Unsaturated fatty acids enhance cell yields and perturb the energy metabolism of an antibody-secreting hybridoma

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Growth of the murine B-lymphocyte cell line CC9C10 and the myeloma SP2/0 was enhanced significantly by the presence of the unsaturated fatty acids, oleic and linoleic acids in serum-free culture. The cellular content of linoleic and oleic acids gradually increased during continuous culture passage, with no evidence of regulatory control. Over 10 culture passages in the presence of these fatty acids, the unsaturated/saturated fatty acid ratio of all cellular lipid fractions increased substantially. Most of the fatty acid accumulated in the polar lipid fraction (more than 74%) and only a small proportion was oxidized to CO_2 (0.5%).

Linoleic acid caused a decrease to one-eighth in the rate of metabolism of glutamine and a 1.4-fold increase in the rate of metabolism of glucose. There was no change in the relative flux of glucose through the pathways of glycolysis, pentose phosphate or the tricarboxylic acid cycle. The changes in energy metabolism were reversed when the cells were removed from fatty acid-supplemented medium. The most plausible explanation for these effects is the observed decrease in the rate of uptake of glutamine into cells loaded with linoleic acid. Growth of the CC9C10 cells in linoleic acid caused the K_m of glutamine uptake to increase from 2.7 to 23 mM, whereas glucose uptake was unaffected.

INTRODUCTION

B-lymphocyte hybridomas are used routinely for the large-scale production of monoclonal antibodies [1,2]. For most production processes the hybridomas are adapted to a serum-free medium formulation that allows batch-to-batch culture consistency and ease of antibody purification [3–7].

Fatty acids are often included in the serum-free medium to replace the growth-promoting properties of the lipid component of serum. However, the effect of fatty acids on the growth of cultured mammalian cells is variable. There are many cases in which fatty acids have been observed to stimulate cell growth [8–10]. However, in other cases fatty acids had no effect [11–13] or were inhibitory to cell growth [14].

Certain fatty acids are known to be essential dietary requirements for mammals [12]. These include polyunsaturated fatty acids of the $n-3$ and $n-6$ group [9] such as linoleic acid ($C_{18:2}$, $n-6$) or linolenic acid ($C_{18:3}$, $n-3$). In contrast, saturated fatty acids or unsaturated fatty acids such as oleic acid ($C_{18:1}$, $n-9$) can be synthesized by normal mammalian cells that possess elongation and desaturation enzymes [15].

The dietary requirement for the essential fatty acids in animals has been associated with the synthesis of eicosanoid hormones such as prostaglandins [16]. Thus linoleic acid has been shown to enhance the proliferation of mouse mammary epithelial cells by metabolism to arachidonic acid, which is a precursor of prostaglandin E_2 [17]. However, the essential fatty acids might also be required for the maintenance of normal membrane structure [15].

Unsaturated fatty acids such as oleic acid and linoleic acid have been found to be particularly effective in enhancing mammalian cell yields in culture [18,19]. However, the mechanism of growth promotion of these fatty acids is unclear but could be related to their importance in the synthesis of cellular membranes [20,21]. The fatty acid composition of the phospholipid component of the membrane can have a significant effect on membrane fluidity [22].

Several reports have indicated the concentration dependence of the growth-promoting activity of unsaturated fatty acids in cell culture. A low concentration (less than 75 μM) can promote growth, whereas a higher concentration (more than 100 μM) can be inhibitory [11,23]. Such a sharp concentration optimum might account for apparently contradictory reports of the growth effects of fatty acids in the literature.

The effect of the unsaturated fatty acids on protein secretion of cultured cells can be dissociated from growth effects. Secretion of individual cytokines from human peripheral lymphocytes is selectively influenced by specific fatty acids [23]. Recombinant protein productivity from BHK cells seems to be stimulated by unsaturated fatty acids independently of cell growth [19].

In a previous report [24] we showed that linoleic or oleic acid enhances significantly the cell yield and monoclonal antibody productivity of a B-lymphocyte hybridoma (CC9C10). However, continued culture passage with the unsaturated fatty acids leads to a lipid-loaded state in which cells maintain a high capacity for growth but a decreased capacity for antibody production. We now present results to show that there are major differences in the energy metabolism of the B-lymphocyte hybridoma after prolonged growth in the presence of linoleic acid.

MATERIALS AND METHODS

Cell line

The murine B-lymphocyte hybridoma (CC9C10), which secretes a monoclonal antibody (IG_1) against bovine insulin, and the parental myeloma (Sp2/0) were obtained from the American Type Culture Collection (ATCC No HB123 and CRL 1581). The cells were shown to be mycoplasma-free by routine testing in an independent laboratory (Rh Pharmaceuticals, Winnipeg, Manitoba, Canada). The cells were adapted from a serum-supplemented medium to a serum-free medium over several

Abbreviations used: mAb, monoclonal antibody; (r)IGF, (recombinant) insulin-like growth factor.

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passages and grown for at least 10 passages before the described experiments involving fatty acid addition.

Culture

The basal medium consisted of DMEM/Ham's F12 in a 1:1 (v/v) ratio (Gibco). This medium was supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 0.1% Pluronic F-68, 100 μM phosphoethanolamine, 10 μM ethanolamine and 10 nM sodium selenite. Preliminary experiments indicated that these concentrations were optimal for cell growth. In the hybridoma (CC9C10), equivalent cell growth was obtained in media in which insulin was replaced by a recombinant analogue of insulin-like growth factor (Long R^3 IGF) at a concentration of 20 ng/ml. Growth yields in the presence of either insulin or recombinant IGF were enhanced by 20%. This form of recombinant IGF has been used previously in mammalian cell culture media as a replacement for insulin [25].

The cultures were supplemented, where indicated, with fatty acid-free BSA (0.1 mg/ml) complexed with a specific concentration of a fatty acid. A BSA/fatty acid concentrate ($\times 100$) was formed by mixing 1 ml of BSA (10 mg/ml) with various volumes (1–10 μl) of fatty acid (200 mg/ml) in ethanol. The concentrate was mixed for 30 min at room temperature before addition to the cultures. Cultures were established in T-flasks or in 24-well plates and grown under a 10% CO_2 non-humidified atmosphere. The basal medium contained a low level of methyl linoleate (0.15 μM). However, this was a minimal concentration compared with the linoleic acid supplements used in our experimental cultures.

Fatty acids

The fatty acids used in this study were: hexanoic ($C_{6:0}$), octanoic ($C_{8:0}$), decanoic ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), margaric ($C_{17:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$, $n-9$), linoleic ($C_{18:2}$, $n-6$), linolenic ($C_{18:3}$, $n-3$) and arachidonic ($C_{20:4}$, $n-6$). All chemicals were purchased from Sigma Chemical Co. unless otherwise stated.

Cell counting

Total cell concentrations were determined by a Coulter counter. Viable cell concentrations were determined by counting a cell suspension diluted 1:1 (v/v) with 0.2% Trypan Blue with a Neubauer haemocytometer.

Intracellular protein content

A cell suspension was sonicated and the protein content was determined by the Bradford assay [26].

Radiolabelled compounds

D-[1-^{14}C]Glucose, D-[6-^{14}C]glucose, D-[3-^{3}H]glucose, D-[U-^{14}C]-glucose, L-[U-^{14}C]glutamine, [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid were purchased from Amersham. Radioactive counts were determined in a scintillation counter (LKB Rackbeta) with Ecolume as a scintillant.

Measurement of CO_2 evolution

Rates of $^{14}CO_2$ release from cell cultures (1 ml) incubated in Konte flasks (10 ml) were determined in growth medium containing 17.5 mM glucose and 6 mM glutamine. Cultures were supplemented with one of the following radioactive components: 0.5 μCi of D-[U-^{14}C]glucose, 0.5 μCi of D-[1-^{14}C]glucose, 0.5 μCi of D-[6-^{14}C]glucose or 0.5 μCi of L-[U-^{14}C]glutamine, 25 μM linoleic acid with 0.2 μCi of [1-^{14}C]linoleic acid, or 25 μM oleic acid with 0.2 μCi of [1-^{14}C]oleic acid. Rates of $^{14}CO_2$ release were measured by methods previously described [27,28].

Rate of glycolysis

This was measured by the rate of release of ^3H_2O from cultures (1 ml) containing 17.5 mM glucose with 1 μCi of D-[3-^{3}H]glucose. The ^3H_2O was separated from D-[3-^{3}H]glucose on an anion-exchange column in the borate form using a method previously described [27,29]. The release of ^3H_2O is a measure of metabolic flux through the aldolase and triose phosphate isomerase reactions, which are committed steps of the glycolytic pathway.

Substrate incorporation into lipid fractions

Cells were incubated with growth medium (50 ml) at 10^5 cells/ml for 24 h in the presence of the radioactive substrates D-[U-^{14}C]glucose (5.3 μCi), L-[U-^{14}C]glutamine (1.3 μCi), [1-^{14}C]linoleic acid (2.6 μCi) or [1-^{14}C]oleic acid (2.6 μCi). The concentrations of glutamine (6 mM) and glucose (17.5 mM) corresponded to the standard growth medium. Oleic or linoleic acids were added at 25 μM where indicated, as a BSA-bound complex. The molar ratio of BSA to fatty acid was 1:16.

Lipid extraction

Lipids were extracted from cells by modification of a process described by Puttmann et al. [30]. Cells (10^8) were centrifuged from growth media, washed with PBS and resuspended in distilled water (2 ml) before sonication. The cell extract was then mixed with 10 ml of isopropanol/n-heptane/2 M phosphoric acid (40:10:1, by vol.). n-Heptane (2 ml) and water (3 ml) were added, the mixture was vortex-mixed and the solvent layers were separated by centrifugation at 1000 ***g*** for 5 min. The upper organic layer was retained and the extraction from the aqueous layer was repeated. The two organic extracts were pooled, dried under a stream of nitrogen and redissolved in chloroform (0.5 ml).

Lipid separation

The cellular lipid extract was separated into three fractions (neutral lipid, free fatty acid and polar lipid) by use of adsorption chromatography [31]. Each column was prepared by introducing 100 mg of aminopropyl glass beads (Sigma) between fibreglass plugs in a Pasteur pipette. The three fractions were separated by sequential elution with 3 ml of chloroform/isopropanol (2:1, v/v), 3 ml of diethyl ether/acetic acid (98:2, v/v) and 4 ml of methanol.

Fatty acid analysis

Lipid fractions were saponified by incubation in 0.27 M methanolic KOH for 20 min at 80 °C and derivatized with *p*-bromophenacyl bromide [30]. The derivatized fatty acids were separated by HPLC (Pharmacia/LKB) with a C_8 column (25 cm $\times$ 4.6 mm; Beckman). Margaric acid ($C_{17:0}$) was used as an internal standard. Derivatized fatty acids were quantified with a spectral detector set at 254 nm and an associated integrator.

Antibody analysis

(1) Monoclonal antibody (mAb) concentrations were determined by HPLC with an immunoglobulin-specific affinity column (ProAnaMabs from Hyclone). (2) An ELISA was also developed to establish the antigen specificity of the mAb secreted by the CC9C10 cells. mAb was purified by a Protein A affinity column from the supernatant of CC9C10 hybridoma cultures. Flat-

bottomed 96-well microtitre plates were coated with varying dilutions (100 μl) of the mAb (0–3 μg) and then washed with PBS containing 0.1 % Tween-20 (PBS-Tween). Non-specific sites were blocked by incubation with 1 % (w/v) BSA in PBS (200 μl per well) for 1 h. All subsequent incubations were at 37 °C unless otherwise stated. Washed plates were incubated with 100 μl of insulin–biotin conjugate (0.1 μg/ml) for 1 h, washed again and incubated for 45 min in 100 μl of a 1 : 5000 dilution of anti-biotin mAb alkaline phosphatase. In a modification of the assay, Long R^3 IGF was added at a concentration range of 0–0.2 μg/ml with the insulin–biotin conjugate (0.1 μg/ml). A final washing step was followed by colour development of the plate with the addition of 100 μl of *p*-nitrophenyl phosphate (1 mg/ml) and incubation for 30 min at room temperature. The absorbance of each well was read at 405 nm with a multiwell plate reader (Thermomax; Molecular Devices). All reagents were purchased from Sigma.

This assay showed a linear response of mAb binding over a concentration range of 0.2–1.0 μg/ml, indicating specificity of the mAb for the insulin conjugate.

Radioactive substrate uptake

The rate of uptake of glucose and glutamine into the cells was determined by a protocol modified from Ash et al. [32]. Cells were washed twice and resuspended at 10^7 cells/ml in sodium-free Hepes-buffered saline (137 mM choline chloride/0.7 mM K_2HPO_4/10 mM Hepes/1 mM $MgCl_2$/1 mM $CaCl_2$). Aliquots (1 ml) were dispensed into a 24-well plate and incubated for 3 min at 37 °C with varying concentrations of glucose (10 μM to 10 mM) with 0.18 μCi of [U-^{14}C]glucose or glutamine (1 μM to 5 mM) with 0.06 μCi of [U-^{14}C]glutamine. After the incubation period the cells were separated by filtration with a Millipore suction manifold. The filters containing cells were washed twice with ice-cold buffer and then added to 1 ml of lysis solution (0.1 % SDS and 0.1 M NaOH) for 2 h before radioactive determination with a scintillation counter. The parameters of uptake (K_m and V_{max}) were determined by hyperbolic regression analysis with the program HYPER. In preliminary experiments linear rates of uptake were established over 5 min in sodium-free Hepes buffer.

Calculation of the specific consumption or production rates (q_c)

Values were determined from culture measurements of cell density and substrate or product concentrations by the following equation [33]:

$$q_c = \frac{\Delta c\,(\ln N - \ln N_0)}{T(N - N_0)}$$

where Δc is the change in concentration of substrate or product, N_0 is the initial cell concentration, N is the final cell concentration and T is the time interval.

RESULTS

Effect of fatty acids on growth in serum-free media

The effect of fatty acids on the growth of the CC9C10 hybridomas was determined by supplementation of cultures with individual fatty acids complexed to BSA (Figure 1). Of the 12 fatty acids tested only oleic and linoleic acid caused significant and substantial growth enhancement (300 %). Oleic acid was optimal at a concentration of 50 μM and linoleic acid at 25 μM. However, at a higher concentration (over 75 μM), the cell yields fall below the level of the control cultures.

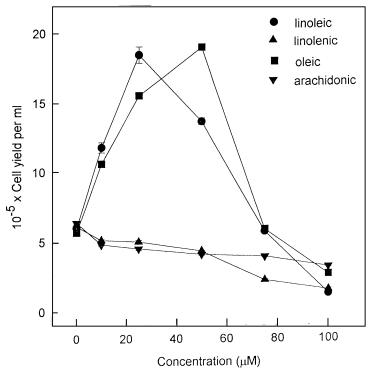

Figure 1 The effect of unsaturated fatty acids on the growth of CC9C10 cells

Cells (10^5/ml) were inoculated into serum-free medium (2 ml) contained in 24-well plates in the presence of linoleic ($C_{18:2}$) (●), oleic ($C_{18:1}$) (■), linolenic ($C_{18:3}$) (▲) or arachidonic ($C_{20:4}$) (▼) acids. The fatty acids (at various concentrations up to 100 μM) were complexed with fatty acid-free BSA (final concentration 0.1 mg/ml) before addition to cultures. Control cultures contained uncomplexed BSA. Cell yields were determined by a Coulter counter after 3 days of growth. Each point represents the mean ± S.E.M. for cell counts from three independent cultures.

Of the other fatty acids tested, hexanoic, lauric, margaric and stearic acids had no effect on cell growth over the concentration range tested. Four fatty acids (arachidonic, linolenic, octanoic and myristic acids) caused a concentration-dependent inhibition of cell growth. Palmitic and decanoic acids enhanced cell growth marginally (9 %) but significantly at 25 μM.

Continuous passage in the presence of linoleic or oleic acids

The growth stimulatory effect of linoleic and oleic acids was determined for hybridoma cultures over prolonged passage. Linoleic acid (25 μM) enhanced growth more than oleic acid (25 μM) but an equimolar mixture of oleic and linoleic acid (25 μM) stimulated growth more than either fatty acid alone (Figure 2A). The growth enhancement effect was shown to be reversible. Thus when cells cultured continuously for several passages in the presence of fatty acids were reintroduced into unsupplemented medium, the growth advantage over control cultures was lost within two or three passages.

The effect of linoleic or oleic acid supplemention on mAb production is shown in Figure 2B. There was a significant initial enhancement of antibody titres in fatty acid-supplemented cultures at passage 1 but this enhancement effect gradually decreased to insignificance by passage 5. Subsequent transfer of the cells to fatty acid-free cultures resulted in a transitory state (passage 8) in which higher mAb titres were re-established for cells previously grown in the presence of the fatty acids. The observed enhancement of mAb titre was significantly higher ($P < 0.01$) in cultures supplemented with linoleic acid or linoleic/oleic acid mix than in those supplemented with oleic acid alone.

Effect of replacing insulin by recombinant IGF (rIGF)

We were concerned to eliminate the possibility that the observed enhancement of growth and antibody production by fatty acid was due to an interaction between the anti-insulin mAb and the insulin present in the medium. Therefore the experiment involving continuous passage of the CC9C10 cells was repeated in cultures in which insulin was replaced by Long R^3 IGF. The CC9C10

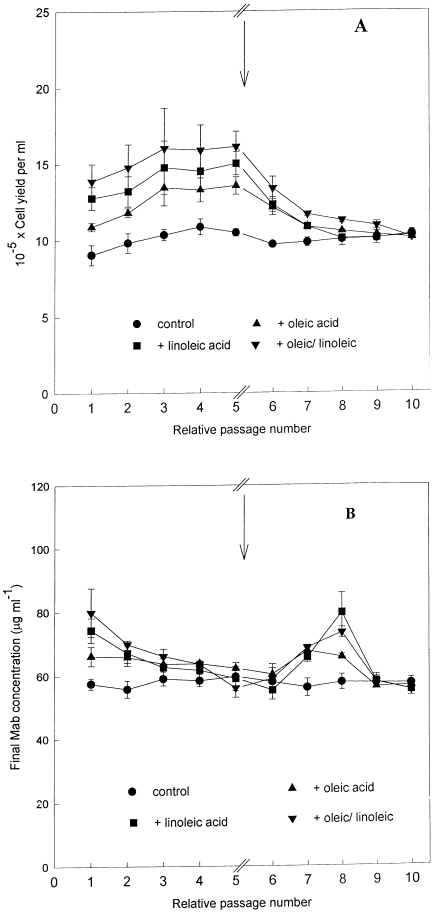

Figure 2 The cell yield (A) and final mAb concentration (B) in CC9C10 cultures over consecutive culture passages

At each passage, cells were inoculated at 10^5/ml into 10 ml of growth medium contained in 75 cm^2 T-flasks. The medium contained no fatty acid (●), 25 μM linoleic acid (■), 25 μM oleic acid (▲) or a 25 μM equimolar mix of linoleic and oleic acids (▼). The final cell concentration was determined after 4 days of growth. The arrow indicates a point at which cells grown in the presence of fatty acids (25 μM) for at least 10 passages were reintroduced into fatty acid-free medium. mAb (Mab) concentrations were determined by an affinity column specific for mouse immunoglobulin. Each point represents the mean ± S.E.M. for cell counts from three independent cultures.

cells were grown over multiple culture passages in the rIGF-supplemented medium with an identical protocol to that described for insulin-supplemented cultures. No significant difference in results was obtained with respect to cell yields or mAb production compared with those shown in Figure 2. In all cases fatty acid supplementation resulted in significantly higher yields. After five passages, cultures containing oleic and linoleic acids (25 μM at a 1:1 mix) attained a cell yield of $(1.51 \pm 0.01) \times 10^6$ cells/ml ($n = 3$) as opposed to $(1.10 \pm 0.03) \times 10^6$ cells/ml ($n = 3$) for control cultures.

Interaction of mAb with insulin or rIGF

The specificity of the CC9C10 mAb was determined by an ELISA based on antibody binding to an insulin–biotin conjugate. Long R^3 IGF had no effect on the linearity or sensitivity of the ELISA. This showed that the CC9C10 mAb was specific for insulin and did not cross-react with Long R^3 IGF. This result was compatible with a previous observation that no anti-insulin antibody has been found to cross-react with IGF [34].

Table 1 Incorporation of unsaturated fatty acids into intracellular lipid fractions

Cells (10^5/ml) were incubated for 24 h in growth medium (50 ml) containing 2.6 μCi of [1-^{14}C]linoleic acid or 2.6 μCi of [1-^{14}C]oleic acid. The cell pellet from each incubation was then separated into polar, non-polar and free fatty acid fractions as described in the Materials and methods section. Cells were grown in 25 μM linoleic acid-supplemented, 25 μM oleic acid-supplemented or unsupplemented (control) cultures for at least five passages before the start of the experiment. Each value (nmol/day per 10^6 cells) is a mean ± S.E.M. for three independent cultures.

	Linoleic acid incorporation (nmol/day per 10^6 cells)		Oleic acid incorporation (nmol/day per 10^6 cells)	
	Control cells	Linoleic acid-grown cells	Control cells	Oleic acid-grown cells
Polar lipid fraction	54 ± 1	49 ± 6	42 ± 4	49 ± 2
Non-polar lipid fraction	10.3 ± 0.7	11.4 ± 0.1	9.7 ± 0.5	13.7 ± 0.3
Free fatty acid fraction	2.1 ± 0.2	2.8 ± 0.3	4.2 ± 0.0	1.8 ± 0.2
Total	66	63	56	65

Effect of linoleic acid on growth of SP2/0 myelomas

The effect of fatty acid supplementation was also determined in cultures of the myeloma SP2/0, which does not secrete antibody. The cells were grown in serum-free media over multiple culture passages in the presence of fatty acids with a protocol similar to that used for the hybridoma cultures. Although the cell yields in the Sp2/0 cultures were consistently lower than those of the CC9C10 cultures, the effect of growth enhancement by linoleic acid was similar. After five passages with linoleic acid (25 μM), cultures attained a cell yield of $(1.07 \pm 0.01) \times 10^6$ cells/ml ($n = 3$), which was significantly higher than the control value of $(0.78 \pm 0.01) \times 10^6$ cells/ml ($n = 3$). The cell yield declined when linoleic acid-grown cells were re-introduced into fatty acid-free medium.

Fatty acid incorporation into intracellular lipid fractions

The pattern of fatty acid utilization was determined by the incorporation of [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid into cellular lipid fractions. There was no significant difference in the total incorporation of linoleic acid or oleic acid between control and fatty acid-grown cells (Table 1). However, in the oleic acid-grown cells a slight but significant difference ($P < 0.05$) was observed in the pattern of incorporation, resulting in a higher accumulation of the fatty acid in the non-polar fraction and a lower accumulation in the free fatty acid fraction compared with control cultures. Most of the fatty acid was incorporated into the polar lipid fraction (more than 74%). This fraction included the phospholipids derived from cell membranes.

Fatty acid composition of the cellular lipid fractions

Over 90% of the fatty acid composition of the cells could be accounted for by linoleic, palmitic, oleic, stearic and arachidonic

Table 2 Fatty acid composition of three cellular lipid fractions from linoleic acid-grown cells

Cells (CC9C10) were grown for one or ten passages in media supplemented with 25 μM linoleic acid. Control cultures contained no added fatty acid. Lipids were extracted and fractionated by adsorption chromatography as described in the Materials and methods section. Individual fatty acids were analysed by HPLC separation of saponified lipid fractions. The fatty acids were derivatized with *p*-bromophenacyl bromide and detected by fluorescence. Peak areas were determined with reference to an internal standard (margaric acid). Values are means ± S.E.M. for three independent cultures.

	Control cells			Linoleic acid-grown passage 1			Linoleic acid-grown passage 10		
Fatty acid	Free fatty acids	Neutral lipids	Polar lipids	Free fatty acids	Neutral lipids	Polar lipids	Free fatty acids	Neutral lipids	Polar lipids
$C_{16:0}$ (mol%)	24.0 ± 0.9	28.0 ± 1.7	17.7 ± 0.1	16.5 ± 0.6	7.6 ± 0.3	4.9 ± 0.2	4.6 ± 1.4	1.4 ± 0.0	0.7 ± 0.0
$C_{18:0}$ (mol%)	33.8 ± 0.6	23.3 ± 0.8	22.9 ± 0.6	8.3 ± 0.1	2.8 ± 0.0	2.1 ± 0.1	8.4 ± 0.4	1.2 ± 0.1	0.6 ± 0.1
$C_{18:1}$ ($n-9$) (mol%)	25.8 ± 0.9	28.1 ± 1.7	37.3 ± 2.4	7.3 ± 0.0	5.3 ± 0.4	3.9 ± 0.4	6.9 ± 0.0	2.0 ± 0.1	1.2 ± 0.0
$C_{18:2}$ ($n-6$) (mol%)	7.4 ± 0.2	8.0 ± 0.5	0.5 ± 0.3	60.1 ± 2.4	79.8 ± 3.5	83.9 ± 1.2	78.4 ± 2.0	95.5 ± 1.3	96.8 ± 1.2
$C_{20:4}$ ($n-6$) (mol%)	2.9 ± 0.9	7.8 ± 0.4	1.8 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.7 ± 0.0	0.03 ± 0.02	0
Total (μmol per 10^6 cells)	5.4	3.3	2.8	6.7	5.8	9.4	13.0	20.9	35.8

Table 3 Fatty acid composition of three cellular lipid fractions from oleic acid-grown cells

Individual fatty acids were analysed by HPLC separation of saponified lipid fractions. The fatty acids were detected by fluorescence and quantified by reference to an internal standard (margaric acid). Cells (CC9C10) were grown for 10 passages in media supplemented with 25 μM oleic acid. Control cultures contained no added fatty acid. Values are means ± S.E.M. for three independent cultures.

Fatty acid	Free fatty acids	Neutral lipids	Polar lipids
$C_{16:0}$ (mol%)	4.4 ± 0.1	1.4 ± 0.1	1.0 ± 0.0
$C_{18:0}$ (mol%)	4.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.0
$C_{18:1}$ ($n-9$) (mol%)	88.5 ± 1.9	97.1 ± 3.2	97.3 ± 2.7
$C_{18:2}$ ($n-6$) (mol%)	1.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
$C_{20:4}$ ($n-6$) (mol%)	0.6 ± 0.0	0.02 ± 0.01	0.01 ± 0.01
Total (μmol per 10^6 cells)	15.6	21.5	25.7

Table 4 Rate of oxidative metabolism of unsaturated fatty acids

The rate of $^{14}CO_2$ release from 0.2 μCi of [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid was measured from cells grown in culture in the absence of fatty acids (control), with 25 μM linoleic acid, 25 μM oleic acid or 25 μM linoleic/oleic acid (1:1 molar ratio). The CC9C10 cells were adapted to each culture medium for at least five passages. The initial linear rates (pmol/min per 10^6 cells) were determined by linear regression of five time point measurements repeated for three independent cultures.

	Initial linear oxidation rate (pmol/min per 10^6 cells)	
Cells	$^{14}CO_2$ from [1-^{14}C]linoleic acid	$^{14}CO_2$ from [1-^{14}C]oleic acid
Control	0.193 ± 0.009	0.233 ± 0.006
Linoleic acid-grown	0.238 ± 0.007	0.191 ± 0.009
Oleic acid-grown	0.263 ± 0.007	0.252 ± 0.005
Linoleic/oleic acid-grown	0.246 ± 0.005	0.203 ± 0.007

acids (Table 2). Minimal quantities of other fatty acids ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{18:3}$) were also determined but were less than 10 mol% in control cells and were decreased to less than 5 mol% after one passage of growth in either oleic or linoleic acid.

In control cells the proportion of linoleic acid varied from 0.5% to 8.0%, whereas oleic acid was present at 26–37% in different lipid fractions. When cells were grown in linoleic acid the content of this fatty acid increased in all three lipid fractions. The increase was particularly significant in the polar lipid fraction, 7.9 μmol per 10^6 cells after one passage and 34.7 μmol per 10^6 cells after 10 passages in linoleic acid-supplemented medium. The linoleic acid content of the polar lipid fraction at passage 10 represented 97% of the total analysed fatty acid. The linoleic acid content of the free fatty acid and non-polar lipid fractions also increased significantly, whereas the content of all other fatty acids declined.

A similar analysis of cells grown over 10 passages in oleic acid-supplemented cultures showed a significant increase in the oleic content of all three lipid fractions (Table 3). This coincided with a decline in the proportion of all other fatty acids in these fractions.

Rate of CO_2 release from the unsaturated fatty acids

A constant rate of $^{14}CO_2$ release from [1-^{14}C]linoleic acid or [1-^{14}C]oleic acid was observed over 80 min in all incubations (Table 4). For linoleic acid-grown cells the rate of $^{14}CO_2$ release from [1-^{14}C]linoleic acid was not significantly different from that from cells grown with oleic acid or the linoleic/oleic acid mix, but was significantly higher than the rate for control cells (0.193 pmol/min per 10^6 cells).

Release of $^{14}CO_2$ from [1-^{14}C]oleic acid occurred at a high rate in those cells grown in the presence of oleic acid compared with control cells. However, the rates of CO_2 release from cells grown in linoleic or the linoleic/oleic acid mix were significantly lower than in control cells. The measured rates of fatty acid oxidation to CO_2 accounted for a small proportion (0.5%) of the total cellular incorporation of either linoleic or oleic acid in all cases.

Effect of fatty acids on the consumption of glucose and glutamine

The pattern of specific consumption of the major carbon substrates (glucose and glutamine) was determined from an analysis of the media harvested in the growth experiment described in Figure 2. The rate of decrease of the concentration of glucose and glutamine was linear over 4 days of culture and neither substrate was completely exhausted from the media. The glucose decreased from an initial value of 17.5 mM to 0.7–3.8 mM, whereas the glutamine decreased from an initial 6 mM to 0.5–2.9 mM.

Although the overall glucose consumption in the fatty acid-containing cultures was significantly higher than in the control,

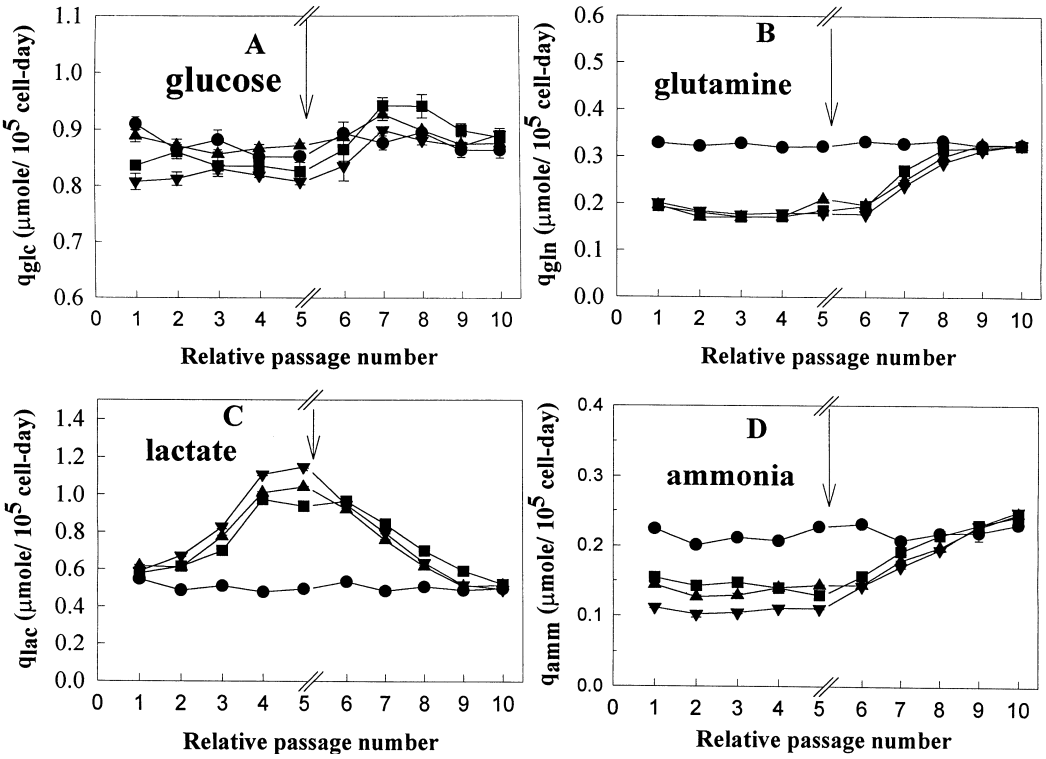

Figure 3 The specific consumption or production rates of (A) glucose, (B) glutamine, (C) lactate or (D) ammonia in CC9C10 cultures

The consumption (q_{glc} and q_{gln}) and production (q_{lac} and q_{amm}) rates were calculated from concentration measurements made at the end of each 4-day culture as described in the legend to Figure 2. The medium contained no fatty acid (●), 25 μM linoleic acid (■), 25 μM oleic acid (▲) or a 25 μM equimolar mix of linoleic and oleic acids (▼). The arrow indicates a point at which cells grown in the presence of fatty acids (25 μM) for at least 10 passages were re-introduced into fatty acid-free medium. Points represent means for three independent cultures. In most cases the error bars were smaller than the symbols used.

the specific glucose consumption rate, q_{glc}, was slightly but significantly lower ($P < 0.01$) in the presence of linoleic acid (passages 1–5 in Figure 3). Reinoculation of the fatty acid-grown cells in fatty acid-free medium caused an increase to the level of the control.

The specific glutamine utilization rate (q_{gln}) decreased to 1/1.8 in fatty acid-containing cultures (Figure 3). Furthermore this change was reversible, as shown by the increase in q_{gln} when the fatty acid-grown cells were inoculated into a fatty acid-free medium.

Measurements of the specific rates of production of the metabolic by-products, ammonia and lactate (q_{amm} and q_{lac}), gave an indication of a significantly different metabolism occurring in the fatty acid-grown cells (Figure 3). The q_{lac} increased significantly in all fatty acid-containing cultures, whereas the q_{amm} decreased significantly. These changes were reversible on removal of the fatty acids.

A similar pattern of glucose and glutamine utilization as well as ammonia and lactate production occurred during the culture of Sp2/0 myeloma cells.

Table 5 Incorporation of glucose and glutamine into intracellular lipid fractions

CC9C10 cells (10^5/ml) were incubated for 24 h in growth media (50 ml) containing (1) 17.5 mM glucose with 5.3 μCi of [U-^{14}C]glucose or (2) 6 mM glutamine with 1.3 μCi of [U-^{14}C]glutamine. The cell pellet from each incubation was then separated into polar, non-polar and free fatty acid fractions as described in the Materials and methods section. Cells were grown in the linoleic acid-supplemented, oleic acid-supplemented or unsupplemented (control) cultures for at least five passages before the start of the experiment. Each value (nmol/day per 10^6 cells) is a mean ± S.E.M. for three independent experiments.

	Glucose incorporation (nmol/day per 10^6 cells)		Glutamine incorporation (nmol/day per 10^6 cells)	
	Control cells	Linoleic acid-grown cells	Control cells	Linoleic acid-grown cells
Polar lipid fraction	236 ± 7	278 ± 7	508 ± 2	348 ± 5
Non-polar lipid fraction	53.9 ± 0.4	51.5 ± 0.9	86 ± 3	77 ± 2
Free fatty acid fraction	91 ± 1	215 ± 3	49.1 ± 0.4	7.9 ± 0.7
Total	381	545	643	433

Incorporation of glucose and glutamine into lipids

The rates of incorporation of [U-^{14}C]glucose and [U-^{14}C]-glutamine into three intracellular lipid fractions were measured by incubation of the CC9C10 hybridomas in media supplemented with specific radiolabelled substrates over 24 h (Table 5). The rate of incorporation of either substrate into the polar lipid fraction was significantly higher than into either of the other fractions. The incorporation rate of [U-^{14}C]glutamine into the non-polar lipids was higher than that into the free fatty acid fraction.

Of particular significance was the difference in overall conversion of glucose and glutamine to the lipid fractions after the growth of cells in linoleic acid. The overall incorporation of

Table 6 Flux through major energy pathways of glucose and glutamine metabolism

Flux values (±S.E.M.) for glucose metabolism in CC9C10 cells were determined from the rate of release of $^{14}CO_2$ or ^3H_2O (glycolysis) as described in the Materials and methods section. The pentose phosphate pathway flux was determined from the difference in gradient measured from the rate of $^{14}CO_2$ release from [1-^{14}C]glucose and [6-^{14}C]glucose. Each value (nmol/min per 10^6 cells) was determined from five time points measurements repeated in four independent experiments.

	Flux value (nmol/min per 10^6 cells)		
Pathway	Control cells	Linoleic acid-grown cells	Linoleic acid-loaded cells grown without linoleic acid
Glycolysis	4.10±0.03	5.24±0.06	4.79±0.05
Pentose phosphate	0.44±0.02	0.62±0.02	0.58±0.02
Tricarboxylic acid cycle	0.0507±0.0006	0.0908±0.0005	0.064±0.001
Total glucose metabolized	4.60	5.95	5.43

[U-^{14}C]glucose into the lipid fractions increased from 381 nmol/day per 10^6 cells in control cells to 545 nmol/day per 10^6 cells in linoleic acid-grown cells. In contrast, the overall incorporation of [U-^{14}C]glutamine into the lipid fractions decreased from 643 nmol/day per 10^6 cells in control cells to 433 nmol/day per 10^6 cells in linoleic acid-grown cells. These differences seemed to be particularly significant for the polar and free fatty acid fractions of the cells.

Metabolic flux of glucose and glutamine

The initial rate of $^{14}CO_2$ release from [U-^{14}C]glucose was 0.14±0.01 nmol/min per 10^6 cells in linoleic acid-grown cells, which was slightly but signficantly greater than the 0.10±0.01 nmol/min per 10^6 cells determined for control cells ($n = 3$). However, the rate of $^{14}CO_2$ release from [U-^{14}C]glutamine was one-eighth of that in linoleic acid-grown cells, at 1.01±0.02 nmol/min per 10^6 cells compared with 8.09±0.09 nmol/min per 10^6 cells for control cells ($n = 3$). Furthermore it was shown that this change was partly reversible after culturing linoleic acid-grown cells for five passages in the absence of supplemented fatty acids, when the rate of $^{14}CO_2$ release from [U-^{14}C]glutamine increased to 4.41±0.04 nmol/min per 10^6 cells. In preliminary experiments it was shown that the addition of linoleic acid immediately before the assay made no difference to these results.

The flux through specific pathways of glucose metabolism was determined by the use of specifically radiolabelled glucose molecules (Table 6). The rate of $^{14}CO_2$ release from [6-^{14}C]glucose is an indication of the glucose-fed tricarboxylic acid cycle flux, whereas the rate of $^{14}CO_2$ release from [1-^{14}C]glucose indicates the sum of metabolic fluxes via the pentose phosphate pathway and tricarboxylic acid cycle. In these experiments the measured rate of CO_2 release was constant for 6 h and indicated a significantly higher rate for linoleic acid-grown cells than for controls. The partial reversibility of this trend was indicated by measurements from the cells that had been re-adapted to control media after growth with linoleic acid.

The glycolytic flux was measured by the rate of ^3H_2O release from [3-^{3}H]glucose. The pattern of flux values determined by this method was similar to those obtained by the $^{14}CO_2$ release experiments. This indicates a significantly higher glucose flux for linoleic acid-grown cells that was partly reversible after re-adaptation to control media.

The metabolic flux data show that there is no significant difference in the pattern of glucose metabolism in any of the three cultures, with glycolysis accounting for 88%, pentose phosphate pathway 10% and the tricarboxylic acid cycle 1% of the glucose utilized. However, the sum of the glucose metabolic flux rates in linoleic acid-grown cells was 5.95 nmol/min per 10^6 cells, which was 30% higher than in the control. The sum of the metabolic flux values determined for glucose metabolism was equated to 0.86 μmol/day per 10^6 cells, which is comparable to the range of q_{glc} values given in Figure 3.

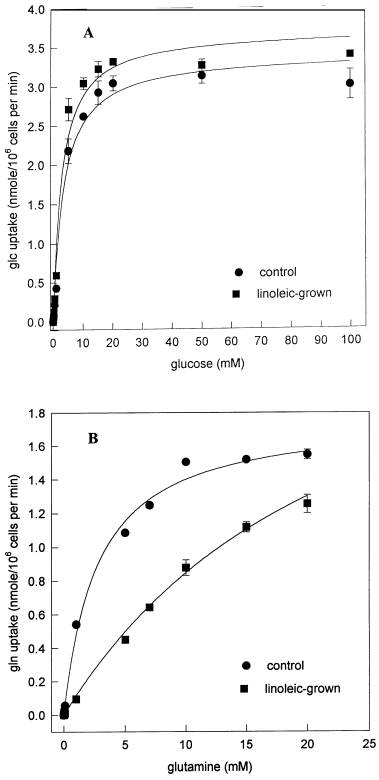

Figure 4 The rate of uptake of (A) glucose and (B) glutamine into CC9C10 cells

Cells grown in the absence of fatty acid (control, ●) or in the presence of 25 μM linoleic acid (■) were suspended in 1 ml of sodium-free Hepes-buffered saline at 10^7 cells/ml. (**A**) Glucose was added at 12 different concentrations between 10 μM and 100 mM with 0.18 μCi of [U-^{14}C]glucose. The points are means for $n = 3$. (**B**) Glutamine was added at 12 different concentrations between 1 μM and 20 mM with 0.06 μCi of [U-^{14}C]glutamine. The points are means for $n = 2$. The rate of uptake was determined by radioactive incorporation into the cells after 3 min of incubation. The hyperbolic curves were drawn from best-fit determinations by Sigmaplot.

Rate of uptake of radioactive substrates

The cellular uptake of glucose and glutamine was determined in a series of radioactive assays of short duration with CC9C10 cells grown in the presence or absence of linoleic acid. Rates were determined from radioactive incorporation into cells over a 3 min incubation period. In separate incubations [U-^{14}C]glucose was added at concentrations up to 100 mM, and [U-^{14}C]-glutamine in concentrations up to 20 mM (Figure 4).

Hyperbolic curve fitting indicated Michaelis–Menten-type kinetics of substrate uptake with respect to concentration (Figure 4). This result shows a significant difference in glutamine uptake between the linoleic acid-grown and control cultures but no significant difference for glucose uptake.

The kinetic parameters of substrate uptake for the control and linoleic acid-grown CC9C10 cells were determined by statistical analysis with HYPER. The apparent K_m and V_{max} values for glucose uptake in the linoleic acid-grown cells were 2.8 ± 0.7 mM and 3.7 ± 0.2 nmol/min per 10^6 cells respectively, neither of which were significantly different from the 3.4 ± 0.9 mM and 3.4 ± 0.2 nmol/min per 10^6 cells determined for control cells.

The uptake rates determined for glutamine were significantly lower in linoleic acid-grown cells at the substrate concentration tested. The apparent V_{max} for glutamine uptake in linoleic acid-grown cells was 2.8 ± 0.4 nmol/min per 10^6 cells, which was slightly but significantly higher than the control value of 1.8 ± 0.1 nmol/min per 10^6 cells. However, the apparent K_m for glutamine uptake in linoleic acid-grown cells, 23 ± 5 mM, was almost an order of magnitude higher than the control value of 2.7 ± 0.5 mM. At the glutamine concentration (6 mM) used in the standard growth medium the glutamine uptake rate in linoleic acid-grown cells was 1/2.5 of that in control cells.

DISCUSSION

Growth stimulation by linoleic and oleic acids was demonstrated for CC9C10 cells and the parental myeloma (SP2/0) in serum-free cultures. The concentration dependence of fatty acid stimulation or inhibition of cell growth has been shown previously for mitogen-stimulated lymphocytes [14,35,36] and hybridomas [6]. However, a requirement by hybridomas or myelomas for lipid supplementation might become apparent in culture only after several passages in serum-free medium after adaptation from serum-based medium [10].

The most likely mechanism for growth enhancement in CC9C10 cells is that the fatty acids are required as components of phospholipids contained in membranes. Other possible explanations can be dismissed from a consideration of the response of the cells to the range of fatty acids tested. A requirement for synthesis of eicosanoids such as prostaglandins would normally be provided by arachidonic acid as well as linoleic acid but not by oleic acid [15,17]. A requirement for substrates of energy metabolism would be provided by a wider range of fatty acids including the saturated fatty acids. This can also be dismissed because of the low rate of oxidation of linoleic or oleic acids to CO_2, which is insignificant compared with the rates of oxidation of glucose and glutamine.

Growth stimulation by fatty acids can be dissociated from effects on synthesis of secreted products [19,23]. For the CC9C10 cells a higher mAb production was observed by initial supplementation with linoleic or oleic acid but this gradually declined over subsequent culture passages. High mAb production was restored by a limited period of growth of the lipid-loaded cells in fatty acid-free medium. This suggests that the optimal intracellular lipid content for mAb production is finely balanced between a diminished and an overloaded state.

Palmitic, stearic, oleic and linoleic acids are the predominant fatty acids in blood serum at a molar ratio of 2:1:3:1 and have been implicated as physiological regulators of DNA synthesis and cytokine release from human lymphocytes [23]. These were also the four predominant fatty acids measured in CC9C10 cells but with an initial molar ratio of 0.9:1:1:0.2 (palmitic/stearic/oleic/linoleic), indicating a considerably higher saturated fatty acid content. An optimal intracellular saturated-to-unsaturated fatty acid ratio for cell growth was proposed by Doi et al. [37], who showed that normal growth of LM cells could be maintained if the unsaturated content of the membrane phospholipid fraction was more than 56%. This is compatible with the observed increase in growth of the CC9C10 cells after the initial addition of linoleic acid to the cultures, which increased the unsaturated content of the cellular lipids from 32% to 88% after one passage.

The energy metabolism of the fatty acid-grown CC9C10 cells was fuelled by an increased utilization of glucose but a decreased utilization of glutamine. However, there was no evidence for a change in the metabolic pathways utilized by either substrate. The decrease in the specific glutamine consumption rate was associated with a corresponding decrease in ammonia production. The measured flux of glucose through all three metabolic pathways (glycolysis, pentose phosphate pathway and the tricarboxylic acid cycle) increased significantly in linoleic acid-loaded cells. A high proportion (more than 88%) of glucose was utilized via the glycolytic pathway with a relatively small proportion via the tricarboxylic acid cycle [27]. This pattern was unaltered in the presence of linoleic acid, suggesting that the relative flux of glucose through the three pathways remained the same. However, there was an increase in lactate production in linoleic acid-grown cells, which resulted in a higher yield coefficient, $Y_{lac/glc}$. This can be explained by a change in the formation of glycolytic end-products. The rate of production of alanine was lower (1/2.2) in linoleic acid-grown cells compared with control cells (results not shown). Alanine is produced from pyruvate by transamination with glutamate in response to the need for the sequestration of excess metabolic nitrogen [38]. It is likely that the decreased glutamine utilization in linoleic acid-grown cells would lower the level of intracellular glutamate. This would in turn decrease the transamination reaction and cause a greater proportion of pyruvate to be converted to lactate.

The most plausible explanation for the fatty acid-mediated changes in cellular metabolism is that the relative capacity of membrane transport for the energy substrates is altered. The K_m of glutamine uptake was lower (1/8.5) in cells grown in linoleic acid whereas the kinetics for glucose uptake was unaffected. The rate of glutamine uptake is known to be a limiting step for the metabolism of glutamine in a hybridoma [27]. Thus it is concluded that the altered growth and energy metabolism of the cells grown with linoleic or oleic acid was most probably caused by a change in the fatty acid composition of the membrane, which significantly decreased the uptake of glutamine.

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