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Inhibition of lymphocyte proliferation by free fatty acids

II. TOXICITY OF STEARIC ACID TOWARDS PHYTOHAEMAGGLUTININ-ACTIVATED T CELLS

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Summary. Studies were performed to further characterize the effects of saturated fatty acids on murine T lymphocyte proliferation. Flow cytometry was used to show that the inhibitory effects of stearic acid (18:0) on [^{3}H]thymidine uptake can be correlated with changes in cellular DNA content. Additional studies using flow cytometry and fluorescein diacetate as a viability stain showed that exogenous 18:0 was toxic for phytohemagglutinin (PHA)-stimulated T cells, whereas the viability of unstimulated T cells was less affected by 18:0. The inhibitory effects of 18:0 on T cell proliferation were evident as early as 4 hr after fatty acid addition and after a 10-hr exposure, the effects of 18:0 could not be reversed by washing the cells or by adding oleic acid (18:1). It is proposed that the inhibitory effects of 18:0 are dependent upon PHA-induced changes in T cell lipid metabolism.

INTRODUCTION

Saturated fatty acids have been shown to suppress a variety of immunological activities *in vitro*. For example, nondecanoic acid (19:0) inhibits (1) receptor mediated phagocytosis and fluid-phase pinocytosis by peritoneal macrophages (Mahoney *et al.*, 1977, 1980); (2) Patching and capping of the histocompatibility (H-2) antigens by the lymphoid tumour line, EL-4 (Mandel & Clark, 1978); (3) cell-mediated cytolysis by cytotoxic memory cells (Gill & Clark, 1980; Bialick *et al.*, 1984); and (4) mitogen-induced T lymphocyte proliferation. The suppressive effects of 19:0 are not unique however, and several other saturated fatty acids are also potent inhibitors of immune functions (Hawley & Gordon, 1976; Weyman *et al.*, 1977; Gill & Clark, 1980). By contrast, mono-saturated fatty acids have generally been reported to be uninhibitory.

While the inhibitory effects of the saturated fatty acids are well documented, the mechanisms by which they influence immune activities have not been defined. In several cases the loss of immune function has been shown to correlate with incorporation of saturated fatty acids into membrane phospholipids (Weyman *et al.*, 1977; Gill & Clark, 1980; Mahoney *et al.*, 1980). This relationship has led previous workers to propose that the saturated fatty acids suppress immune cell activities by adversely changing membrane physical properties (Weyman *et al.*, 1977; Mahoney *et al.*, 1977). However, despite the attractiveness of this model, subsequent studies have demonstrated membrane fluidity changes in macrophages (Mahoney *et al.*, 1980), but not in lymphocytes (Stubbs *et al.*, 1980) or in the tumour line EL-4 (Poon, Richards & Clark, 1981; McVey *et al.*, 1981). This discrepancy suggests that saturated fatty acids sup-

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press lymphocyte-mediated immune activities by some other mechanism, perhaps by inhibiting membrane-bound enzymes (Poon, Richards & Clark, 1981).

We have been using mitogen-induced lymphocyte proliferation as an *in vitro* model system to determine how fatty acids modulate immune function. In the previous paper (Buttke, 1984), it was shown that stearic acid (18:0) was a potent inhibitor of PHA-induced T cell activation, and that the simultaneous addition of oleic acid (18:1) overcame the effects of the saturated fatty acid. In order to understand the mechanisms by which saturated fatty acids block lymphocyte proliferation, it was important to further characterize the effects of 18:0 on PHA-induced T cell activation. Therefore, in the present study we have examined the effects of 18:0 on T cell viability, determined the length of exposure time necessary for 18:0 to inhibit lymphocyte proliferation, and re-examined the reversibility of 18:0 inhibition by 18:1.

MATERIALS AND METHODS

Mice

Male and female BALB/c mice, 8–12 weeks of age, were obtained from a colony maintained at the University of Mississippi Medical Center.

Fatty acids

Stearic acid (18:0), oleic acid (*cis* 18:1^9), bovine serum albumin (BSA) and diatomaceous earth were obtained from Sigma Chemical Co., St. Louis, MO. The fatty acids were adsorbed onto diatomaceous earth and complexed to BSA as described previously (Buttke, 1984). For the present studies, fatty acid/BSA ratios of 3·5–4·0 were routinely used.

Lymphocyte isolation and culture

The procedures used for isolating and culturing splenic lymphocytes and purified T lymphocytes have been described previously (Buttke, 1984; Buttke, Mallett & Cuchens, 1983).

Proliferation assays

Lymphocytes were cultured in 96-well microtiter plates (Linbro; Flow Laboratories, Inc., McLean, VA) at a concentration of 5×10^5 cells per 0·2 ml of culture medium. T lymphocytes were stimulated with PHA (Difco Laboratories, Detroit, MI) at a final mitogen concentration of 1 μg/well. The effects of BSA-complexed fatty acids on mitogen-induced proliferation were determined by adding the various supplements to the culture medium at the indicated times. Mitogen-induced DNA synthesis was assayed by labelling cells in triplicate with 0·5 μCi/well of [methyl-^{3}H]thymidine (New England Nuclear Corp., Boston, MA) during the final 6 hr of incubation. The labelled cells were subsequently harvested onto glass fibre filters using a multichannel automated harvester (Brandel Biomedical, Gaithersburg, MD) and the amount of [^{3}H]thymidine incorporated was measured by liquid scintillation counting. The mean counts per minute and standard deviation were calculated for each culture condition, and in every case the standard deviation was $\leq 10\%$ of the mean. All experiments were performed at least twice and in many cases three times. The data presented are from representative experiments. In some cases, the data are expressed as the percentage of control, where % of control = (ct/min of mitogen-stimulated cultures with fatty acid × 100)/(ct/min of mitogen-stimulated cultures with BSA).

Reversibility studies

To determine the effects of short term exposure to 18:0, lymphocytes were cultured in 96-well microtiter plates as described above, and supplemented with 18:0/BSA. At 0 hr, half of the cultures were stimulated with PHA while the remainder were left unstimulated. At various times, the cells were pelleted by centrifugation (200 ***g*** for 5 min). Culture supernatants containing 18:0 were removed and replaced with culture medium containing PHA but lacking 18:0. The cultures were subsequently incubated at 37°C for the duration of the experiment.

The reversibility of 18:0-inhibition was also determined by adding 18:1/BSA at various times to cultures which had previously been stimulated with PHA in the presence or absence of 18:0.

Cell cycle analyses by flow cytometry

To determine the effects of 18:0 on cellular DNA content, 2×10^6 cultured T cells were collected by centrifugation and resuspended in 0·5 ml of phosphate buffered saline (PBS) containing 0·03 M NaN_3. An equal volume of a solution consisting of 0·05 mg/ml propidium iodide, 0·1 M citrate and 1·0% Nonidet P-40 was added and the cell suspension was vortexed vigorously (Mond *et al.*, 1983). The stained cells were subsequently analysed for fluorescence intensity using a Cytofluorograf 50HH and 2150 computer (Ortho Diagnostic Systems Inc., Westwood, MA). All fluo-

rescence measurements were obtained using the 488 nm emission of an argon laser with a constant laser output (250 mW) and constant photomultiplier gain settings. Fixed calf thymocytes (Ortho) served as a standard diploid reference. For each sample a flow rate of 200 cells/sec was maintained. Dead cells (i.e., cells with a less than normal diploid DNA content) were eliminated from the analysis by gating on the cells with $\geq$normal diploid DNA content, based on reference measurements of fresh cell isolates with $>95\%$ viability as judged by trypan blue exclusion or fluorescein diactetate staining (see below). In all analyses fluorescence area versus peak signal cytograms were used in order to identify cell doublets, i.e., fluorescent events with twice the area values but with single peak values. The number of doublets routinely represented less than 2% of the fluorescent events with $\geq$diploid staining characteristics.

Flow cytometric analyses of lymphocyte viability
To determine the effects of 18:0 on T lymphocyte viability, 1×10^6 cells in 0·4 ml of culture medium were diluted to 1·0 ml with PBS. Fluorescein diacetate (FDA), a widely used viability stain (Rotman & Papermaster, 1966; Buttke, *et al.*, 1983), was added to the cell suspension to give a final FDA concentration of 5 μg/ml. After a 10-min incubation at room temperature, lymphocyte viability was assessed by flow cytometry as described above. The electronic signals were ungated in order to reflect the total number of fluorescent (live) and non-fluorescent (dead) cells during the analysis. Each analysis was performed on 10,000 cells at a flow rate of 200 cells/sec.

RESULTS

In the previous paper it was shown that stearic acid (18:0) inhibited the incorporation of [^{3}H]thymidine into PHA-stimulated mouse lymphocytes by greater than 95% (Buttke, 1984). Since there has recently been some question as to the validity of assaying DNA synthesis solely by measuring [^{3}H]thymidine uptake (Betel, Martünse & van der Westen, 1979; Steen & Lindmo, 1979), it was important to assess the inhibitory effects of 18:0 on DNA synthesis by another technique. For this purpose we chose to use flow cytometric techniques to assess cellular DNA content. Purified T cells were cultured with PHA in the presence or absence of 50 μM 18:0 (18:0/BSA$=$4·0) and assayed for their DNA content. As shown in Fig. 1, T lymphocytes stimulated with PHA in the absence of 18:0 contained a distinct population of cells in $S+G_2$ (region 2) that did not exist in lymphocytes stimulated with PHA in the presence of 50 μM 18:0. These findings demonstrate that DNA synthesis in the 18:0-treated cells was in fact inhibited, and that the fatty acid did not simply reduce thymidine transport. From the data shown in Fig. 1 it appears that 18:0 arrested the majority of the lymphocytes in either G_0 or G_1, and not in S phase.

During our flow cytometric studies, it became apparent that the addition of 18:0 to PHA-stimulated T cells and to a lesser extent, unstimulated T cells, led to an increased number of cells which, based on their staining characteristics with propidium iodide, had a less than normal (diploid) DNA content (region 3). Such an abnormally low DNA content has been associated with cell death (Steen & Lindmo, 1979). However, it should be noted that as described under Materials and Methods, cells with a very low fluorescence intensity were for the most part electronically excluded. Therefore, in order to critically examine the effects of 18:0 on T cell viability, purified T cells were cultured in the presence or absence of 50 μM 18:0 and either stimulated with PHA or left unstimulated. At various times samples were subsequently removed and stained with FDA, and the viability was assessed using flow cytometry (Fig. 2). In the absence of 18:0, purified T cells displayed a rapid loss of viability with the greatest decrease in cell number occurring during the first 24 hr in culture. The rate at which viability declined was the same for both PHA-stimulated and unstimulated T cells. The addition of 18:0 to unstimulated T lymphocytes had little effect on cell viability, but adding the saturated fatty acid to PHA-stimulated T cells led to a further decrease in lymphocyte viability. The toxicity of the 18:0-PHA combination was evident as early as 17 hr after 18:0 and PHA addition, but with further incubation the effects of the fatty acid and mitogen became more pronounced. These results confirmed the findings we obtained with propidium iodide-stained cells, and clearly demonstrate that 18:0 is toxic for PHA-stimulated T lymphocytes.

The results presented in Figs 1 and 2 show that an exposure time of 17–24 hr is sufficient for 18:0 to affect DNA synthesis and cell viability. Therefore, it appears that 18:0 can exert its inhibitory effects within a relatively short period of time. To examine this possibility in greater detail, two approaches were

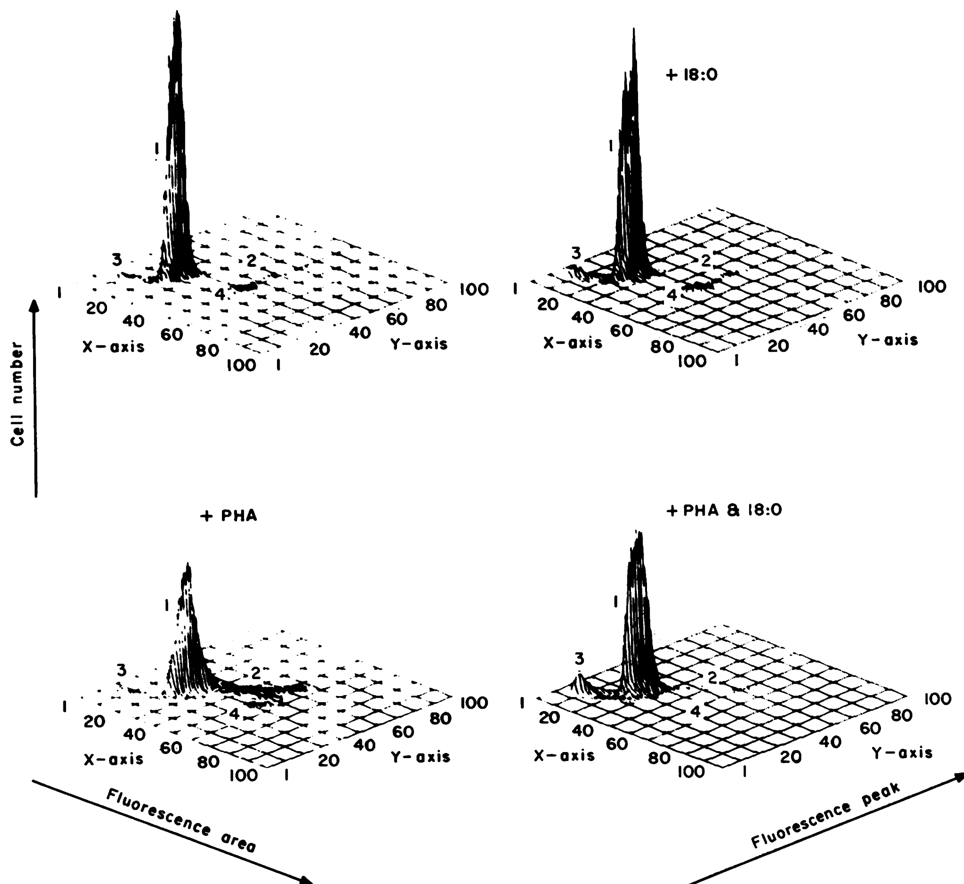

Figure 1. Cell cycle analysis of T lymphocytes left unstimulated, or stimulated with PHA in the presence or absence of 18:0. Purified T cells were cultured in the presence or absence of PHA and 50 μM 18:0. After 48 hr, samples were removed from each of the culture conditions and the cells were assayed for their DNA content. By using calf thymocytes as a diploid standard, fluorescence intensity was correlated with lymphocyte DNA content to determine the distribution of the cells in the cell cycle. Region 1 defines cells with a diploid DNA content (G_0+G_1), region 2 defines cells with a >diploid DNA content ($S+G_2$), region 3 defines cells with a <diploid DNA content (dead cells), and region 4 corresponds to cell doublets. Each profile represents 50,000 cells.

taken. First, splenic lymphocytes were cultured in the presence of 50 μM 18:0 and either stimulated with PHA, or left unstimulated. At daily intervals, cells from each of the culture conditions were washed to remove excess 18:0, followed by a second incubation, during which PHA was added to both PHA prestimulated and unstimulated cultures. After 42 hr, all cultures were labelled with [^{3}H]thymidine (6 hr incubation) to determine their ability to undergo PHA-induced proliferation (Fig. 3). As expected, PHA-stimulated cells cultured in the complete absence of 18:0 demonstrated a normal proliferative response, whereas cells cultured in the continuous presence of 18:0 and PHA did not (Fig. 3a). Exposing PHA-stimulated cells to 18:0 for 4 hr did not prevent PHA-induced proliferation, although these cells incorporated only 65% as much labelled thymidine as cells that were not treated with PHA during the 4-hr exposure to 18:0 (Fig. 3b). By contrast, lymphocytes treated with PHA and 18:0 for 20 hr did not undergo

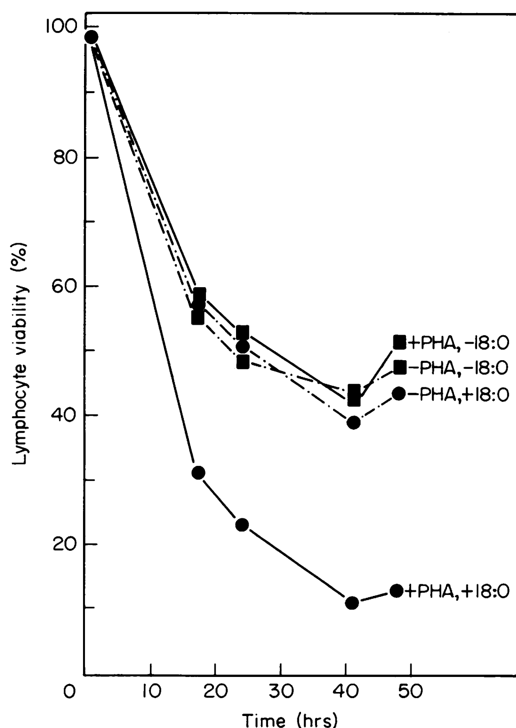

Figure 2. Effect of 18:0 on the viability of cultured T lymphocytes. Purified T cells were cultured in the presence or absence of PHA and 50 μM 18:0. At the indicated times, samples were removed, stained with FDA and cell viability was assessed using flow cytometry.

PHA-induced proliferation following the removal of 18:0 (Fig. 3c). Since cells that were left unstimulated during their 20-hr exposure to 18:0 were still capable of undergoing subsequent PHA-induced proliferation (Fig. 3c), it would appear that the irreversibility of 18:0 inhibition is confined to mitogen-activated lymphocytes. This finding is in agreement with the results of our previous experiments regarding lymphocyte viability (Fig. 2). It should be mentioned that exposing mixed lymphocytes to PHA and 18:0 for 20 hr did not prevent them from undergoing subsequent LPS-induced proliferation (121% of control, data not shown). Since both B and T cells are agglutinated by PHA, the toxic effects of 18:0 are not merely due to the binding of PHA to cell surface molecules.

The second approach that was used to determine the importance of exposure time in the reversibility of 18:0 inhibition was to add oleic acid (18:1) to cells which had previously been treated with PHA and either 25 or 50 μM 18:0 (Fig. 4). As described previously (Buttke, 1984), the simultaneous addition of 18:0 and 18:1 (added at 0 hr in Fig. 4) prevented the normal inhibitory effects of 18:0 on PHA-induced DNA synthesis. However, if 18:1 was added as early as 5 hr after 18:0 and PHA addition, subsequent DNA synthesis was reduced by 30–50%, depending upon the concentration of 18:0 used. With 50 μM 18:0 the cells were irreversibly inhibited within 10 hr (94% inhibition), whereas with 25 μM, the same level of inhibition was not achieved until 20 hr after PHA and 18:0 addition. Collectively, the data presented in Figs 2–4 imply that 50 μM 18:0 is significantly toxic towards PHA-activated T cells within 5 hr of addition, and is completely inhibitory within 10 hr.

Lastly, it was of interest to determine whether the inhibitory effects of 18:0 were confined to the early stages of activation or whether cells further along in the activation process would also be inhibited. Splenic lymphocytes were stimulated at 0 hr with PHA, and at various times after mitogen addition, 18:0 was added to give a final concentration of 50 μM. During the initial 20 hr of incubation, the addition of 18:0 led to a near total (99%) inhibition of DNA synthesis. When the addition of 18:0 was delayed for as late as 45 hr after PHA addition, the fatty acid inhibited [^{3}H]thymidine uptake by 60% (data not shown). Thus, the inhibitory effects of 18:0 are not limited to very early proliferative events.

DISCUSSION

As part of the present study we examined the effects of 18:0 on T lymphocyte proliferation by measuring cellular DNA content using flow cytometry. In agreement with our earlier observations (Buttke, 1984), T cells stimulated with DNA in the presence of 18:0 displayed a reduced DNA content, indicating an actual inhibitory effect of the saturated fatty acid on PHA-induced T lymphocyte proliferation. An unexpected finding of our flow cytometric studies was that 18:0 is selectively toxic for PHA-stimulated T cells. Previous studies have shown that saturated fatty acids, at concentrations approximating the doses used here, retard the patching of H-2 antigens, suppress cell-mediated cytotoxicity and inhibit receptor-mediated phagocytosis while having little or no effect on cell viability (Mahoney *et al.*, 1977; Mandel & Clark, 1978;

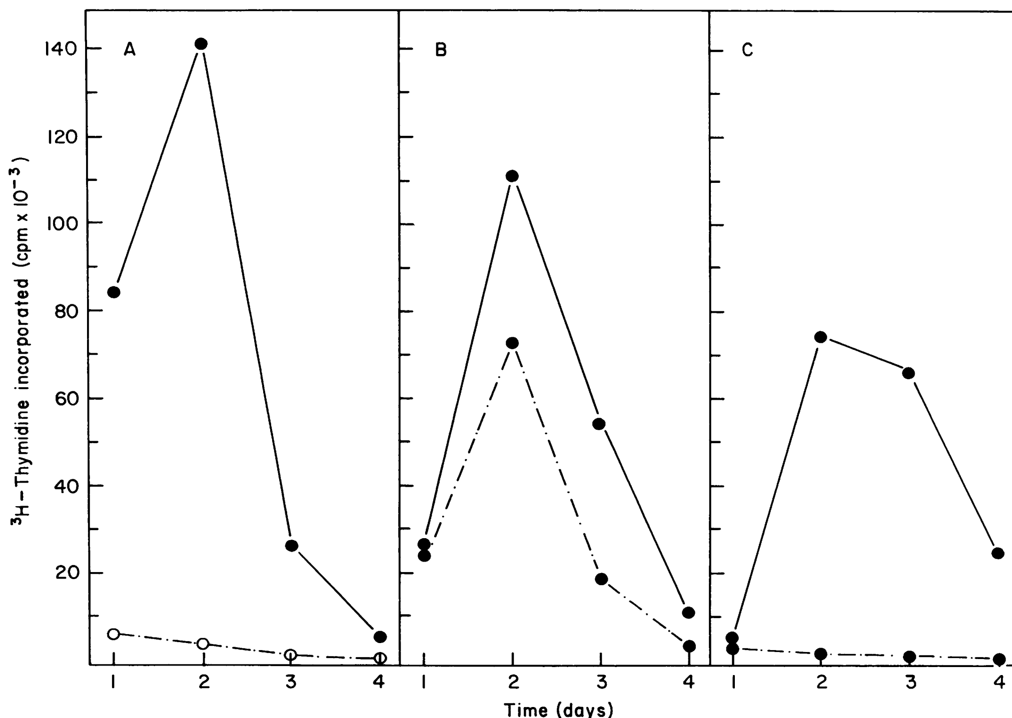

Figure 3. Effects of short-term exposure to 18:0 on PHA-induced lymphocyte proliferation. Splenic lymphocytes were cultured in the presence or absence of 18:0 and PHA under a variety of conditions: (A) Cells were incubated in the continuous absence (solid lines) or presence (dashed lines) of 50 μM 18:0 and stimulated with PHA. At the times indicated, samples were removed and DNA synthesis was measured by assaying [^{3}H]thymidine uptake. (b) Cells were incubated either in the presence of 50 μM 18:0 and PHA (prestimulated, dashed line), or in the presence of just 50 μM 18:0 (unstimulated, solid line). After 4 hr, the cells were washed to remove exogenous 18:0 and both cultures were stimulated with PHA. At the indicated times, samples were labelled with [^{3}H]thymidine to assess DNA synthesis. (c) Same protocol as in (b), except that the exposure time to 18:0 was increased to 20 hr.

Gill & Clark, 1980). At the present time we do not know whether the inhibition of DNA synthesis by 18:0 is solely the result of reduced T cell viability, or whether 18:0 can continue to block proliferation under conditions where cell viability is preserved.

While it would be premature to propose a mechanism whereby saturated fatty acids inhibit T cell proliferation, some of our findings may nonetheless help in defining the site(s) of inhibition. First, since delaying the addition of 18:0 for as late as 45 hr still led to a reduction in DNA synthesis, the specific site of inhibition, whatever it is, must be constitutively expressed throughout the cell cycle. Secondly, with 50 μM 18:0 maximum inhibition of DNA synthesis occurred within 10 hr. Thus, if an activation-dependent metabolism of 18:0 is responsible for the fatty acid's inhibitory effects, then conversion of 18:0 to a toxic lipid species must occur rather rapidly. Based upon these two considerations, the most logical target site for 18:0 inhibition is at the level of the cell membranes. Previous workers have proposed that saturated fatty acids may inhibit immune activities via their incorporation into phospholipids with resultant changes in membrane structure that might adversely affect the activities of membrane-bound enzymes (Weyman *et al.*, 1977; Mahoney *et al.*, 1977; Gill & Clark, 1980; Poon, Richards & Clark, 1981; Szamel & Resch, 1981). Consistent with such a mechanism are the findings of Resch & Ferber (1972) and Stubbs & Owawa (1983) showing that although both stimulated and unstimulated T cells incorporate exogenously-supplied fatty acids, mitogen-stimulated lymphocytes

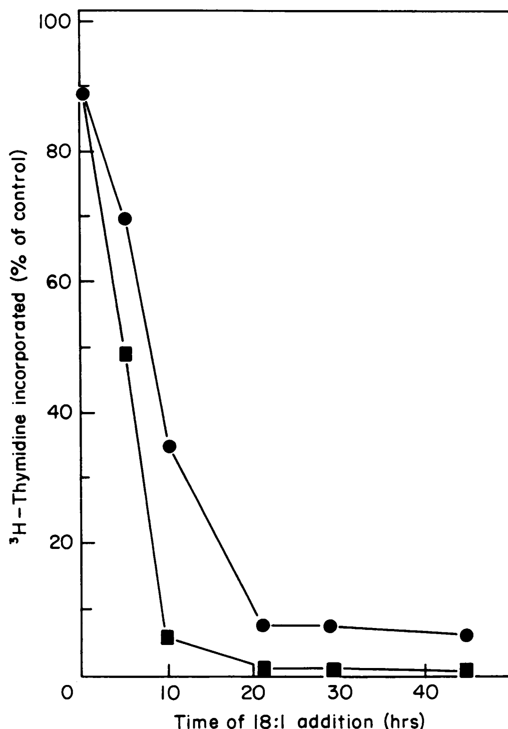

Figure 4. Reversibility of 18:0-inhibition of PHA-induced DNA synthesis by 18:1. Splenic lymphocytes were cultured in the absence (control) or presence of either 25 μM 18:0 (circles) or 50 μM 18:0 (squares). At the indicated times, cultures received either 25 μM (circles) or 50 μM (squares) 18:1. After 45 hr, cells were labelled for 6 hr with [^{3}H]thymidine to assess PHA-induced proliferation. The amounts of [^{3}H]thymidine incorporated were 161,516 c.p.m. in the absence of 18:0, 10,535 c.p.m. in the presence of 25 μM 18:0, and 978 c.p.m. in the presence of 50 μM 18:0.

incorporate the fatty acids for a longer period of time. In similar studies we have found that unstimulated T cells incorporate exogenously-supplied 18:0 into cellular phospholipids for about 6 hr, whereas PHA-stimulated T cells incorporated 18:0 into phospholipids for an additional 6–8 hr (T. M. Buttke, unpublished observations). Thus, the length of time that activated cells must be exposed to 18:0 in order for DNA synthesis to be inhibited correlates very well with PHA-enhanced phospholipid synthesis. It remains to be seen whether the toxic effects of 18:0 towards activated T cells stem from altered membrane properties (e.g., fluidity or permeability) or from an inhibition of essential membrane-bound enzymes.

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