# Molecular basis for the immunosuppressive action of stearic acid on T cells

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## SUMMARY

Studies were performed to determine the mechanism by which stearic acid (18:0) selectively inhibits T-dependent immune responses *in vitro*. Incubation of mitogen-activated B and T cells with 18:0 resulted in dissimilar patterns of incorporation of the saturated fatty acid into their membranes. High-performance liquid chromatography (HPLC) analyses of T cells showed an accumulation of desaturated 18:0-containing phosphatidylcholine (PC) that replaced normal cellular PC. Less significant quantities of the same PC species were seen to accumulate in B-cell membranes; rather, they increased their proportion of oleic acid (18:1)-containing PC. The different lipid compositions of the lymphocyte cell membranes after exposure to 18:0 were correlated with their plasma membrane potentials. In T cells, the accumulation of desaturated, 18:0-containing PC coincided with a rapid (within 8 hr) collapse of membrane integrity, as determined by flow cytometry. The collapse of membrane integrity was found to be time and dose dependent. No such depolarization was observed in B cells which, by virtue of their desaturating ability, were able to avoid incorporating large amounts of desaturated 18:0-containing phospholipids into their membranes. It is proposed that a lack of stearoyl-CoA desaturase in T cells precludes them from desaturating exogenously derived 18:0, thus leading to increased proportions of 18:0-containing desaturated PC in their cell membranes. The increased abundance of this PC species may enhance membrane rigidity to an extent that plasma membrane integrity is significantly impaired, leading to a loss of membrane potential and ultimately cell function and viability.

## INTRODUCTION

Various saturated and unsaturated fatty acids have been reported to modulate the immunological role of lymphocytes both *in vitro* and *in vivo* (Erickson, 1986; Gurr, 1983; Meade & Mertin, 1978). Of particular interest is the finding that some fatty acids can selectively inhibit T-cell-mediated functions in the relative absence of effects on B cells. For example, stearic acid (18:0) has been shown to be a potent inhibitor of phytohaemagglutinin (PHA)-dependent T-lymphocyte proliferation while having little effect on lipopolysaccharide (LPS)-induced B-cell proliferation (Buttke, 1984). Further, 18:0 suppresses primary *in vitro* antibody responses to T-dependent but not T-independent antigens (Pourbohloul, Mallett & Buttke, 1985). T-helper cells are suggested to be the principle target of such immunosuppression, wherein interleukin-2 (IL-2) production is inhibited (Pourbohloul & Buttke, 1990). Lastly, 18:0 has been shown to be cytotoxic for T cells but not B cells (Buttke & Cuchens, 1984).

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Previous workers have proposed that saturated fatty acids may, via their incorporation into phospholipids, perturb the membrane structure, thus adversely affecting membrane-bound enzymes (Stubbs & Smith, 1984; Mahoney *et al.*, 1977; Tsang, Weyman & Smith, 1977). However, the differential effects of 18:0 on T and B cells suggest a fundamental difference between the two lymphocyte types in either lipid metabolism or membrane lipid composition. Indeed, further investigation has revealed that B cells can desaturate 18:0 to 18:1, whereas T cells can not (Buttke *et al.*, 1989). This finding suggests that B cells are better able to maintain a functional balance between saturated and unsaturated fatty acid levels in their membranes by virtue of their having higher levels of stearoyl-CoA desaturase, the enzyme responsible for converting 18:0 to 18:1 (Buttke, *et al.*, 1989). Such results may signify an important difference in the regulation of lipid metabolism during B- and T-lymphocyte maturation, which may be related to their *in vivo* function.

The present studies attempted to define the biochemical basis for the selective cytotoxic effect of 18:0 on T cells. Since it has been shown previously that the inability of T cells to convert 18:0 to 18:1 coincides with an accumulation of desaturated

phospholipid species (Buttke *et al.*, 1989), it seemed possible that such changes in membrane lipid composition might lead to impaired membrane integrity (Kuypers *et al.*, 1984; Lange *et al.*, 1980). Indeed, the accumulation of desaturated 18:0-containing phospholipid species results in the loss of membrane potential in a significant proportion of T cells, implying perturbation of membrane integrity and loss of viability.

## METHODS AND MATERIALS

### Isolation and culture of lymphocytes

Male and female BALB/c mice, 8–12 weeks of age, were obtained from Charles River Breeding Laboratories (Raleigh NC). Spleen cell suspensions were depleted of erythrocytes and macrophages by $NH_4Cl$ lysis and passage of cells through glass-wool columns, respectively (Buttke, Mallett & Cuchens, 1983). The lymphocyte-enriched suspension was further purified into immunoglobulin-positive ($Ig^+$) (B cells) and $Ig^-$ cell populations by incubation on rabbit anti-mouse Ig (Accurate Chemical Co.)-coated Petri plates (Buttke *et al.*, 1983). The non-adherent $Ig^-$ population was subsequently incubated in suspension with a monoclonal mouse alloantisera directed against the Thy-1·2 antigen, followed by a second round of selection on the rabbit anti-mouse Ig-coated Petri plates (Buttke *et al.*, 1983). The adherent populations recovered from the two rounds of positive selection were >95% viable, and considered to be highly enriched populations of B and T cells based on several criteria (Buttke *et al.*, 1983).

Lymphocytes were cultured in 96-well, round-bottomed, microtitre plates (Linbro, Flow Laboratories Inc., McLean, VA) at a concentration of $2·5 \times 10^6$ cells per ml of culture medium. The culture medium consisted of RPMI-1640 containing 0·2% $NaHCO_3$, penicillin (50 U/ml), streptomycin (50 μg/ml) and 5% fetal bovine serum. T lymphocytes were stimulated with phytohaemagglutinin (PHA; Difco) at a concentration of 5 μg/ml and B lymphocytes with lipopolysaccharide (LPS; Difco) at 50 μg/ml. Cell cultures were incubated at 37° in a humidified atmosphere containing 6% $CO_2$ 94% air.

### Albumin-complexed fatty acids

Stearic acid (18:0), bovine serum albumin (BSA) and diatomaceous earth were obtained from Sigma Chemical Co. (St Louis, MO). The fatty acid was adsorbed onto diatomaceous earth and subsequently complexed to BSA to yield fatty acid: BSA ratios of ∼3·5 (Buttke, 1984).

### Phospholipid extraction and analysis

Aliquots of $5 \times 10^7$ B or T lymphocytes were collected by centrifugation at 250 *g* for 10 min, and the pellets were washed once with ice-cold culture medium. Lipids were extracted by the addition of 1 ml of methanol and 2 ml of chloroform (Folch, Lees & Sloan-Stanley, 1957). After 1 hr, 0·6 ml of 0·1 M KCl was added and the suspension was centrifuged at 200 *g* for 5 min to separate the aqueous and organic phases. The lower chloroform layer was recovered and the solvent evaporated under nitrogen in a 37° water bath. Recovered lipids were dissolved in 0·1 ml of 2:1 (v/v) chloroform-methanol prior to analysis.

Total lymphocyte phospholipids were separated by thin-layer chromatography (TLC) on silica gel 60 plates developed with chloroform-methanol-acetic acid-$H_2O$ (75:45:12:5·5). TLC plates were predeveloped with acetone followed by heating

at 110° for at least 1 hr to overcome adverse effects of humidity on phospholipid separation. Once spotted with sample, the TLC plates were incubated for a second time at 110° for 30 min. The plate was then transfered directly to a pre-equilibrated (approximately 1 hr) TLC chamber for development. Separated phospholipids were localized using $I_2$ vapour.

Phosphatidylcholine (PC) fractions were eluted from the silica gel with 10 ml of methanol, converted to diacylglycerides by phospholipase C digestion (Mavis, Bell & Vagelos, 1972), and dinitrobenzylated (Takamura *et al.*, 1986). The resultant diacylglycerobenzoates were separated into molecular species using a Beckman System Gold HPLC fitted with a $4·6 \times 45$ mm precolumn and a Beckman $4·6 \times 250$ mm analytical column, both packed with 5 μm C-18 Ultrasphere (Beckman). Elution with 1 ml/min acetonitrile:2-propanol (70:30 v/v for the first 5 min, followed by 75:25) allowed separation of 11 molecular species. Quantification of individual molecular species was carried out by monitoring UV absorption at 230 nm. Molecular species were identified by comparison of their retention times to those of known authentic standards, by gas-liquid chromatography of their acyl chains, and in some cases by conversion to diacyglyceroacetates and subsequent argentation TLC (Buttke *et al.*, 1989).

### Flow cytometric measurements of relative membrane potential

Membrane potential measurements were made using the catio-nic potential-sensitive dye dihexyloxacarbocyanine iodide [$DiOC_6(3)$], obtained from Molecular Probes Inc. (Eugene, OR). A stock solution of $DiOC_6(3)$ was prepared in dimethyl sulphoxide and stored at $-20°$. Just prior to use, aliquots were thawed, diluted to 50 μM with phosphate-buffered saline (PBS) and added to $1 \times 10^6$ cells in 1 ml PBS. The final $DiOC_6(3)$ concentration attained (125 nm) is reported to be non-toxic for lymphocytes (Damjanovich *et al.*, 1987). After a 15-min incubation at room temperature, cells were analysed using a Becton-Dickinson FACS 440 flow cytometer. The laser was tuned to an excitation wavelength of 488 nm at an output of 400 mW. Emission of fluorescence was assayed within a band ranging from 515–545 nm as a measure of relative membrane potential (Shapiro, Natale & Kamentsky, 1979). Control and gramicidin-treated (20 μg/ml) lymphocyte populations were used to determine fluorescence values for fully polarized and totally depolarized cells, respectively. At each time-point, the number of cells in the polarized region for each culture condition was divided by the total number of cells analysed ($\sim 10^4$) to derive the relative proportion of polarized cells. The ratio obtained at each time-point was multiplied by 100 to obtain the percentage of polarized cells in each sample.

## RESULTS

### PC molecular species of mitogen-activated B and T cells

It has been shown previously that incubation of mitogen-stimulated B and T cells in the presence of 50 μM 18:0 leads to substantial differences in the PC molecular species (Buttke *et al.*, 1989). The present studies were therefore performed to determine if such lipid changes could be correlated with decreased lymphocyte viability. In the initial phase of this study, purified B and T cells were analysed separately for their PC molecular species. To account for any anomalies of membrane composition which might have occurred as a result of culture, B and T
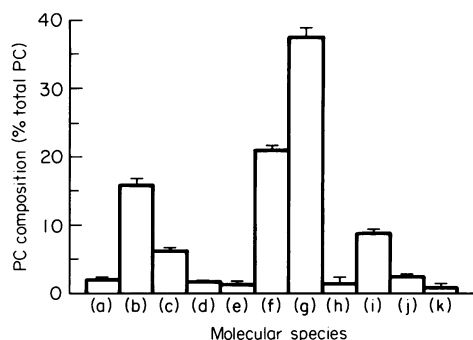
**Figure 1.** PC molecular species of mitogen-activated B and T lymphocytes. Purified B and T cells were stimulated with LPS or PHA, respectively. Cells were harvested and analysed for their PC molecular species by HPLC. Quantities of each molecular species are shown as a percentage of total PC. Data shown represent the mean (±SD) of three separate experiments. Molecular species: (a) 18:0–22:6 and 18:1–18:2; (b) 16:0–18:2; (c) 18:0–20:4; (d) 18:0–22·5; (e) 18:1–18:1; (f) 16:0–18:1 and 18:0–18:2; (g) 16:0–16:0; (h) 18:0–18:1; (i) 16:0–18:0; (j) 16:0–20:1; (k) 18:0–18:0.

cells were first stimulated for 6 hr with PHA or LPS, respectively, in the absence of 18:0.

Mitogen-activated B and T cells contained 11 separable PC molecular species, with the proportions of individual species being nearly identical within the two cell types. The results for each lymphocyte type were therefore combined and in Fig. 1 the level of each individual PC species is shown as the proportion of total PC. A comparison of the results shown in Fig. 1 with the data previously reported for mouse B and T cells (Buttke *et al.*, 1989) reveals a much higher level of dipalmitoyl (16:0–16:0) PC in the present study. Based on additional observations (T. M. Buttke and S. Van Cleave, unpublished data), the differing levels of 16:0–16:0 may have resulted from a change in murine diet. It is interesting to note that 16:0–16:0 PC has also been observed in human tonsil lymphocytes (Morimoto & Kanoh, 1980).

In addition to the desaturated molecular species, lymphocytes also contained several molecular species having the more expected composition of one saturated and one unsaturated fatty acid per PC molecule. PC species containing at least one molecule of 16:0 comprised the majority (~80%) of PC molecular species, the remainder containing primarily 18:0 as their saturated moiety.

## Modulation of PC molecular species in response to 18:0 supplementation

Next the PC molecular species of mitogen-stimulated B and T cells that had been incubated for 6 hr in the presence of 60, 120 or 180 μM albumin-complexed 18:0 were examined. Selection of the lowest dose was based on previous studies showing that 50 μM 18:0 irreversibly inhibited T-cell proliferation by >90% in 10 hr while having much less effect on B-cell proliferation (Buttke & Cuchens, 1984). The higher doses of 120 and 180 μM 18:0 were used in an attempt to amplify 18:0-induced changes in membrane lipid composition. The major effects of 18:0 supplementation on B- and T-cell PC molecular species are shown in Fig. 2.
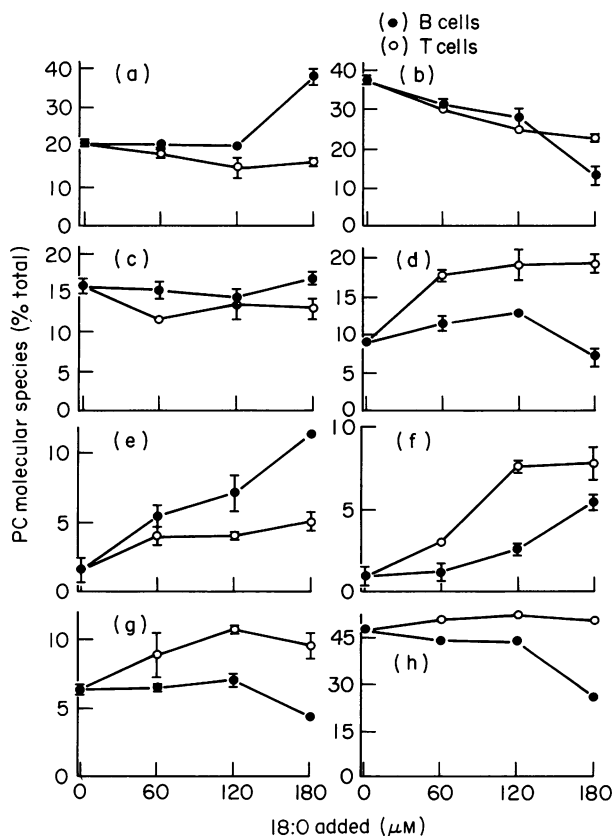


**Figure 2.** Effect of 18:0 addition on PC molecular species of B and T lymphocytes. Purified B and T lymphocytes were stimulated with PHA or LPS, respectively, in the absence or presence of 60 μM, 120 μM or 180 μM 18:0. After 6 hr the cells were harvested and analysed for their PC molecular species. Data are shown for the major molecular species of PC only and are represented as the mean (±SD) of three separate experiments. (a) 16:0–18:1 and 18:0–18:2; (b) 16:0–16:0; (c) 16:0–18:2; (d) 16:0–18:0; (e) 18:0–18:1; (f) 18:0–18:0; (g) 18:0–20:4; (h) total desaturates.

The addition of 18:0 was accompanied by changes in the levels of six of the seven major PC molecular species, with 16:0–18:2 being the singular exception (Fig. 2c). Because of their low levels (1–2%), it was not possible to reliably test for 18:0-induced changes in the four molecular species not shown in Fig. 2. In both B and T cells, levels of 16:0–16:0 PC declined with increasing doses of 18:0 added (Fig. 2b). However, whereas the proportion of 16:0–16:0 decreased from 40% to 25% in T cells exposed to 180 μM 18:0, a further reduction to 15% was observed in similarly treated B cells. The decline in 16:0–16:0 PC in T cells was largely accompanied by increased levels of 16:0–18:0 (Fig. 2d) and 18:0–18:0 (Fig. 2f), which resulted in the overall preservation of similar levels of total desaturated molecular species (Fig. 2h). By contrast, in B cells 16:0–16:0 was replaced by 18:0–18:1 (Fig. 2e) and 16:0–18:1 and 18:0–18:2 (Fig. 2a), resulting in a significant decline in total desaturated PC (Fig. 2h). Overall, with increasing doses of 18:0, T cells were found to substitute 18:0 for 16:0 with little change in the total amount of desaturated species. B cells, however, showed a paradoxical decrease in desaturated species as a consequence of 18:0 exposure.
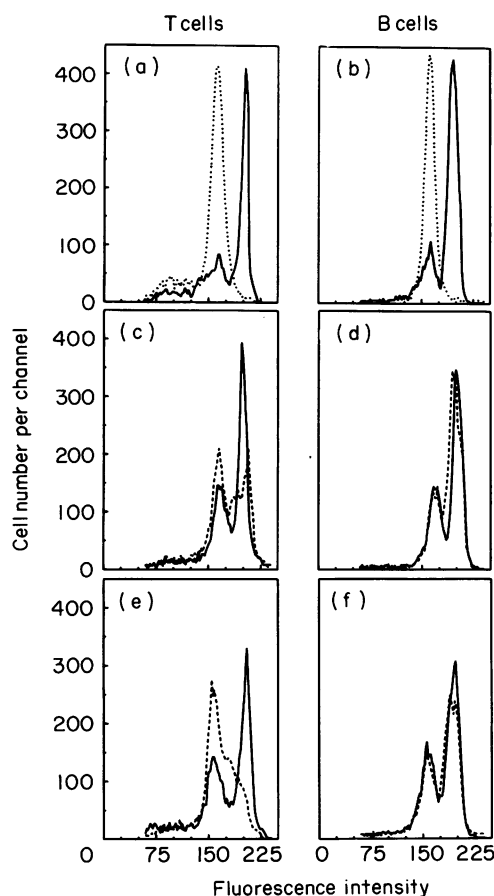
Figure 3. Relative membrane potential of B and T cells exposed to 18:0. Purified B and T cells were stimulated with PHA or LPS, respectively, and cultured in the absence (solid lines) or presence (dashed lines) of 180 $\mu$M 18:0. Aliquots were removed after 0 hr (a, b), 4 hr (c, d) and 8 hr (e, f), the cells stained with DiOC$_6$(3), and analysed by flow cytometry. Gramidicin S was also used to determine the fluorescence intensity of totally depolarized cells (dotted lines, a, b).
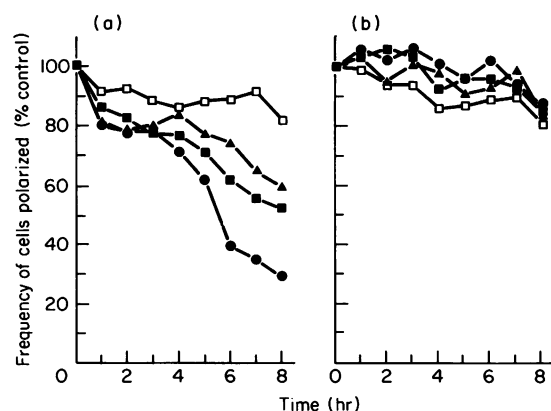


Figure 4. Disruption of T-cell membrane potential by 18:0 is time and dose dependent. Purified B (b) and T (a) cells were mitogen stimulated and cultured in the absence or presence of various doses of 18:0. Aliquots were removed at each hourly time-point and the cells were stained with DiOC$_6$ (3) prior to flow cytometric analysis. Fluorescence intensity values were assigned to depolarized and polarized cells based upon Gramicidin-treated and untreated cell populations. Data are expressed as the percentage of cells displaying fluorescence intensities comparable to control (polarized) cells; each data point is based upon the analysis of $10^4$ cells. Symbols: ($\square$) no 18:0; ($\blacktriangle$) 60 $\mu$M 18:0; ($\blacksquare$) 120 $\mu$M 18:0; and ($\bullet$) 180 $\mu$M 18:0.

## Effect of 18:0 supplementation on lymphocyte membrane potential

Previous studies with erythrocytes have determined that cell haemolysis results after replacement of only 25% of native PC with 18:0–18:0 PC (Lange *et al.*, 1980; Kuypers *et al.*, 1984). An accumulation of the desaturated lipid species was thought to increase membrane rigidity and leakiness, and thus promote haemolysis. To test the effect of 18:0 exposure on the integrity of B- and T-cell membranes, experiments were undertaken to correlate 18:0 supplementation with changes in membrane potential, this being a marker for a functionally intact plasma membrane.

Upon incubation with the membrane potential-sensitive dye, DiOC$_6$(3), both B and T lymphocytes displayed a level of fluorescence intensity expected for cells having an electronegative intracellular milieu (solid line, Fig. 3a,b). Gramicidin S, an antibiotic known to depolarize cells (Damjanovich *et al.*, 1987), was used to define cells in a totally depolarized state. At a concentration of 20 $\mu$g/ml, a maximum reduction in fluorescence intensity of both cell types was observed (dotted line, Fig. 3a,b).

The proportion of viable lymphocytes has previously been reported to decrease with time even under optimal cell culture conditions (Buttke & Cuchens, 1984). This effect is also seen in the histograms shown in Fig. 3b–f at time-points of 0, 4 and 8 hr, respectively. Incubation of B and T cells with 180 $\mu$M 18:0 (dashed line, Fig. 3) resulted in a further time-dependent decrease in the proportion of polarized T cells to an extent comparable to that seen with Gramicidin S. By comparison, 18:0 had no significant effect on the proportion of polarized B cells.

A more detailed analysis of the effects of varying doses of 18:0 on T and B cell-membrane potential were subsequently carried out. Purified B and T cells were separately stimulated with LPS or PHA, respectively, in the absence or presence of 60–180 $\mu$M 18:0. Following incubation at 37°, aliquots were removed from each of the eight cultures at hourly intervals and the cells were stained with DiOC$_6$(3) prior to analysis by flow cytometry. The proportion of polarized lymphocytes was subsequently determined as a function of both time and 18:0 dose (Fig. 4). The relative membrane potential of B cells was not altered by the addition of 18:0 to the culture medium, but T cells displayed a reduced membrane potential as early as 5–6 hr after exposure to 60 $\mu$M 18:0. Higher doses of 18:0 had more pronounced effects, reducing the number of polarized cells to < 30% at the 8 hr time-point. The results presented are in agreement with previous findings that the inhibitory effects of a similar dose (50 $\mu$M) of 18:0 on mitogen-induced DNA synthesis were manifest within 4–10 hr after exposure to 18:0 (Buttke & Cuchens, 1984).

It was attempted to discern a correlation between membrane lipid composition and membrane integrity by analysing data obtained from Fig. 2 as a function of data derived from Fig. 4. Although the level of total desaturated molecular species in B and T cells could not be correlated with the reduced membrane potential, an excellent correlation ($r = -0.85$) was observed
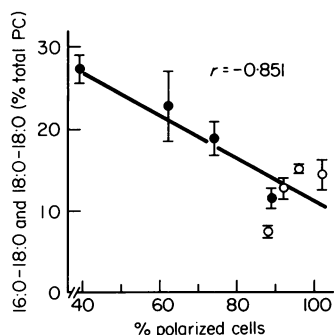
**Figure 5.** Correlation between membrane lipid composition and membrane integrity. The sum of 18:0–18:0 plus 16:0–18:0 PC molecular species in B (open circles) and T (solid circles) cells exposed to 0–180 $\mu$M 18:0 for 6 hr (derived from Fig. 2) was plotted as a function of the proportion of polarized cells at the 6 hr time-point (derived from Fig. 4). A best fit line was determined by linear regression analysis.

when the percentage of polarized cells was plotted versus the sum of 16:0–18:0 and 18:0–18:0 PC (Fig. 5). This suggests that the replacement of 16:0–16:0 with desaturates containing one or more 18:0 moiety per molecule are primarily responsible for 18:0-induced T-cell death.

## DISCUSSION

The studies described in this report were designed to determine the mechanism by which 18:0 selectively kills murine T cells (Buttke & Cuchens, 1984). To this end, a model system for the study of saturated fatty acid uptake and subsequent metabolism by B and T lymphocytes has been developed. The system uses highly enriched populations of B and T lymphocytes ($>95\%$) and albumin-complexed fatty acids. Both conditions were essential for defining the basis of 18:0 inhibition. Previous workers have used heterogenous leucocyte populations in which comparative studies of lipid metabolism between B and T lymphocytes were precluded. Further, in studies wherein the fatty acids were delivered as ethanolic solutions, large intracellular pools of non-esterified fatty acids were shown to accumulate (Klausner *et al.*, 1980). By contrast, other studies have shown that 18:0 provided as an albumin complex is efficiently taken up and esterified into cellular phospholipids, with $<10\%$ remaining unesterified (Yang, Cuchens & Buttke, 1986; Buttke *et al.*, 1989). Furthermore, using [$^{14}$C]18:0 it has been shown that the majority of exogenously supplied albumin-complexed 18:0 is incorporated into PC (Buttke *et al.*, 1989). Thus analyses of PC molecular species serves as a useful parameter for assessing the overall effects of 18:0 on B- and T-cell membranes.

As shown previously (Buttke *et al.*, 1989) and in this study, incubation of B and T cells with albumin-complexed 18:0 results in marked changes in the membrane lipid compositions of both cell types. Although both B and T cells take up similar quantities of 18:0 into PC (Buttke *et al.*, 1989), following 18:0 exposure the two cell types display substantial differences in their PC molecular species. The addition of 18:0 to B cells leads to an unexpected increase in unsaturated species at the expense of desaturates. Presumably, this shift in unsaturation is due to their ability to desaturate the exogenously supplied 18:0 using the stearoyl-CoA desaturase enzyme. Conversely, T cells, which are uniquely deficient in stearoyl-CoA desaturase (Buttke *et al.*,

1989) cannot similarly avoid the incorporation of 18:0 into their membrane phospholipids. As a result, the T cells are forced into increasing their levels of phospholipid species containing 18:0, in particular 16:0–18:0 and 18:0–18:0 PC. Importantly, the demonstrated changes were dose-dependent and occurred within 6 hr, a time sufficient to induce T-cell death (Buttke & Cuchens, 1984).

The ultimate effect of the observed changes in the membrane composition of 18:0-treated T cells may have been indicated previously. In studies with erythrocytes, Lange *et al.* (1980) and Kuypers *et al.* (1984) showed that stochiometric replacement of native erythrocyte PC with 18:0–18:0 was accompanied by increased osmotic fragility, resulting in haemolysis. Thus it was determined whether the previously observed accumulation of 18:0–18:0 PC in T cells (Buttke *et al.*, 1989) could also be associated with increased membrane leakiness. The ability of B and T cells to maintain a membrane potential was assayed as an indicator of plasma membrane integrity. It was found that 18:0 exposure led to a dose-dependent decline in the proportion of T cells capable of maintaining a membrane potential. A similar collapse of the plasma membrane permeability barrier was not observed in B cells. The concept that 18:0-induced depolarization is responsible for reduced T-cell viability is in agreement with the relatively rapid (4–10 hr) cytotoxic effect of the fatty acid. These studies do not prove a cause-and-effect relationship between membrane lipid changes and disruption of the membrane potential. Nevertheless, they do show a strong correlation ($r = -0.85$) between the proportion of polarized B and T cells and their levels of 16:0–18:0 plus 18:0–18:0 PC.

Data obtained in this study and elsewhere (Buttke & Cuchens, 1984; Buttke *et al.*, 1989) collectively suggest the following paradigm for the inhibition of T-dependent immune responses by 18:0: B cells incorporate 18:0, desaturate a portion of it to yield 18:1, and insert both fatty acids into phospholipids to maintain a functional level of membrane fluidity. T cells, however, due to their lack of stearoyl-CoA desaturase, cannot convert 18:0 to 18:1. Consequently, the T cells replace much of their 16:0 and olefinic moieties with 18:0, and accumulate significant amounts of both 16:0–18:0 and 18:0–18:0 PC. Replacement of 16:0–16:0, and perhaps other species, by 16:0–18:0 and 18:0–18:0 would be expected to decrease membrane fluidity and promote the formation of gel-like membrane domains. Such alterations in the physical properties of T-cell membranes may lead to a collapse of plasma membrane potential and, ultimately, cell death.

Lastly, the selective toxicity of 18:0 for T cells and its rapid mechanism of action may have clinical relevance in allograft or autoimmune situations. Immunosuppressive agents such as cyclosporin A are widely used for delaying the onset of allograft rejection (Borel *et al.*, 1976). The therapeutic value of cyclosporin derives from its marked selectivity toward T cells (Cohen *et al.*, 1984). Like 18:0, cyclosporin has also been shown to depolarize T cells (Damjanovich *et al.*, 1987), and both agents block IL-2 production (Kronke, Leonard & Depper, 1984; Pourbohloul & Buttke, 1990). Since cyclosporin has numerous potential side effects, including nephrotoxicity, hepatotoxicity and malignant lesions (Kahan *et al.*, 1986), alternative immunosuppressive agents are required. If the effects of 18:0 on T cells could be retained *in vivo*, the fatty acid could effectively and rapidly immunosuppress cell-mediated responses, but without the serious side-effects of cyclosporin.

## ACKNOWLEDGMENTS

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