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Inhibition of dimethyl sulfoxide-stimulated Friend cell erythrodifferentiation by hydrocortisone and other steroids

(Friend leukemia virus/globin mRNA/glucocorticoids/pretranslational control)

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ABSTRACT **Erythrodifferentiation and hemoglobin synthesis in dimethyl sulfoxide-stimulated Friend erythroleukemia cells were inhibited by hydrocortisone (HC) and four other steroids: dexamethasone, deoxycorticosterone, corticosterone, and aldosterone. The effect was specific, because no significant cytotoxicity occurred with any of these compounds at the concentrations that were inhibitory. The mechanism of action of HC was studied in detail. In the absence of dimethyl sulfoxide, it had no effect on hemoglobin levels; but, in the presence of this inducer, the synthesis of heme and globin were each inhibited by approximately 90%. There was no alteration in the synthesis of any major protein other than globin, as determined by gel electrophoresis of cell lysates. The activities of two enzymes in the heme biosynthetic pathway, δ-aminolevulinate dehydratase and uroporphyrinogen-I synthase, were inhibited by 80% and 70%, respectively. Globin mRNA induction was reduced by approximately 90%. This demonstrated that the HC inhibition of globin synthesis occurred at a pretranslational step. The dimethyl sulfoxide-induced single-stranded breaks in DNA, which have been suggested to play a role in Friend leukemia cell differentiation, were reduced in number but not eliminated. HC reduced the dimethyl sulfoxide-stimulation of virus release into the medium by approximately 50%. HC treatment in the absence of dimethyl sulfoxide doubled the production of virus.**

Alterations in DNA metabolism (1, 2) and structure (3) are among the many changes that occur in Friend leukemia (FL) cells undergoing dimethyl sulfoxide (Me_2SO)-induced erythrodifferentiation (4). The increase in single-stranded breaks in DNA, detected early after Me_2SO treatment (5, 6), before globin mRNA appeared (Scher, unpublished), suggested that these breaks might play a role in differentiation in this system as postulated in lens differentiation (7, 8) and in transcription (9, 10). Since Me_2SO is also known to affect the activity of lysosomes (11, 12), the possibility that it was causing the release of specific DNases that might be involved in the production of these scissions was considered. Therefore, a study of the effect of lysosome-stabilizing agents on FL cells was undertaken. Since some compounds known to stabilize lysosomes were found to inhibit erythrodifferentiation of FL cells and others did not, this property did not appear to be correlated with the ability of an agent to inhibit the Me_2SO effect.

The inhibitory compounds were dexamethasone, hydrocortisone (HC), deoxycorticosterone, corticosterone and aldosterone. The most potent inhibitors were HC and dexamethasone. The mechanism of action of HC, a naturally occurring steroid, was studied further. It markedly inhibited both hemoglobin (Hb) synthesis and globin mRNA levels up to 90% without cytotoxicity, indicating that it acted at a pretranslational step(s). Me_2SO-stimulated virus release was also reduced.

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MATERIALS AND METHODS

FL cell clone 5-86, derived from clone 745 of DBA/2J mouse origin (4, 6, 13) and with similar properties, was used in all experiments unless otherwise noted. T3C12 cells of DDD mouse origin (14) were obtained from Y. Ikawa. Cells were passed twice weekly in prewarmed basal metabolic Eagle's medium containing Earle's salts (Grand Island Biological Company, Grand Island, NY) supplemented with 250 units of penicillin/ml/200 μg of streptomycin/ml/and 15% (vol/vol) fetal bovine serum (Reheis Chemical Company, Kankakee, IL) in Falcon flasks. The cultures were then flushed with 5% CO_2 in air for approximately 20 sec prior to closing tightly. Cells in the log phase of growth were seeded at 10^5/ml. The doubling time of 5-86 cultures under these conditions was 12 hr. Cell numbers were determined with the aid of a hemocytometer. Cell viability was estimated by the trypan blue exclusion test. Me_2SO (Lot 770514, Fisher Scientific, Springfield, NJ) was prepared in complete medium on the day of use to yield a final concentration of 2.0% (vol/vol). Me_2SO and other compounds tested were added at the time of seeding.

RESULTS

A number of agents that influence lysosome activity or inflammatory responses and some structurally related compounds were studied to determine their effect on Me_2SO-stimulated erythrodifferentiation of FL cells (Table 1). HC and dexamethasone were found to be most effective in inhibiting Hb synthesis. Aldosterone, corticosterone, and deoxycorticosterone were moderate inhibitors of the Me_2SO effect. In preliminary experiments (performed by T. Stematsky), compounds not listed in Table 1, acetyl salycilic acid (aspirin), an antiinflammatory agent, and vitamin A, a lysosomal labilizing agent (15), were ineffective.

We studied in detail the effect of HC, the most potent of the naturally occurring steroids tested, in an effort to determine the level at which it exerted its inhibitory effect. HC, over a wide range of concentrations, was not cytotoxic and did not affect the cell saturation density (Fig. 1). However, an early inhibition of cell multiplication occurred occasionally. After 24–48 hr of growth, cultures containing 1.8 μM HC plus Me_2SO contained 7–19% less cells per ml than those grown with Me_2SO alone. By 72 hr, there was no difference in cell density (data not shown). Me_2SO stimulation of Hb synthesis in FL cells was inhibited by HC and was dose-dependent (Fig. 1). After 118 hr of treatment with both compounds, the percentage of Hb-

Abbreviations: B+, benzidine-stained; Me_2SO, dimethyl sulfoxide; FL, Friend leukemia; Hb, hemoglobin; HC, hydrocortisone; ALA-D, δ-aminolevulinate dehydratase; URO-S, uroporphyrinogen-I synthase; C_rt, initial concentration of total RNA (moles of nucleotide/liter) × time (seconds).

Table 1. Effect of steroids and antiinflammatory compounds on Me_2SO-stimulated differentiation of FL cells

Compound	Concentration, μM	Inhibition, %*	ID_{50},† μM
Dexamethasone	0.1	94	0.0025
Dexamethasone	1	97	0.0025
Hydrocortisone	1	91	0.11
Aldosterone	1	64	0.34
Aldosterone	10	62	0.34
Corticosterone	1	68	0.55
Corticosterone	10	75	0.55
Deoxycorticosterone	1	41	3.2
Etiocholanolone	1	0	ND
Etiocholanolone	10	29	ND
Progesterone	1	9	ND
17β-Estradiol	1	2	ND
Testosterone	1	1	ND
Cortisone	10	0	ND
Indomethacin	10	18	ND
Chloroquine	1	0	ND
Primaquine	1	0	ND

ND–Not determined.

* Compounds were added together with 1.9% Me_2SO at time of seeding the culture. Chloroquine, cortisone, deoxycorticosterone, dexamethasone, indomethacin, primaquine, progesterone, and testosterone proprionate were obtained from Sigma Chemical Company, St. Louis, MO; corticosterone and etiocholanolone, from CalBiochem, La Jolla, CA; 17β-estradiol, from Nutritional Biochemicals Corporation, Cleveland, OH; and aldosterone, from Steraloids Inc., Wilton, NH. On day 4 or 5, the percent of benzidine-stained (B+) cells (Hb-containing cells) in cultures treated with a combination of Me_2SO and the test compounds was compared to cultures treated with Me_2SO alone, in which 75–90% of the cells were B+. The percent of B+ cells in untreated cultures (0–2%) was substracted from each value prior to the calculation.

† ID_{50} is the approximate concentration of each compound that inhibited Me_2SO induction of B+ cells by 50%.

containing (B+) cells was decreased by 77% and heme levels by 97% as compared to the cultures treated with Me_2SO alone. Heme appeared to be more sensitive to the action of HC, because 50% inhibition of heme content and of B+ cells occurred at approximately 15 and 74 nM HC, respectively. A similar inhibition by HC and dexamethasone of Me_2SO-stimulated heme and hemoglobin synthesis occurred in T3-Cl-2 cells (data not shown).

In addition, the activities of two enzymes in the heme biosynthetic pathway, δ-aminolevulinate dehydratase (ALA-D) and uroporphyrinogen-I synthase (URO-S), were also found to be inhibited (Fig. 2*A*). After 93 hr of treatment with Me_2SO and 1.8 μM HC, inhibition of ALA-D and URO-S activities was 80% and 71%, respectively. Since the activity of these enzymes reached a peak about 48 hr after the cells had been exposed to Me_2SO (16), experiments were performed to ascertain the effect of HC at this time point. The level of inhibition was found to be as great at 45 hr as it was at 93 hr (data not shown). The concentrations of HC that led to 50% reduction in the levels of ALA-D and URO-S, respectively, were approximately 2.3 and 4.9 nM at 93 hr. Fifty percent inhibition in Hb-containing cells detected by immunofluorescent antibody assay was observed at approximately 30 nM HC (Fig. 2*B*).

To determine if HC was exerting a block at a pretranslational or translational level, we compared the globin mRNA content of cells treated for 4 days with Me_2SO to that of cells treated with Me_2SO and HC (Fig. 3). HC inhibited globin mRNA content to approximately the same extent as it inhibited Hb synthesis (90%), as estimated by comparing the C_rt values at 50% of maximum hybridization. Concomitantly, the level of

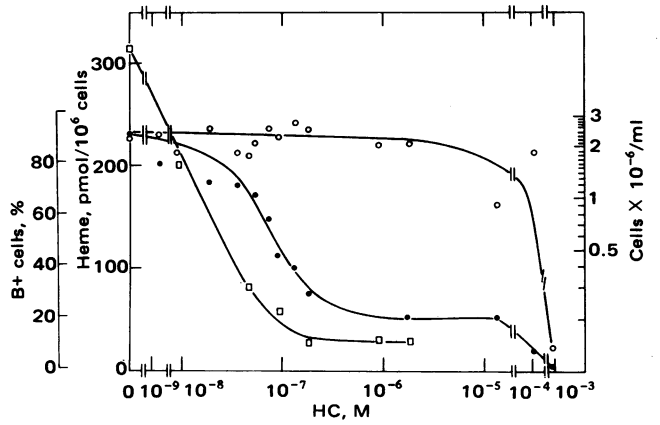

FIG. 1. Effects of HC on Me_2SO-treated FL cells. The percentage of B+ cells (●), heme content (□), and cell number (○) were determined after 118 hr of growth in the presence of 2.0% Me_2SO with the HC concentrations indicated. HC, lot FFH (the free alcohol) (Upjohn, Kalamazoo, MI), dissolved at 0.6 mg/ml in complete medium, was sterilized by filtration. Dilutions (1:10) in medium of this solution were prepared and then stored at 4° until used. The heme content of the cells was determined in triplicate samples by a modification of a described fluorescence method (16). The cells were sedimented at 200 × *g* for 10 min at 4°, washed in 0.95% (wt/vol) NaCl, and resedimented to eliminate fluorescence due to phenol red. The percentage of cells containing Hb (B+ cells) was estimated by the modification of the benzidine method of Orkin *et al.* (17), in which the concentration of benzidine was decreased to 0.67% (wt/vol) and that of H_2O_2, to 0.2% (vol/vol). At least 300 cells were counted in each determination.

intracellular globin was reduced to approximately that of untreated cells (compare lanes 4 and 5 to lane 3 of the gels in Fig. 4). The increased globin content (observed by Coomassie blue staining; Fig. 4*A*) and globin synthesis (observed in radioautographs of [^{35}S]methionine-labeled proteins; Fig. 4*B*) in cultures treated with Me_2SO alone are shown for comparison. Globins with molecular weights of approximately 15,500 migrate ahead of cytochrome C (12,000) and the histone region (11,000–15,000). The nonlinearity of the mobilities in sodium dodecyl sulfate acrylamide gels of some proteins, including globin, when compared to their molecular weights, has been previously reported (23–25). Other than the globin band, no proteins were found to be consistently altered in the presence of the inhibitor. In the histone region, there were at least four bands with the approximate molecular weights of 11,300, 13,000, 14,000, and 15,000 daltons. These appear to be H4, H2a, H2b, and H3, which have been previously demonstrated in FL cells (26–30). Steroids also have no effect on the level or synthesis of these proteins in other systems (e.g., 31–34).

Since the single-strand breaks in DNA of Me_2SO-stimulated FL cells may be associated with differentiation (5, 6), it was of interest to see whether HC would block this process. In neutral gradients, the DNA sediments as "folded genomes" (6, 35). Single-strand nicks in the DNA relax some of the supercoils and result in a more unfolded or ballooned-out structure that has a lower sedimentation value. As seen in Fig. 5*A*, the peak sedimentation of the "folded genome" of the Me_2SO-treated sample lags two fractions behind that of the untreated sample and the trailing edge of the peak lags even further. This is equivalent to approximately a 15% reduction in the peak S value, which, in the untreated sample, is approximately 4500 (6, 36). The combined treatment with HC and Me_2SO resulted in a reduction in the S value of the "folded genomes" but did not completely block its occurrence (compare Fig. 5*A* with *B*).

Since the mechanism by which Me_2SO enhances virus production in this system appears to be independent from that by

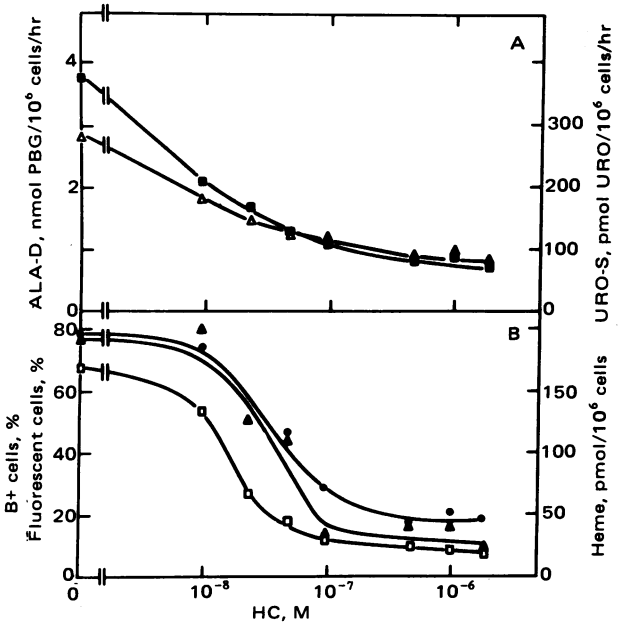

FIG. 2. The effect of HC on heme and hemoglobin metabolism of Me_2SO-treated FL cells. ALA-D (■) and URO-S (△) activities were determined in duplicate as described (16). Heme content (□) and the percentage of B+ cells (●) were assayed as in Fig. 1. Hb-containing cells (▲) were also estimated by indirect immunofluorescence (18) of cells deposited on glass slides (Cytocentrifuge, Shandon Instruments, Sewickley, PA). Goat anti-rat Hb serum (0.1 ml), Cappel Lab., Downington, PA, and fluoresceinated, purified immunoglobulin from pigs immunized with goat immunoglobulins (0.1 ml of a 1:20 dilution in phosphate buffered saline), obtained through the courtesy of J. Gruber, Special Cancer Program, were used. The slides were examined within a few hours with a Zeiss reflected fluorescence microscope, using a BG12 excitation filter, barrier filters (44 and 50), and a high pressure mercury light source (HBO 200). Two hundred cells were counted for each sample, and the percentage of cells exhibiting cytoplasmic fluorescence was determined. Cells exposed to phosphate buffered saline instead of antiserum and mouse erythrocytes served as negative and positive controls, respectively. PBG, porphobilinogen; URO, uroporphyrinogen.

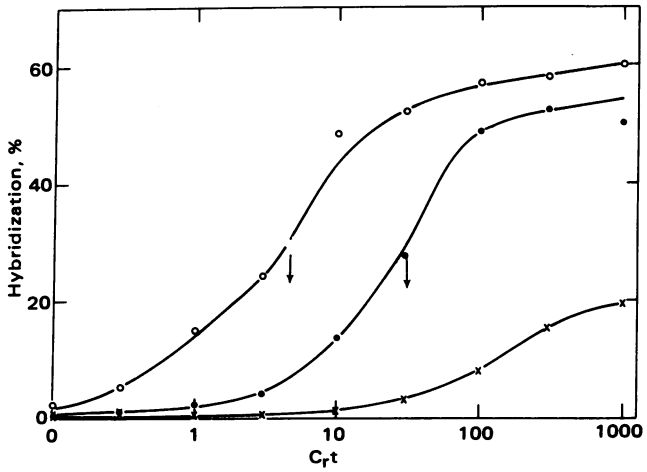

FIG. 3. The effect of HC on the level of globin mRNA sequences in Me_2SO-treated FL cells. The percent of globin cDNA hybridized to cytoplasmic RNA extracted from cells grown for 93 hr in medium containing no supplement (x), 2.0% Me_2SO (○), or 2.0% Me_2SO plus 1.82 μM HC (●) at the C_rt (see below) values indicated was determined. The arrows indicate the half-maximal hybridization values. The preparation of Swiss mouse globin mRNA, its cDNA, and FL cell cytoplasmic RNA and the hybridization of globin cDNA to FL cell RNA have been described (19). The data are given as C_rt values (the initial concentration of total RNA in moles of nucleotide per liter multiplied by time in sec). The percent B+ cells in untreated, Me_2SO-treated, and Me_2SO plus HC-treated cultures at the time of harvest were 1.0, 74.0, and 6.3, respectively.

which Hb production is stimulated (14, 38–40), the effect of HC on virus synthesis was examined. We determined reverse transcriptase activity to estimate the amount of virus released in cultures containing Me_2SO after 4 days and in those not treated with Me_2SO after 3 days of growth. Virus titers were generally at their peak at these times under each of these conditions (40). The relative amount of virus released from FL cells is shown in Fig. 6. Stimulation by Me_2SO in cultures without inhibitor (Fig. 6*A*) was approximately 6 times that of the untreated control (see Fig. 6 legend). The inhibition by HC of Me_2SO-stimulated virus proliferation (which generally ranged from 30–50%) was not as marked as that of Hb synthesis. HC alone was stimulatory. The level of reverse transcriptase in the medium was approximately double that of the untreated control (Fig. 6*B*). There was no effect of HC alone on the percentage of B+ cells, which remained at the level of the untreated cells.

DISCUSSION

This investigation was initiated to explore the effect of agents that affect lysosomes or have anti-inflammatory activity. Various steroid hormones, dexamethasone and HC (anti-inflammatory glucocorticoids), deoxycorticosterone and aldosterone (mineralocorticoids), and corticosterone [the major naturally occurring murine glucocorticoid (41)] were found to inhibit Me_2SO stimulation of Hb synthesis. While this work was in progress, three of the compounds we tested—HC (42), dexamethasone (42, 43), and progesterone (43)—were noted to inhibit Me_2SO-stimulated Hb synthesis. It was also reported that estradiol (43), testosterone (43), and etiocholanolone (42) were not inhibitory. Taken together, these results show no clear correlation between the inhibitory effect and the biological properties of the compounds studied here or elsewhere (42, 43). For instance, some anti-inflammatory agents tested—HC, dexamethasone, and corticosterone—were inhibitory whereas others—cortisone, chloroquine, and indomethacin—were not.

The mechanism of action of HC was studied in detail. Inhibition of the stimulation of erythrodifferentiation in Me_2SO-treated FL cells was linearly related to HC concentration. HC in general was not cytotoxic and did not reduce the final cell density, although a slight reduction in cell number up to the 48th hr of growth was occasionally observed. By the fourth day, Hb, globin, and heme levels were each reduced by approximately 90% in the presence of 2 μM HC. Globin mRNA production was inhibited to approximately the same extent, indicating that HC acts at a pretranslational step. The activities of two enzymes related to Hb synthesis were also tested in cultures containing Me_2SO and HC. ALA-D activity was decreased by 80% and URO-S by 71%. Whether or not the synthesis of these enzymes is also inhibited at a pretranslational step is not yet known.

The fact that HC in combination with Me_2SO did not affect cell multiplication, yet caused an almost complete inhibition of Hb production, indicates a high degree of specificity in its action. This is clearly shown by the gel patterns of cell lysates on which only a trace of the globin band was seen in the HC plus Me_2SO-treated sample, whereas no other major structural protein band appeared to be altered. It remains to be determined whether this faint band of globin represents one or more of the multiple types of globins that this line of FL cells has been shown to synthesize (44).

McClintock *et al.* (42) also noted an inhibition of hemoglobin

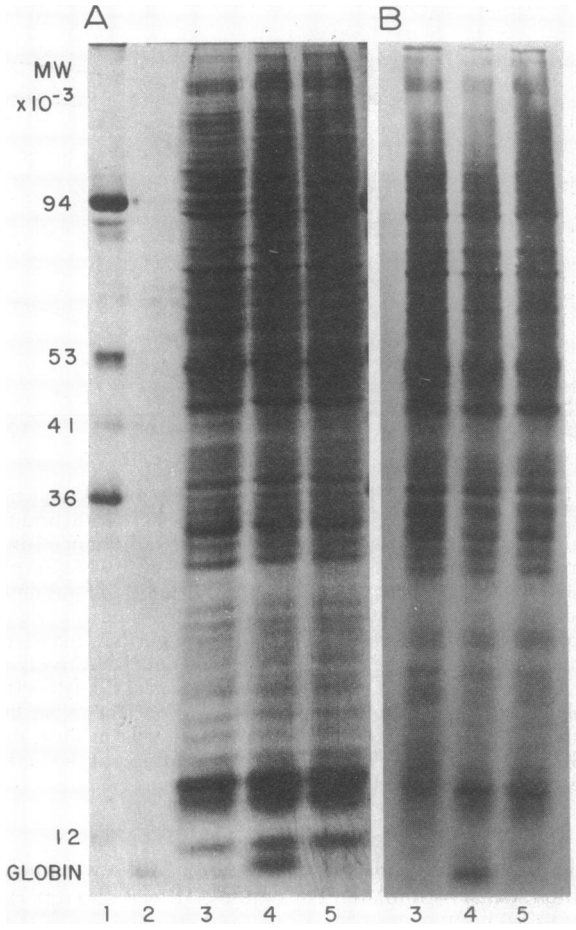

FIG. 4. The effect of HC on globin level and synthesis in Me_2SO-treated FL cells. Gel patterns of extracts from cells grown for 97 hr are shown. L-[^{35}S]methionine, 40 μCi/ml, 0.65 Ci/mol (The Radiochemical Center, Amersham) was present in the medium during the last 17 hr. The sample lysates were prepared by centrifugation of 3×10^6 cells at $200 \times g$ at 4° for 10 min. The cells were washed in 0.95% (wt/vol) NaCl and resedimented. Protein concentration was estimated by a Folin method (20) with bovine albumin as a standard. The cells were suspended in 0.125 ml of 2% (wt/vol) sodium dodecyl sulfate/1.0% (wt/vol) 2-mercaptoethanol/0.025 ml 60% (wt/vol) glycerol/0.3% (wt/vol) bromophenol blue. Erythrocyte lysates were prepared in the same way except that erythrocytes from one mouse were centrifuged, washed in saline, lysed in 0.4 ml H_2O, and clarified by centrifugation at $12,000 \times g$ for 15 min. The samples were stored frozen and boiled 1–2 min just prior to use. Equal amounts of protein were placed in each gel lane in approximately 40-μl volumes. Electrophoresis on sodium dodecyl sulfate linear gradient polyacrylamide gels (stacking gel, 3%, and separating gel, 10–15% wt/vol acrylamide, respectively) was performed by described methods (21, 22) with minor modifications (V. Racaniello, personal communication). The running buffer (22) and the Coomassie blue staining (*A*), destaining, and drying of the gels have also been described (21). The gels were radioautographed (*B*) with Kodak X-R1 film. Lane 3, no supplements; lane 4, 2% DMSO; lane 5, 2.0% DMSO plus 1.82 μM HC. Lane 1 contained molecular weight standards; cytochrome C, 12,000; lactate dehydrogenase, 36,000; alcohol dehydrogenase, 41,000; glutamate dehydrogenase, 53,000; and phosphorylase, 94,000. Lane 2 contained DBA/2J erythrocyte lysate.

synthesis by HC, but their results are at variance with ours in that they observed neither a decrease in globin mRNA nor an effect on virus production. The reason for these differences remains to be resolved but may possibly be due to the fact that different clones were used.

An analysis of breaks in DNA of Me_2SO-treated cells grown in the presence and absence of HC was also undertaken, because single-stranded scissions in DNA may play a role in differentiation (5–8) and transcription (9, 10). HC reduced, but did not eliminate, the number of breaks induced by Me_2SO. This finding suggests that either breaks at only a few specific sites may be important in the control of differentiation or that DNA schisms are unrelated to the process.

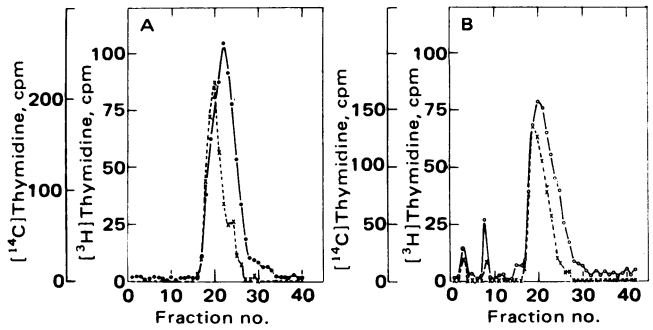

FIG. 5. The effect of HC on the Me_2SO-induced reduction in the sedimentation of FL cell "folded genomes". Cells were grown with [*methyl*-^{3}H]thymidine, 54.0 Ci/mole, 0.25 μCi/ml (Schwarz/Mann, Orangeburg, NY) or [*methyl*-^{14}C]thymidine, 57.0 Ci/mole, 0.25 μCi/ml (Amersham Searle, Arlington Hts., IL) for 15.5 hr and then diluted (approximately 1:4) with nonradioactive medium to the original concentration of 10^5 cells per ml. The ^{14}C-labeled cells were diluted with medium containing either Me_2SO (●) or Me_2SO plus HC (○) to yield 2.0% Me_2SO and 1.8 μM HC. Twenty-five hours later, equal numbers (2×10^4) of untreated ^{3}H-labeled (x) and treated ^{14}C-labeled (○,●) cells were mixed, lysed, and centrifuged in neutral 15–30% (wt/vol) linear sucrose gradients at 8000 rpm for 45 min in a Beckman SW 50.1 rotor (6, 26). Fractions were collected from the bottom of the tubes, precipitated with trichloroacetic acid, and counted in a scintillation spectrometer (6). Sedimentation is from right to left. The percentage of B+ cells in the cultures, determined after 125 hr of growth, was 0.3, 82.0, and 23.0% in the untreated, Me_2SO-treated, and Me_2SO plus HC-treated cultures, respectively.

The effect of corticosteroids on Me_2SO-stimulated virus synthesis was also studied, because they have been reported to

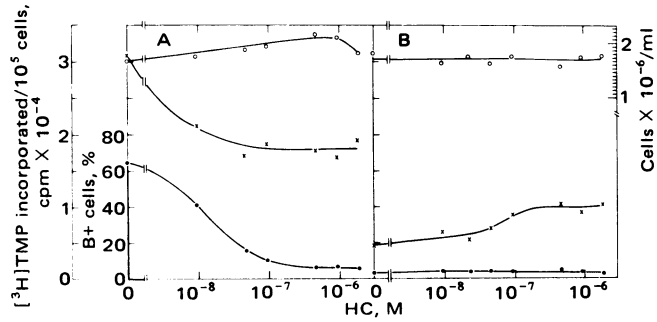

FIG. 6. The effect of HC on reverse transcriptase activity in the presence or absence of Me_2SO. Reverse transcriptase activity in the medium was determined by a modification of a described method (36). Cultures were centrifuged at $200 \times g$ and 4° for 10 min. The supernatant fluid was stored at −70° until used. The reaction mixture (100 μl) contained 50 μl of the supernatant fluid, 50 mM Tris·HCl (pH 8.0), 30 mM NaCl, 2.0 mM dithiothreitol, 0.1 mM dATP, 1.0 mM $MnCl_2$, 0.02% (vol/vol) Nonidet P40 (Shell Oil Co., NY), 10 μg/ml $(rA)_n \cdot (dT)_{10}$ (Collaborative Res. Inc., Waltham, MA), 3 μCi [^{3}H] dTTP (51 Ci/mmol, New England Nuclear, Boston, MA), and 0.01 mM unlabeled dTTP. The mixture was incubated at 37° for 3 hr, then placed on ice after 20 μl of 0.2 M EDTA had been added. The entire mixture was placed on a 25-mm DE-81 filter paper disk (Whatman Inc., Clifton, NJ), which was washed, dried, and counted as described (37). At 94 hr the untreated control culture contained 0.573×10^{-4} cpm [^{3}H]-dTMP incorporated/10^5 cells, 0.7% B+ cells, and 25.4×10^5 cells per ml. The reverse transcriptase activity (x), the percentage of B+ cells (●), and the cell number (○) were determined after growth for 94 hr in the presence of Me_2SO (*A*) and after 69 hr in the absence of Me_2SO (*B*).

affect retrovirus production in the absence (e.g., refs. 45, 46) and presence of halogenated pyrimidines (e.g., refs. 47, 49). HC reduced Me_2SO-stimulated viral reverse transcriptase by 54% when compared to cultures treated with Me_2SO alone. Although the effect of HC on virus synthesis was not as marked as that on Hb synthesis, the decrease in virus release appeared to be as specific because it also occurred at concentrations that did not affect cell multiplication. HC alone, however, was found, as in other systems (e.g., refs. 45–48), to stimulate virus production. The effect of other steroids on virus production will be communicated separately.

In comparing the present results with those obtained with a halogenated pyrimidine, BrdUrd, another well-studied inhibitor of FL cell differentiation (e.g., refs. 49–52), it appears that each of these inhibitors may act at similar pretranslational levels in controlling Hb synthesis. However, they appear to act at different molecular sites in affecting virus synthesis. In the presence of Me_2SO, HC was inhibitory, whereas BrdUrd increased the level of virus released (39, 53; unpublished data¶).

The regulation of gene expression by steroids is not understood. Most, if not all, classes of these hormones are thought to act at the level of transcription after being "placed" in contact with chromatin by specific receptors. The steroid may then bind to chromatin by intercalating between specific bases (54, 55). Whether this type of mechanism occurs in FL cells remains to be determined.

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