Response to Prednisolone of Cultured Immunoglobulin-producing Human Lymphoid Cells

By James E. Strong and Benjamin Drewinko

The survival of asynchronously growing lymphoid cells (T₁ cells) in vitro decreased to 50% viability after 1 hr treatment with concentrations of prednisolone above 10 µg/ml. Treatment with prednisolone for 24 hr produced a decrease in cell survival to a plateau of 30% viability for concentrations above 10 µg/ml. After 48 hr treatment, 10 µg/ml prednisolone reduced viability to 12%. Synchronously growing T₁ cells were least sensitive to prednisolone treatment (50 µg/ml) during S phase (DNA synthesis period) and most sensitive during G₁ (pre-DNA-synthesis period). This cell cycle specificity is postulated to be an explanation for the presence of prednisolone-resistant cells in asynchronously growing populations. Immunofluorescence studies on the effect of prednisolone (100 µg/ml) on immunoglobulin production indicated there was no correlation between the length of drug treatment and reduction in the percentage of immunoglobulin-producing cells. These results suggest that the mechanism of prednisolone-induced immunosuppression in lymphoid cells is primarily lymphopenia.

CORTICOSTEROIDS HAVE BEEN shown to produce a general suppression of the immune response, probably caused by the rapid atrophy of lymphoid structures after administration of the drug.¹ This is particularly evident in the thymus where cortisone treatment destroys approximately 95% of the lymphocytes (T cells).² However, the remaining cortisone-resistant T cells are tenfold more reactive in producing graft-versus-host responses than whole thymus cells from untreated donors.³ Lymphoid cells of the thymus-independent series (B cells) also exhibit variations in corticosteroid sensitivity. B cells found in the bone marrow are cortisone resistant, while B cells of peripheral lymphoid organs are cortisone sensitive.⁴ ⁵

To better understand the effect of corticosteroids upon lymphocytes, we have investigated the response of an immunoglobulin-producing human lymphoma cell line grown in vitro to treatment by a corticosteroid analog, prednisolone.

MATERIALS AND METHODS

Cell Line

A human lymphoma cell line (T₁ cells) was derived from the tumorous lymph node of a patient with the diagnosis of poorly differentiated lymphocytic lymphoma.⁶ These cells synthesize immunoglobulin of the IgA K type⁷ and display phenotypic variations described as reticular cells, lymphoid cells, and plasmacytoid cells which are considered morphologic expressions of different functional stages.⁸ This cell line is considered to represent an in vitro model for human im-

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munoglobulin-producing cells.9 All experiments were conducted on T1 cells grown in Ham's F-10 medium supplemented by 20% fetal calf serum, glutamine, vitamins, and antibiotics. Methods for serial propagation have been previously discussed.10 Kinetic values for T1 cells have been established as follows: doubling time, 52 hr; generation time, 27 hr; G1 (pre-DNA-synthesis period), 3.5 hr; S (DNA-synthesis stage), 13.5 hr; G2 (post-DNA-synthesis stage), 10 hr; and M (mitosis), 0.43 hr.11 The plating efficiency for these experiments was about 20%.

Synchronization of T1 Cells

Cells synchronized at the beginning of S phase were obtained using a single treatment of excess TdR (3 mM) in the medium for 24 hr. The excess TdR medium was then discarded and the cells washed twice in fresh medium and incubated in normal medium. The degree of synchrony was monitored by 30-min pulse-labeling replicate cultures with 3H-Tdr (1 μCi/ml, specific activity, 3.0 Ci/m mole) at 2-hr intervals. These cells were harvested, made hypotonic with 1% sodium citrate, fixed with Carnoy's solution, and processed for radioautography by the liquid emulsion technique (Ilford K5). The labeling index (LI) and the mitotic index (MI) were determined for all samples by counting a minimum of 500 cells. At the end of TdR treatment, 85%-90% of the cells were in S phase, while no mitotic cells were observed. These cells moved synchronously into G2 phase as indicated by the LI and MI. After 16 hr, the MI rose to a peak of 10%-12%, and the cells entered G1 synchronously. The proportion of labeled cells increased again after 28 hr.

Drug

Prednisolone sodium succinate (Meticortelone, Schering Corp., Bloomfield, N. J.) was obtained in lyophilized powder form. This was dissolved in 0.9% NaCl sterile solution immediately before use, and dilutions were prepared using fresh medium.

Survival of Cells After Drug Treatment

T1 cells were inoculated into 60-mm Petri dishes containing fresh medium (106 cells per dish) and incubated 48 hr at 37°C in a 5% CO2 humid atmosphere to achieve asynchronous exponential growth. The medium was decanted, and appropriate concentrations of freshly prepared prednisolone (1-100 μg/ml) in fresh medium were added to the cells for the indicated time intervals. Following the drug treatment period, the medium was decanted and cells were washed twice with fresh medium. Cells were harvested, counted in an electronic particle counter (Coulter Model F Counter, Coulter Electronics, Hialeah, Fla.), and replated in known cell densities so that 50-100 colonies would appear after 21 days of incubation. Colonies were briefly rinsed with 0.9% NaCl solution, stained with 0.5% crystal violet in 95% ethanol, and examined under a stereomicroscope. A cell was considered viable if it formed a colony greater than 50 cells. Synchronized cells were similarly treated with a single concentration of prednisolone (50 μg/ml) for 1 hr at 2-hr intervals throughout the cell cycle. The viability assay procedure was identical to that used for asynchronous cells.

Immunofluorescence Studies

Cells were grown 4 days on microscope slides in Petri plates containing fresh medium. Prednisolone (100 μg/ml) was added to the medium, and slides were removed at periodic intervals for 24 hr thereafter. The slides were rinsed in saline, air dried, fixed in acetone, and incubated 30 min in a moist chamber with goat antihuman gamma globulin conjugated with fluorescein isothiocyanate (FITC). After rinsing the slides in fluorescent-tagged antibody (FTA) buffer (Difco), they were washed twice for 30 min in FTA buffer. Then, slides were counterstained with 0.006% Evan's blue dye in FTA buffer. Cells were examined under a Zeiss fluorescent microscope with a uv Osram HB-200 lamp, using UG 1 exciting filters, a 41 barrier filter, and a dark-field condenser. A positive apple-green fluorescence was distinguishable from autofluorescence and was indicative of immunoglobulin synthesis. Control cells were treated similarly, except that no prednisolone was added to the medium.
RESULTS

Survival of Asynchronously Growing T, Cells Following Prednisolone Treatment

Figure 1 demonstrates the lethal effect produced by increasing concentrations of prednisolone on asynchronously growing T, cells. Steep exponential decreases in viability were obtained for treatment with up to 10 μg/ml of the drug for all incubation intervals. The 1-hr treatment resulted in a decreased cell survival (Do, mean lethal dose = 21 μg/ml, estimated by extrapolation) to a plateau of about 50% viability for concentrations above 10 μg/ml. Treatment with prednisolone for 24 hr produced a decrease in cell survival (Do = 9 μg/ml) to a plateau of 30% viability for concentrations above 10 μg/ml, and prednisolone treatment for 48 hr produced a steep exponential decrease in cellular viability (Do = 4 μg/ml) to 12% with 10 μg/ml prednisolone. Drug concentrations above 10 μg/ml for 48 hr produced a shallow downward slope in the viability curve (Do = 112 μg/ml, estimated by extrapolation).

The survival of T, cells given a single concentration of prednisolone (50 μg/ml) for up to 48 hr indicates a positive correlation between the decrease in survival and the duration of treatment (Fig. 2). The steepest decrease in survival occurred during the first hour, with an exponential decrease occurring thereafter.

The Effect of Prednisolone on Immunoglobulin Production

We found that, during asynchronous growth, about 42% ± 12% of T, cells in an untreated population produce immunoglobulin as measured by the fluorescent antibody technique (Fig. 3). The percentage of immunoglobulin-producing cells in an untreated control population was set equal to 1.0, and the per-
The effect of a single concentration of prednisolone as a function of duration of treatment. The upper part of the figure represents the percentage of fluorescent cells incubated with 100 μg/ml of prednisolone and normalized in reference to control cells. The bottom part of the figure depicts the survival of T1 cells incubated with 50 μg/ml prednisolone.

Percentage of immunoglobulin-producing cells in prednisolone-treated populations (100 μg/ml) was normalized to that control value. The effect of prednisolone treatment on the percentage of immunofluorescent cells for up to 24 hr is depicted in Fig. 2. Variation in the proportion of immunoglobulin-producing cells was within the range that would be expected for an untreated population. There was no correlation between the length of drug treatment and reduction in the percentage of immunoglobulin-producing cells.

Fig. 3. T1 cells exposed to FITC-labeled goat anti-human gamma globulin antibody (x 450).
The Effect of Prednisolone on Synchronized T$_i$ Cells

Treatment of T$_i$ cells at regular intervals following removal of excess TdR produced an age-dependent response (Fig. 4), with a maximum survival during early to mid-S phase and a minimum during G$_1$. The transition from S to G$_2$ produced a sharp decline in survival to about 30% in G$_2$ and 25% in G$_1$. There was about a fourfold difference in survival of cells between early S and G$_1$ phases.

DISCUSSION

The differential effect of corticosteroids on lymphocytes has been examined by Esteban.$^{12}$ He found that by injecting $^3$H-thymidine into a rat, using appropriate schedules, "short-" and "long-" lived lymphocytes could be selectively labeled. The results from analyzing percentages of labeled small lymphocytes in lymph organs following corticosteroid injection indicated that short-lived, small lymphocytes were more susceptible to corticosteroid than long-lived, small lymphocytes.

The T$_i$ lymphoid cells are considered an in vitro model for human B cells.$^9$ They resemble short-lived lymphocytes because of their rapid rate of replication during growth. The results obtained following prednisolone treatment of T$_i$ cells should be similar to the effect which would occur in vivo once prednisolone reaches the rapidly proliferating lymphocytes.

We found that asynchronously growing lymphoid cells in vitro present populations with differential sensitivities to prednisolone (Fig. 1). Our explanation for this result is that prednisolone is only effective during certain stages of the cell cycle. Mammalian cells often display differences in their drug sensitivity throughout the generation cycle. These patterns for age response of cells to drugs show some common features: mitotic poisons, chemical mutagens, and inhibitors of DNA synthesis appear to be most effective on cells in S phase; alkylating agents, in M and G$_1$ phase; inhibitors of protein synthesis, at the G$_1$/S transition; and inhibitors of RNA synthesis plus x-rays, in M and at the
$G_1/S$ transition.$^{13}$ $T_1$ cells show an age-dependent response to prednisolone characterized by a relative resistance in the $S$ period and a relative sensitivity in the $G_1$ and $G_2$ stages (Fig. 3), similar to the response of $T_1$ lymphoid cells to x-irradiation.$^{14}$ Thus, the primary site of action for prednisolone does not appear to be interference with DNA synthesis.

Sinclair and Morton$^{15}$ have demonstrated that the survival curve of asynchronous populations closely approaches the one obtained from summation of survival curves for each stage of the cell cycle times the proportion of cells in that stage. The proportion of $T_1$ cells in $G_1$ phase in asynchronous populations is 58%, that in $S$ phase is 26%, and that in $G_2$ is 16%. If these values are multiplied by the average survival of $T_1$ cells treated for 1 hr with 50 $\mu$g/ml prednisolone during each stage of the cell cycle, the survival of asynchronous $T_1$ cells should be 42%. This estimate is reasonably close to the 47% survival experimentally obtained by treatment of asynchronously growing $T_1$ cells with 50 $\mu$g/ml prednisolone for 1 hr (Fig. 1).

Ernst and Killman$^{17}$ have investigated the effect of prednisolone on bone marrow lymphoblasts in patients with lymphoblastic leukemia. It was found that prednisolone decreased the influx from $G_1$ phase into $S$ phase for proliferating lymphoblasts. They suggested that this was caused by either a depopulation of the $G_1$ pool or an arrest of cells in $G_1$ phase. Our results indicate that rapidly growing immunoglobulin-producing lymphoid cells in vitro are most sensitive to prednisolone during $G_1$ and $G_2$ phases. This would explain the results obtained by Ernst and Killman in vivo and suggests that depopulation of the $G_1$ pool, rather than arrest of cells in $G_1$ phase, produces the decreased influx from $G_1$ phase into $S$ phase.

Studies on the immunosuppressive effect of corticosteroids indicate that corticosteroids are most effective when administered before antigen stimulation.$^{18}$ This might be explained if corticosteroids suppress de novo antibody production through a reduction in lymphocyte viability but do not inhibit ongoing antibody synthesis. Our results indicate that treatment of lymphoid cells with prednisolone caused a progressive reduction in viability with increasing length of treatment (Fig. 2). However, the number of immunoglobulin-producing cells was not significantly altered during the same treatment period. Although an alteration in the amount of antibody synthesis by each cell may have occurred, our technique was not sensitive enough to detect such quantitative changes. Despite that possibility, our results suggest that prednisolone treatment has little effect on ongoing immunoglobulin synthesis but causes immunosuppression primarily by lymphopenia.

Our data also provide a rationale for exploring new approaches to corticosteroid therapy. In the past, protocols for immunosuppressive therapy$^{19}$ and treatment of leukemia$^{20}$ using such drugs have not been based on cell cycle specificity considerations. Our results indicate that lymphoid cells exhibit an age-dependent sensitivity to prednisolone. This sensitivity could be exploited to achieve maximum chemotherapy efficacy. Protocols for lymphoreticular neoplasia and immunosuppressive therapy could be reformulated to utilize prednisolone in conjunction with cell cycle blocking agents and other age-dependent drugs.
IMMUNOGLOBIN-PRODUCING LYMPHOID CELLS

REFERENCES

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