Studies on the Mechanism of Corticosteroid-induced Lymphocytolysis

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SUMMARY

When cells of the thymus or corticosteroid-sensitive mouse lymphosarcoma P1798S are treated in vitro with 0.27 μM cortisol in tissue culture medium with 10% serum or 0.5% human albumin, nuclear damage occurs that ends with karyorrhexis. The steroid-resistant subline P1798R does not show these changes.

Corticosteroid-sensitive lymphocytes that undergo lysis differ from the resistant subline in several respects with regard to changes in fatty acid metabolism. Corticosteroid treatment in vivo for 2 hr raised the free fatty acid (FFA) pool in thymus and P1798S cells, while decreasing it in P1798R cells. In vitro, cortisol had no effect on the uptake of 14C-labeled palmitic acid but decreased oxidation of this acid by 46 and 17% in thymus and P1798S, respectively, while increasing it 9% in P1798R.

After sensitive P1798S cells were incubated in a medium that contained FFA calculated to be equivalent to that accumulated after steroid treatment, electron microscopy revealed that certain effects of corticosteroids could be reproduced by fatty acids of chain length C-9 and higher, namely, nuclear edema, focal dissolution and disintegration of the nuclear membrane and, ultimately, karyolysis. Steroid-resistant cells show cytological changes only at 10-fold higher concentrations of FFA.

On the basis of these results, the following scheme is proposed as the mechanism by which cytolysis occurs in corticosteroid-sensitive lymphoid tissues:

\[
\text{Triglyceride} \rightarrow \text{FFA} \rightarrow \text{Accumulation} \rightarrow \text{Nuclear damage} \rightarrow \text{Cell lysis}
\]

INTRODUCTION

Adrenal corticoids induce dose-dependent regression of lymphatic organs (8, 9). This cytolytic action also occurs following the addition of corticosteroids to lymphocytes and lymphosarcoma cells in vitro (7, 13, 15). A regression in thymus is accompanied by an increase in acid RNase activity (39), as well as by a concurrent decrease in nucleoside precursor uptake into RNA and DNA (14, 15). In thymocytes there is also decreased glucose utilization (16, 21, 22). In P1798S, the corticosteroid-sensitive form of mouse lymphosarcoma P1798, effects similar to those observed in thymus are seen after glucocorticoid administration; however, these effects are not seen in P1798R, the steroid-resistant line of this lymphosarcoma (2, 27). The inhibition of glucose uptake by the cell and the decrease in nucleoside incorporation into RNA and DNA brought about by corticosteroid administration probably cannot alone account for the lysis of thymus or corticosteroid-sensitive mouse lymphosarcoma P1798 cells that begins after 4 hr.

This study is concerned with the mechanism of the cytolytic action of corticosteroids, and stems from earlier reported observations in this and other laboratories. These include (a) the low concentration of FFA's3 that damage cells (4, 5); (b) the fact that in vitro FFA's induce pycnosis of thymocytes and of the corticosteroid-sensitive mouse lymphosarcoma P1798, which is temperature dependent (A. F. Burton, unpublished results); and (c) that corticosteroids are capable of inducing the release of FFA from triglyceride stores (20). This study is therefore focused mainly on the metabolism of FFA's by lymphocytes in order to determine whether a relationship exists between FFA metabolism and the sensitivity or resistance of malignant lymphocytes to corticosteroids.

A preliminary account of these results has appeared elsewhere (35).

MATERIALS AND METHODS

Chemicals

All chemicals used in this study, unless otherwise noted, were purchased from the Fisher Chemical outlet in Vancouver, British Columbia, Canada. Hyamine, PPO, and POPOP were obtained from the Packard Instrument Company, Downers Grove, Ill. Azelaic acid, palmitic acid, and carnitine were obtained from Calbiochem, Los Angeles, Calif., and cortisol was from the Sigma Chemical Company, St. Louis, Mo.

1 The studies reported are from a thesis submitted to the University of British Columbia at Vancouver in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Recipient of a Medical Research Council of Canada Studentship. Present address: Department of Experimental Therapeutics, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, N. Y. 14203.

2 Recipient of a Killam Predoctoral Fellowship.

Received February 21, 1972; accepted October 18, 1972.

3 The abbreviations used are: FFA, free fatty acid; KEP buffer, Krebs-Eggleston phosphate buffer.
Solvents

All solvents used were of reagent grade and were purified by redistillation before use.

Radiochemicals

The following radiochemicals were purchased from Amersham/Searle Corporation, Don Mills, Ontario, Canada, with specific activities as indicated: palmitic acid-1-14C (35.2 to 56.9 mCi/m mole); sodium bicarbonate-14C (56.9 mCi/m mole); acetic acid-1-14C (52.9 mCi/m mole); sodium n-octanoate-1-14C (17.5 mCi/m mole); and n-hexadecane-1-14C (120.8 mCi/mole). All radiochemicals were used upon receipt or were purified routinely by thin-layer or paper chromatography.

Materials

Plastic center wells, 1-hole rubber serum stoppers, and 25-ml Erlenmeyer flasks used in the collection of 14CO2 were purchased from Kontes Glass Company, Vineland, N. J. Scintillation vials were obtained from Fraser Medical Supplies, Vancouver, B.C.

Animals

The BALB/cJ and DBA/2J mice used in these experiments were obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals were fed a diet of Purina breeder chow and water ad libitum.

The corticosteroid-sensitive and -resistant mouse lymphosarcomas were maintained s.c. as solid tumors in the area of the 1st inguinal gland. We transplanted the tumors routinely every 15 to 17 days by grinding 100 mg tumor in a tissue grinder with 5 ml 0.9% NaCl solution and injecting 0.1 ml of the cell suspension s.c. into BALB/cJ mice.

The murine leukemias L5178Y and LI210 were maintained in DBA/2J mice as ascites tumors. Every 7 to 10 days, ascites fluid was withdrawn from mice, and 0.1 ml of this fluid was injected into recipients.

Biochemical Methods

Cell Suspensions. Thymus or tumor tissue was removed from BALB/cJ mice and ground in a test tube with a loosely fitting glass pestle in 0.9% NaCl solution. The suspension was filtered through 8 layers of surgical cotton gauze and centrifuged at 1200 g for 2 min. It was washed once with 5 ml of 0.9% NaCl solution and pelleted. The pellet was resuspended, and an aliquot of this suspension was diluted with Fischer's medium (Grand Island Biological Company, Berkeley, Calif.), containing 10% horse serum (Hyland Div. Costa Mesa, Calif.) or 0.5% human albumin (Sigma), to give a final concentration of 0.5 to 4 million cells/ml, as determined by a hemacytometer.

Incubations. Incubations were carried out in standard conical glass centrifuge tubes sealed with silicone rubber stoppers; when 14CO2 was being assayed, incubations were carried out in 25-ml Erlenmeyer flasks. Samples were incubated without agitation in an oven or water bath at 38° for the required time. Cortisol was dissolved in ethanol at a concentration of 2 mg/ml and was diluted with 0.9% NaCl solution before it was added to samples. Palmitic and azelaic acids, as ethanol solutions, were added to the samples before incubation. At no time did the ethanol concentrations in incubated samples exceed 1%.

Determination of Radioactivity. The sample that was to be counted was placed in a scintillation vial, and 10 ml of liquid scintillation fluid containing 4 g PPO per liter and 100 mg POPOP per liter were added. For aqueous samples, an appropriate amount of Hyamine solubilizer was added. Samples were counted with either a Packard Tri-Carb Model 3003 liquid scintillation spectrometer or a Nuclear-Chicago Unilux III ambient temperature liquid scintillation counter. Sample quenching was monitored by the channels-ratio method (6).

Assay for 14CO2 Production. Nine-tenths ml of either Fischer's medium or KEP buffer, pH 7.4 (18), was placed in 25-ml flasks, and to this was added labeled materials, cortisol, FFA's and other chemicals, as the experimental regimen dictated. One-tenth ml of the cell suspension was added, and the flasks were closed with serum stoppers equipped with a center well. Hyamine (0.3 ml) was placed in the center well via a syringe and needle. The flasks were then placed in an oven at 37° for the length of time required. At the end of the incubation period, flasks were removed from the oven and 0.3 ml 2 N H2SO4 was added to the incubation mixture via syringe through the serum stopper. Flasks were then placed in a water bath at 37° and shaken at 120 cpm for at least 2 hr. At the end of this time, the center wells were removed, cut from the stems, and placed in scintillation vials. Another 0.5 ml of Hyamine was added, as well as 10 ml of scintillator, and radioactivity was determined. Samples were corrected for quenching by the method of channel ratios (6).

Electron Microscopic Methods

The preparation of cell suspensions and incubations of the tumor P1798 was identical to that described previously for biochemical studies, except that the final concentration of cells was adjusted to 1 to 2 million cells/ml, as determined by a hemacytometer. Two techniques of initial cell fixation were used (11, 26). The procedures gave identical results. After a buffer wash, samples fixed by each method were dehydrated in an ascending ethanol series and embedded in Epon 812 (19) or Maraglas (32). Sections were cut on an MT2 Sorval ultramicrotome. Thick sections (0.5 μm) were stained with toluidine blue (34). Thin sections were mounted on uncoated copper grids, stained with aqueous uranyl acetate and lead citrate, and examined in a Siemens-Elmiskop I electron microscope.

RESULTS

Observations were made on cells with the use of a system that was developed in this laboratory for maintaining the viability of thymus cells in culture for periods up to 24 hr (7). Preliminary experiments were carried out in which 14C-labeled palmitic acid was incubated with 2 steroid-sensitive tissues, thymus and P1798S; and 3 steroid-resistant tumors, P1798R,
Cortisol appeared to increase the disappearance of the label in all resistant tissues, while decreasing it in sensitive cells. This was also true when corticosteroids were added. These findings suggested a difference in the metabolic activity of fatty acids in the 2 types of cells, possibly in the oxidation to CO₂.

**Oxidation of Palmitic Acid-1-¹⁴C to ¹⁴CO₂.** In order to determine whether there was a difference in the oxidative capacity of corticosteroid-resistant and sensitive cells, we studied the oxidation of FFA to ¹⁴CO₂. Thymus, P1798S, and P1798R cells were incubated with palmitic acid-1-¹⁴C, and ¹⁴CO₂ was collected for various times up to a maximum of 2 hr. The production of ¹⁴CO₂ increased over the 1st 30 min and was maximal between 30 and 60 min. Because all of the curves showed a linear increase in ¹⁴CO₂ production over the 1st 30 min, all subsequent incubations were for 30 min. Experiments were carried out in a complete tissue culture medium (Fischer's), which is the medium in which the cytolytic action of corticosteroids can be demonstrated (7). The oxidation of labeled palmitic acid in this medium was less than that in simple buffer solution in all 3 cell lines (Table 1). The oxidation of palmitic acid was considerably greater in the corticosteroid-sensitive thymus and P1798S than in the corticosteroid-resistant thymus and P1798R.

Numerous experiments were undertaken so that we might examine the effects of cortisol and corticosteroids upon the oxidation of palmitic acid in complete medium. While the replication in any one experiment was good, the effect of added constituents was variable from one experiment to the next. Since the labeled substrate was added only in tracer amounts, the metabolism of a minute amount of fatty acid would be influenced by the size of the endogenous fatty acid pool (a variable factor) at any given moment. Great variability has been encountered in this laboratory and also by other workers in the uptake of labeled fatty acids by tumor cells in vivo and in vitro (10). When the labeled FFA was diluted with unlabeled carrier, the variability was greatly reduced and reproducible results were obtained. The effect of unlabeled substrate could also give information on the size of the endogenous pool and would more likely reveal the true oxidative capacity of the cells. Results of such experiments are shown in Table 2. Expressed as a percentage of the control values in each case, cortisol reduced the oxidation of palmitic acid in sensitive thymus and P1798S while increasing it slightly in the resistant P1798R line. The addition of 78 µM palmitic acid depressed ¹⁴CO₂ production 25% in both sensitive cell lines, but it only slightly decreased that of the resistant cell line. This result with the resistant line was interpreted as indicating a larger endogenous pool and/or a greater capacity for oxidation. Cortisol produced a further decrease in the sensitive cells with 78 µM palmitic acid, while again a slight stimulation was observed in the P1798R line.

Direct measurement of the FFA pool in thymus and tumors before and after steroid treatment was then made. Mice were given injections of dexamethasone, and the thymus or tumor tissue was examined 2 hr later. As shown in Table 3, an increase in FFA content occurred in the thymus and the sensitive tumor, while a decrease was observed in the resistant line.

Whether the increased levels of FFA were a reflection of increased release of FFA's from endogenous fat stores or whether they reflected an increased uptake of plasma FFA by these sensitive tissues in vivo could not be ascertained from the results in Table 3. The effect of cortisol on fatty acid uptake in vitro was therefore determined. The results are expressed in Table 4. The uptake of palmitic acid was not affected significantly by cortisol in any of the tissues studied. It would appear that the increased endogenous levels of FFA come from the lipid stores of the lymphoid cells.

**The Oxidation of Short-Chain Fatty Acids.** Spector and Steinberg (31) have reported that tumor cells are limited in their capacity to oxidize short-chain fatty acids as compared with long-chain fatty acids. Accordingly, thymus and mouse lymphosarcoma cells were incubated with equivalent

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>Comparison of palmitic acid-1-¹⁴C oxidation in different media</strong></td>
</tr>
<tr>
<td>Cells were incubated in Fischer's medium or KEP buffer, pH 7.4, for 30 min, and ¹⁴CO₂ produced from oxidation of 0.5 µM palmitic acid-1-¹⁴C was collected. Each value is the mean ± S.E. of 4 or 5 samples.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>¹⁴CO₂ recovered (dpm/10⁶ cells)</th>
<th>KEP buffer</th>
<th>Fischer's medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>5847 ± 286</td>
<td>576 ± 67</td>
</tr>
<tr>
<td>P1798S</td>
<td>3479 ± 563</td>
<td>446 ± 52</td>
</tr>
<tr>
<td>P1798R</td>
<td>551 ± 73</td>
<td>111 ± 12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td><strong>Effect of cortisol and/or palmitic acid on the oxidation of palmitic acid-1-¹⁴C to ¹⁴CO₂</strong></td>
</tr>
<tr>
<td>Cells were incubated in Fischer's medium at 37° for 30 min with 0.5 µM palmitic acid-1-¹⁴C. Unless otherwise indicated, each value is the mean ± S.E. of 4 or 5 samples.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymus</th>
<th>P1798S</th>
<th>P1798R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cortisol (2.7 µM)</td>
<td>56 ± 1.0</td>
<td>83 ± 2.4</td>
<td>109 ± 2.5</td>
</tr>
<tr>
<td>Palmitic acid (78 µM)</td>
<td>75 ± 1.6</td>
<td>73 ± 1.6</td>
<td>90 ± 1.0</td>
</tr>
<tr>
<td>Cortisol (2.7 µM) + palmitic acid (78 µM)</td>
<td>50 ± 2.0</td>
<td>66 ± 1.6</td>
<td>95 ± 3.3</td>
</tr>
</tbody>
</table>

* In this instance, 6 samples were used.

<table>
<thead>
<tr>
<th>Table 3</th>
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<tbody>
<tr>
<td><strong>Effect of dexamethasone on endogenous FFA levels</strong></td>
</tr>
<tr>
<td>BALB/c mice were treated either with 1 mg dexamethasone in 0.1 ml sesame oil or with vehicle alone. Two hr later, animals were sacrificed, tissue was removed, and FFA levels were determined by microtitration by the method of Kelley (17). Each value is the mean ± S.E. of 5 to 7 determinations.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level of FFA (µEq/g)</th>
<th>Tissue</th>
<th>Control</th>
<th>Dexamethasone treated</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>9.55 ± 0.97</td>
<td>16.88 ± 2.17</td>
<td>+76</td>
<td></td>
</tr>
<tr>
<td>P1798S</td>
<td>3.43 ± 0.24</td>
<td>3.90 ± 0.21</td>
<td>+14</td>
<td></td>
</tr>
<tr>
<td>P1798R</td>
<td>3.90 ± 0.29</td>
<td>3.02 ± 0.10</td>
<td>-22</td>
<td></td>
</tr>
</tbody>
</table>
concentrations of labeled palmitic acid or sodium octanoate, and 14CO2 production was monitored. The results (Table 5) indicate that both normal and malignant lymphoid cells have a greater capacity to oxidize short-chain fatty acids than long-chain fatty acids; this is true also of various normal tissues (29).

Effect of FFA on Mouse Lymphosarcoma Cell Survival. The results of isotope dilution experiments (Table 2) indicated that cells sensitive to corticosteroid might accumulate FFA. This was confirmed by direct measurement (Table 3). In order to determine whether this accumulation of FFA's could cause damage to cells, we carried out incubations in which we studied the effects on cell viability of fatty acids added over a period of 3 to 6 hr. As can be seen in Table 6, both palmitic acid, a C-16 monocarboxylic acid, and azelaic, a C-9 dicarboxylic acid (used because of its greater solubility in aqueous solution), increased the death of steroid-sensitive mouse lymphosarcoma P1798 cells by FFA and its effects on resistant cells were examined in greater detail by means of the electron microscope. Fig. 1 shows the integrity of normal fine structure of untreated corticosteroid-sensitive mouse lymphosarcoma P1798 cells incubated in Fischer's medium for 4 hr. Maintenance of normal fine structure was also observed in untreated thymocyte suspensions and in corticosteroid-resistant mouse lymphosarcoma P1798 suspension, with or without added fatty acids (1). The presence of capsid-like structures, both partially formed, in close association with the endoplasmic reticulum membrane, and more completely formed, in an intracisternal position (Fig. 1, inset; Fig. 7), was noted in P1798R cells. These appeared identical to the so-called A-particles reported in other murine leukemias and in the normal tissue of neonatal mice (3).

Electron Microscopic Examination of the Effects of FFA on Mouse Lymphoid Cells. The nature of the damage to sensitive cells by FFA and its effects on resistant cells were examined in greater detail by means of the electron microscope. Fig. 1 shows the integrity of normal fine structure of untreated corticosteroid-sensitive mouse lymphosarcoma P1798 cells incubated in Fischer's medium for 4 hr. Maintenance of normal fine structure was also observed in untreated thymocyte suspensions and in corticosteroid-resistant mouse lymphosarcoma P1798 suspension, with or without added fatty acids (1). The presence of capsid-like structures, both partially formed, in close association with the endoplasmic reticulum membrane, and more completely formed, in an intracisternal position (Fig. 1, inset; Fig. 7), was noted in P1798R cells. These appeared identical to the so-called A-particles reported in other murine leukemias and in the normal tissue of neonatal mice (3).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Experiment</th>
<th>Control</th>
<th>Cortisol-treated (2.7 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>1</td>
<td>1967 ± 196</td>
<td>1753 ± 371</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>900 ± 19</td>
<td>834 ± 22</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>978 ± 36</td>
<td>1045 ± 15</td>
</tr>
<tr>
<td>P1798S</td>
<td>1</td>
<td>923 ± 59</td>
<td>1039 ± 15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>609 ± 11</td>
<td>605 ± 21</td>
</tr>
<tr>
<td>P1798R</td>
<td>1</td>
<td>1955 ± 59</td>
<td>1872 ± 42</td>
</tr>
</tbody>
</table>

*Mean ± S.E. for 8 samples.*

| Table 5 | Oxidation of long- and short-chain fatty acids
<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>Tissue</td>
<td>Palmitic acid-l-14C Sodium n-octanoate-l-14C</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Thymus</td>
<td>799 ± 64</td>
</tr>
<tr>
<td>P1798S</td>
<td>1,539 ± 87</td>
</tr>
<tr>
<td>P1798R</td>
<td>701 ± 18</td>
</tr>
</tbody>
</table>

| Table 6 | Effect of FFA's on the corticosteroid-sensitive mouse lymphosarcoma P1798 in vitro
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Viable cells (X10⁶ cells/ml)</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Control</td>
<td>1.75 ± 0.109</td>
</tr>
<tr>
<td>Palmitate-treated (78 μM)</td>
<td>1.117 ± 0.217</td>
</tr>
<tr>
<td>Azelaic acid-treated (53 μM)</td>
<td>1.083 ± 0.174</td>
</tr>
</tbody>
</table>

*All values are mean ± S.E.*

When thick sections of control and treated P1798 cells were incubated for 4 hr and stained with toluidine blue, there was a marked increase in cell degradation and necrosis in the treated sections (Figs. 2 to 4). Degenerative changes especially evident were the swelling and vesiculation of the cytoplasm. Cells undergoing necrosis were characterized by pyknosis and karyolysis. The fine structure of P1798R cells after 4 hr of treatment confirmed the progressive nature of the degenerative process. Dilation, swelling and subsequent vesiculation of the endoplasmic reticulum (Figs. 5 to 8), especially in Golgi regions (Fig. 7), preceded cell death (Fig. 9). Noteworthy was the almost complete absence of membrane-bound ribosomes in

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those cells exhibiting minimal pathological changes at that time. Marked dilation of the perinuclear cisterna (Fig. 6) usually progressed concomitantly with the vesiculation of the endoplasmic reticulum (36). Mitochondrial alterations, except in prenecrotic cells, were not synchronous, but they included only a portion of those within the cell (Fig. 5). Large dense granules (Fig. 8) appeared in the cytoplasm of treated but not control cells at 4 hr. They were especially conspicuous in cell debris that remained after cytolysis.

Detailed events in the degenerative changes in nuclear fine structure were difficult to assess because of the variable morphology of the nuclei of this tumor and because of the asynchronous development of degeneration within cell populations. However, as a general rule, as cytoplasmic degeneration proceeded, the granularity of euchromatin became less pronounced, and its usual sharp demarcation from heterochromatin became less marked (compare Fig. 1 with Figs. 6 and 8).

**DISCUSSION**

The possibility that an accumulation of FFA might be involved in corticosteroid-induced cytolysis (8) appeared to be supported by preliminary observations. When the oxidation of $^{14}$C-labeled palmitic acid to $^{14}$CO$_2$ was examined, we found that the corticosteroid-sensitive tissues, thymus and P1798S, produced more $^{14}$CO$_2$ than did the resistant P1798R tissues. This was true in simple KEP buffer and in Fischer's medium (Table 1), but oxidation was reduced in the latter medium in all 3 cell lines. Since Fischer's medium contains glucose, it was felt that the utilization of FFA for energy would be less and that the data might be reflecting the relative dependency of the cells upon FFA as a source of energy. In order to test the capacity of cells to metabolize FFA, we felt it necessary to examine the oxidation of larger quantities, rather than tracer amounts. When this was done (Table 2), the dilution of counts in $^{14}$CO$_2$ was much greater in sensitive cells and cortisol produced a further decrease whereas, in the resistant line, cortisol actually increased oxidation. This was interpreted as indicative of a greater capacity of resistant cells to oxidize FFA, which interpretation, is consistent with the thesis that the steroid might be causing an accumulation of FFA in sensitive cells.

The data in Table 3 indicate that in intact mice, following hormone administration, there is a large significant increase in the FFA content of thymus ($p < 0.01$) and a slight increase in the pool in the P1798S tumor. In contrast to these steroid-sensitive tissues, the pool size decreases by 22% in the steroid-resistant P1798R tumor ($p < 0.02$) after this treatment.

It appears from the results shown in Table 4 that FFA uptake is not likely to be affected by treatment with cortisol; the FFA increase is probably due to release from intracellular lipid stores.

That an accumulation of FFA does in fact damage sensitive cells is illustrated in Table 5. A parallel between resistance to lysis induced by corticosteroids and by palmitic and azelaic acid is seen in Table 6, where more than a 10-fold increase in the concentration of azelaic acid that destroys sensitive cells is required to cause lysis of the resistant line.

Short-chain fatty acids are transferred into the mitochondrion by a different transferase than C-16 or C-18 fatty acids (12, 24). Spector and Steinberg (31), Weinhouse et al. (37), and Scholefield et al. (30) have reported that tumor cells appear to have little capacity to oxidize short-chain fatty acids. The mouse lymphosarcomas P1798S and P1798R do not follow the pattern observed by these workers. Like thymus (Table 4) and other peripheral tissues (29), these cells have a greater capacity for the oxidation of short-chain than long-chain fatty acids, which are generally thought to be more efficiently used in lipid synthesis than in energy production (29).

Electron micrographic observations show that an accumulation of FFA in corticosteroid-sensitive cells would have pronounced cytolytic effects. The degenerative change set in motion by FFA causes concomitant nuclear and cytoplasmic alterations. The progressive loss of normal dichotomy between heterochromatin and euchromatin 4 hr after treatment is strikingly similar to that induced by corticosteroid (7). Bessis (5) has termed this type of change nuclear edema. Indeed, due to dilation of the endoplasmic reticulum and vesiculation in FFA-treated P1798S cells, the possibility of secondary alteration in ion and water balance within the cell seems likely. The structural changes observed here are probably manifestations of the following retrograde metabolic alterations reported in corticosteroid-induced degeneration: (a) a decrease in DNA synthesis (33) and in DNA-dependent RNA polymerase activity (23); (b) an increase in DNase II activity (27); and (c) changes in the histone complement (38).

The primary event in FFA-induced cytolysis is unknown. However, the similar effects of FFA and corticosteroids on corticosteroid-sensitive lymphocytes suggest a strong likelihood of a common basis. FFA administration seems to affect neither the morphology of mitochondria nor of the cell membrane, primarily.

Our observations that FFA's are cytotoxic are supported by 2 other recent observations. Sahler and Glick (28) observed that lipid from mouse liver chromatin was highly toxic to L1210 mouse leukemia cells. The cytotoxic effect was caused by FFA's especially oleic acid. A concentration of 3µg FFA per ml caused 100% cell lysis in the L1210 leukemia. A cytotoxic factor has also been identified recently in a lymph node extract (25). This "cellular lytic factor" was cytotoxic to tumor cells such as MM2, Friend, SR61, Ehrlich, Sarcoma 180, and AH-66F. It also lysed the erythrocytes of sheep, guinea pigs, rats, rabbits, and mice. Infrared spectra and thin-layer, gas-liquid, and ion-exchange chromatography established that the cellular lytic factor is an unsaturated long-chain fatty acid. The results showed that the lytic activity was probably due to the presence of oleic acid, which is a monoenoic acid (18:1). The effects of this lytic factor in causing cytolysis were temperature dependent, as were those previously observed in this laboratory.

In conclusion, the observations made in this paper demonstrate that there is a similarity in the events induced by corticosteroids and those by FFA's. The low concentration of FFA at which these effects occurred is especially significant. The data strengthen the hypothesis (Chart 1) that the
UPTAKE

Chart 1. Proposed scheme of possible factors leading to cytolyis. The scheme proposes that FFA in steroid-responsive tissues would lead to nuclear damage and ultimately cell lysis. Steroid-resistant tissues would remove FFA by oxidation and possibly by reesterification. TG, triglyceride.

The cytolytic action of corticosteroids on normal and malignant lymphocytes is mediated by FFA. The fatty acids could be released from endogenous triglyceride stores by the action of a corticosteroid-sensitive lipase. In sensitive tissues, an accumulation of FFA leads to nuclear damage and ultimately cell lysis. Resistance of malignant lymphocytes to the cytolytic action of corticosteroids might be partially attributed to either the failure of the FFA to be released from endogenous fat stores, lower fat stores in the steroid-resistant tissues, or the greater capacity of resistant cells to oxidize FFA. The fact that these cells are resistant to the lytic effect of exogenously added fatty acid strengthens the likelihood that oxidation is the most important factor in rendering cells resistant to the action of corticosteroids.

ACKNOWLEDGMENTS

The advice and support of Dr. R. L. Noble, Director of the Cancer Research Centre, and the facilities of the Centre are gratefully acknowledged. We wish to thank Dr. W. L. Dunn, Head of the Department of Pathology, for the use of the electron microscope facilities. The technical assistance of Mrs. Brenda Hochachka, Mrs. Denise McClellan, and Mrs. Nora Moorhead was greatly appreciated.

REFERENCES

Fig. 1. Untreated control P1798S cells after 4 hr of incubation. The structural integrity of the incubated cells is well preserved. Maraglas, X 21,640. Inset, membrane-associated and intracisternal capsid-like structures that are commonly found in the cytoplasm throughout the cell population. X 5,840.

Fig. 2. Untreated control P1798S cells after 4 hr of incubation. Good preservation of cell population is evident (section is 0.5 μm thick). Toluidine blue stain, X 1,350.

Fig. 3. Palmitic acid-treated P1798S cells after 4 hr of incubation. Cytoplasmic vesiculation (arrows) is very obvious. Extensive necrosis is evidenced by pycnosis. Toluidine blue, X 1,350.

Fig. 4. Azelaic acid-treated P1798S cells after 4 hr of incubation. Extensive cell necrosis has occurred. Cytolysis is a predominating event. Toluidine blue, X 1,350.

Fig. 5. Palmitic acid-treated P1798S cells after 4 hr of incubation. Swelling of the endoplasmic reticulum, especially in Golgi regions, is prominent in the early stages of degeneration. Note the almost complete absence of membrane-bound polysomes. Alterations in mitochondria and nuclear membrane are not obvious at this time. Maraglas, X 29,120.

Fig. 6. Palmitic acid-treated P1798S cells after 4 hr of incubation. Massive vesiculation of the endoplasmic reticulum and swelling of the perinuclear cisterna are prominent events in the late stage of degeneration. Note the focal alterations in the mitochondria and the loss of sharp dichotomy between euchromatin and heterochromatin. Maraglas, X 28,430.

Fig. 7. Azelaic acid-treated P1798S cells after 4 hr of incubation. Early swelling of Golgi zones and focal alterations in mitochondria are evident. Note the presence of capsid-like structures (arrow) within the endoplasmic reticulum. Upper right, Cell debris, a consequence of cytolysis. Maraglas, X 24,880.

Fig. 8. Azelaic acid-treated P1798S cells after 4 hr of incubation. Progressive degeneration of the cytoplasm is typified by swelling of both endoplasmic reticulum (arrow) and perinuclear cisterna. Isolated mitochondria show matrix-staining alterations. Demarcation between euchromatin and heterochromatin is reduced. The dense body at the left is found commonly in the treated cells at 4 hr but not in untreated cells. Maraglas, X 19,260.

Fig. 9. Azelaic acid-treated P1798S cells after 4 hr of incubation. Terminal structural degeneration in prenecrotic cells is apparent by its progressive nature. The integrity of the nuclear membrane is lost. Note the membrane profiles within the nucleus (arrow). Degenerate mitochondria, vesiculated cisternae, and degranulation of the endoplasmic reticulum are characteristic of this terminal stage. Maraglas, X 29,850.
Studies on the Mechanism of Corticosteroid-induced Lymphocytolysis

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