trioleoyl	70701 ± 4441	98113 ± 7357
Trilinoleoyl	32026 ± 8098**	31859 ± 4341***
Tri(α)linolenoyl	23345 ± 3171***	24674 ± 4565***
Triarachidonoyl	13248 ± 2425***	21149 ± 2173***

glycerols did not account for all of the triacylglycerol glycerol that should have been released calculated from the decrease in triacylglycerol concentration (Table 2). This suggests that lymphocytes may be capable of utilizing glycerol. This was studied by incubating lymphocytes with radioactively labelled glycerol.

Glycerol from the culture medium was incorporated into lymphocyte lipids (Figure 1). This confirms that lymphocytes are able to utilize free glycerol. Such utilization would require the presence of glycerol kinase, the enzyme responsible for the conversion of glycerol to glycerol 3-phosphate prior to triacylglycerol and phospholipid synthesis [30]. Indeed, rat lymph node lymphocytes possess a glycerol kinase activity of 0.6 ± 0.1 nmol of glycerol phosphorylated/min per mg of protein ($n = 3$), which is about 10% of the activity measured in rat liver [24]. This activity of glycerol kinase could account for the glycerol incorporation by lymphocytes (Figure 1).

The effect of triacylglycerols on *in vitro* lymphocyte proliferation

The effect of different concentrations of a number of triacylglycerols upon ConA-stimulated lymphocyte proliferation was investigated (Table 5); in all cases the total concentration of emulsion was kept constant and thymidine incorporation was measured over the final 18 h of a 66 h culture period. In the absence of emulsion, [^{3}H]thymidine incorporation was 109420 ± 8246 d.p.m./well ($n = 6$). Thymidine incorporation was not affected by addition of emulsion which did not contain triacylglycerol (110650 ± 5197 d.p.m./well; $n = 6$). Triacylglycerols containing saturated or monosaturated fatty acids (myristic, palmitic, stearic or oleic acids) did not affect lymphocyte proliferation, except for trimyristoylglycerol and tripalmitoylglycerol at the highest concentration used (100 μM). In contrast, triacylglycerols containing PUFAs (linoleic, α-linolenic or arachidonic acids) inhibited lymphocyte proliferation at concentrations as low as 10 or 30 μM (Table 5). At a concentration of 100 μM, such triacylglycerols inhibited lymphocyte proliferation by up to 85%.

The effect of triacylglycerols on lymphocyte proliferation was also investigated at an earlier period in culture (Table 6); in all cases the triacylglycerol concentration was 100 μM and thymidine incorporation was measured over the final 18 h of the culture period. At the earlier time point (42 h) the triacylglycerols containing PUFAs inhibited proliferation (by up to 80%), but triacylglycerols containing other fatty acids had no effect (Table 6).

DISCUSSION

This study investigates, for the first time, triacylglycerol metabolism by lymphocytes. In addition, the effect of triacylglycerols on a key immune function, lymphocyte proliferation, was examined *in vitro*.

Using a standard assay [20], lymphocytes isolated from lymph nodes, spleen and thymus were shown to contain the enzyme lipoprotein lipase, which had an activity of approx. 10 units/mg of cellular protein (Table 1). This enzyme is responsible for the hydrolysis of circulating triacylglycerols in specific tissues so that released fatty acids are taken up by the cells of that tissue. The activity of lipoprotein lipase is high in adipose tissue, heart and red skeletal muscle [31]; lower activities are reported in other tissues such as liver, kidney, lung and spleen [32,33]. The enzyme is synthesized by a variety of cells including adipocytes, myocytes, epithelial cells and skeletal and smooth muscle cells [34,35]. These cells release the lipase which then becomes attached to local endothelial cells via heparan sulphate proteoglycans [31,36]. This locates the enzyme on the luminal surface of the endothelial cells in the tissue in which it is synthesized. Hence it catalyses the hydrolysis of triacylglycerols in circulating lipoproteins.

Macrophages and monocytes synthesize large amounts of lipoprotein lipase [26–28,37], much of which is secreted into the

medium when the cells are in culture [26–28,37]. The activity of the enzyme in murine peritoneal macrophages has not been previously reported; it was found to be approximately 100 units/mg cellular protein. This activity is in general agreement with previous reports for the activity in human peripheral blood monocytes [28], rabbit alveolar macrophages [37] and a macrophage-like cell line [37]. It has been shown that, in addition to secreting lipoprotein lipase into the culture medium, macrophages also attach the enzyme to the outer surface of their plasma membrane [38]. It is not known whether the lipoprotein lipase of lymphocytes detected in the present study is intracellular, and destined for secretion, or whether it is bound to the cell surface.

Since the lipoprotein lipase activity of macrophages is high, the enzyme activity detected in lymphocyte preparations could have been due to a small proportion of macrophages. However, thoracic duct lymphocytes, which are devoid of macrophages [18,29], and lymph node lymphocytes depleted of macrophages, exhibited lipoprotein lipase activities similar to those of the tissue lymphocyte preparations.

Consistent with the apparent ability of lymphocytes to produce lipoprotein lipase, triacylglycerols added to lymphocyte culture media were hydrolysed (Table 2). However, the proportion of triacylglycerol degraded was less than 10%, suggesting that only a small amount of the enzyme is secreted by these cells. Indeed, the activity of lipoprotein lipase in the culture medium of lymphocytes which had been cultured for 48 h was only 5–10% of the cellular activity (results not shown). This is in contrast to the situation found with macrophages, where the lipoprotein lipase activity of the culture medium can exceed that of the cells within 2 h of the onset of culture, and can ultimately be 10 or 20 times greater than the cellular activity [28].

Triacylglycerols containing PUFAs were hydrolysed to a greater extent than those containing saturated fatty acids or oleic acid (Table 2). This suggests that the lymphocyte lipoprotein lipase has the ability to differentiate between substrates, possibly on the basis of both fatty acid chain length and degree of unsaturation. Although there are reports that lipoprotein lipase does not exhibit specificity with respect to the acyl chains of its substrate [39,40], the finding of greater hydrolysis of triacylglycerols containing PUFAs than of those containing saturated fatty acids is in accordance with a previous study of the substrate specificity of the enzyme from human milk [41]. That study showed that triacylglycerols containing linoleic or linolenic acids are hydrolysed at a rate up to four times greater than the rate of hydrolysis of triacylglycerols containing saturated fatty acids (myristic, palmitic or stearic acids) [41]. However, this earlier study found that triacylglycerol was the most rapidly hydrolysed triacylglycerol of those tested [41]; in the current study trioleoylglycerol was hydrolysed at a rate comparable with that of hydrolysis of triacylglycerols containing saturated fatty acids (Table 2).

Since it is unlikely that physiologically available triacylglycerols will be composed of three identical fatty acids, it would be of interest to investigate the ability of lymphocyte lipoprotein lipase to hydrolyse triacylglycerols containing fatty acids of more than one type. However, lipoprotein lipase also exhibits specificity with respect to the position of fatty acid chains in the triacylglycerol, with the ester bond at position 1 being attacked in preference to that at position 2 [41]; this could make the interpretation of studies comparing rates of hydrolysis of 'mixed' triacylglycerols difficult.

Not all of the triacylglycerol that was hydrolysed in lymphocyte culture medium could be accounted for by the appearance of free glycerol and fatty acids (Table 2), suggesting that the cells are capable of utilizing both products of triacylglycerol hydrolysis. Indeed, lymphocytes were capable of incorporating glycerol (Figure 1) and fatty acid derived from triacylglycerols (Tables 3 and 4) from the medium into cellular lipids. Between 70 and 90% of the fatty acid released from triacylglycerols was utilized by lymphocytes (Table 2), and, based upon the experiments with radioactively labelled triacylglycerol-fatty acids, most (more than 95%) of the utilized fatty acid was incorporated into lymphocyte lipids. The rate of incorporation of triacylglycerol-fatty acid differed between the two triacylglycerols tested. The rate of oleic acid incorporation from trioleoylglycerol was lower than that of linoleic acid from trilinoleoylglycerol, and also that of non-esterified fatty acids in similar culture conditions (200–400 nmol/mg of protein per 64 h [42]). The rate of linoleic acid incorporation from trilinoleoylglycerol was similar to that of non-esterified fatty acids added to the culture medium [42]. The intracellular fate of triacylglycerol-fatty acids differed according to their origin. Most (more than 70%) triacylglycerol-oleic acid was incorporated into cellular triacylglycerol, with only a small proportion (less than 10%) being incorporated into the phospholipid fraction (Table 3). In contrast, up to 45% of triacylglycerol-linoleic acid was incorporated into phospholipids with less than 40% being incorporated into cellular triacylglycerols (Table 4). In this respect, the fate of linoleic acid incorporated from triacylglycerol resembles the incorporation of non-esterified fatty acids [42]. A greater proportion (40–65%, depending upon the fatty acid) of non-esterified fatty acid taken up by lymphocytes is incorporated into phospholipids than in cellular triacylglycerols (20–45%, depending upon the fatty acid) [42]. This indicates not only that the ability of lymphocytes to utilize different triacylglycerol-fatty acids is different, but also that the intracellular handling of fatty acids may differ according to their origin. The mechanism underlying this difference is not known.

In contrast to a lipoprotein lipase-mediated mechanism for uptake of the components of triacylglycerols, an alternative mechanism could be uptake of entire triacylglycerols, as has been shown to occur with fibroblasts in culture [43]. However, the experimental data do not support this mechanism, in that trioleoylglycerol and trilinoleoylglycerol were utilized by lymphocytes at significantly different rates and had different fates (Tables 3 and 4). If entire triacylglycerols were taken by a non-specific mechanism, then the rates of uptake of both triacylglycerols should be the same and the major intracellular fate would be triacylglycerol. However, much of the trilinoleoylglycerol is incorporated into phospholipid, even after only 18 h of culture.

The findings of this study are important in relation to the use of dietary lipid manipulation as an immunosuppressive therapy in inflammatory diseases. Many studies have shown immunosuppressive effects of non-esterified unsaturated fatty acids *in vitro* (see the Introduction). It is often assumed that these effects are due to eicosanoids synthesized from the unsaturated fatty acids. However, it has been shown that such immunosuppressive effects occur even when eicosanoid synthesis is inhibited [12]. It seems likely that the effects of fatty acids are due to their incorporation into membrane phospholipids, altering the fatty acid composition and fluidity [42]. In other tissues and cell types such changes have been shown to affect receptor activity (see [44]) and signal transduction mechanisms (see [45,46]). If this is the mechanism by which dietary lipids cause immunosuppression, then it must be shown that lymphocyte phospholipids are accessible to triacylglycerol-fatty acids in the circulation, which have in turn been formed from digested and absorbed dietary lipids. This study shows that lymphocytes, at least *in vitro*, have

the ability to take up fatty acids from triacylglycerols and to incorporate them into phospholipids. This indicates that the membrane phospholipid composition of lymphocytes may indeed be sensitive to the manipulation of circulating, and therefore dietary, triacylglycerol-fatty acids. Of possible particular importance in this respect is the apparent ability of lymphocytes to incorporate PUFA from triacylglycerols at a greater rate than monosaturated (and perhaps saturated) fatty acids.

Although effects of non-esterified fatty acids upon a number of key immune functions have been demonstrated *in vitro*, direct effects of triacylglycerols have been rarely reported. This study shows that triacylglycerols, particularly those containing PUFAs, are potent inhibitors of mitogen-stimulated lymphocyte proliferation *in vitro* (Tables 5 and 6). Such an effect has not been previously reported. Recently other effects of PUFA-containing triacylglycerols upon *in vitro* lymphocyte functions have been described. For example, it has been shown that trieicosapentaenoylglycerol inhibits the antigen-presenting function of murine spleen cells [47] and that both trieicosapentaenoylglycerol and tridocosahexaenoylglyerol inhibit the natural killer cell activity of human peripheral blood lymphocytes [48]. Such direct immunosuppressive effects of triacylglycerols may explain why, in some clinical trials, PUFA-rich diets have been successful in improving the clinical condition of patients suffering from inflammatory diseases.

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