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Induction of differentiation of human promyelocytic leukemia cell line HL-60 by retinoyl glucuronide, a biologically active metabolite of vitamin A

(retinoids/myeloid differentiation/glucuronosyl conjugate)

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ABSTRACT **We examined the differentiation activity of retinoyl β-D-glucuronide, a biologically active physiological metabolite of vitamin A, using the human promyelocytic leukemic cell line HL-60, which can be induced to differentiate with retinoic acid. Retinoyl β-D-glucuronide (1 μM) inhibited HL-60 cell proliferation by 55–75%, inhibited tritiated thymidine incorporation into DNA by 63–80%, and induced 38–50% of the cells to differentiate into mature granulocytes. The potency of growth inhibition and induction of differentiation by retinoyl β-D-glucuronide was similar to that of all-*trans*-retinoic acid. The continuous presence of either retinoyl β-D-glucuronide or all-*trans*-retinoic acid was not required to obtain maximum growth arrest and differentiation: a 1-hr exposure of HL-60 cells to the retinoids gave the same response (measured after a total incubation time of 48 hr) as a 24-hr or 48-hr continuous treatment. Retinoyl β-D-glucuronide (0.1–0.2 mM) was 50% less cytotoxic to HL-60 cells than all-*trans*-retinoic acid at an equimolar concentration. Retinoyl β-D-glucuronide was not significantly metabolized to other retinoids; retinoic acid was not formed during incubation. We conclude that retinoyl β-D-glucuronide can arrest HL-60 cell proliferation and induce their differentiation into mature granulocytes; it may act by itself or by being hydrolyzed to retinoic acid, which could be immediately utilized and metabolized. The therapeutic use of this retinoid as an antineoplastic agent is suggested.**

Vitamin A is an essential micronutrient involved in normal proliferation and differentiation of cells of mesodermal, endodermal, and ectodermal origin (1–4). Vitamin A compounds, their biologically active metabolites, and their synthetic derivatives, collectively called retinoids, are also potent anticarcinogenic agents in many experimental models: they suppress or delay the process of carcinogenesis *in vivo* and *in vitro*. In many *in vitro* systems vitamin A active compounds inhibit growth of transformed neoplastic cells and induce their differentiation and maturation (2–5).

Vitamin A compounds are involved in normal and abnormal hemopoiesis (12). Acute myeloid leukemia is attributed to the propagation of a clone of cells that have a block in their differentiation pathway to functional mature granulocytes or monocytes. The human cell line HL-60, developed by Collins *et al.* (6), has been extensively used to examine compounds that might induce maturation of these cells and that might be used clinically. Breitman *et al.* (7, 8) demonstrated that retinoic acid inhibits proliferation of HL-60 cells and induces them to differentiate into morphologically functional mature granulocytes. This finding has been substantiated in other laboratories (9–14). The well-established anticarcinogenic effect of retinoids in experimental systems has suggested a potential for clinical use of retinoids as cancer preventive or chemotherapeutic agents. Retinoic acid is among the more promising candidates for successful clinical application (5, 9, 15, 16). Treatment of leukemia patients with retinoic acid has resulted in an increased number of maturing bone marrow cells and myeloid cells in the peripheral blood of the patients (17–19). However, clinical usefulness of retinoids as antineoplastic agents is limited, due to their toxicity and teratogenicity at the relatively high concentrations that are required for their effectiveness in chemotherapy (20, 21). It is, therefore, important to search for antineoplastic agents with properties more suited for clinical use.

We report here for the first time that retinoyl β-D-glucuronide, a biologically active glucuronic acid conjugate of retinoic acid, inhibits proliferation of promyelocytic leukemia HL-60 cells and induces their terminal differentiation into mature granulocytes. While the antineoplastic biopotency of retinoyl glucuronide in this system was similar to that of the free retinoic acid, this physiologically active metabolite of vitamin A is water soluble and is less cytotoxic.

MATERIALS AND METHODS

Chemicals and Solvents. All biochemicals and solvents were of reagent or HPLC grade. [*methyl*-^{3}H]Thymidine ([^{3}H]dThd; 25 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Nitroblue tetrazolium was purchased from Sigma. Phorbol 12,13-dimyristate was obtained from P-L Biochemicals. Saccharo-1,4-lactone monhydrate was obtained from Calbiochem–Behring. Calf serum was purchased from HyClone (Logan, UT); RPMI 1640 medium and horse serum were obtained from GIBCO. β-D-Glucuronidase (bovine liver), phenolphthalein glucuronide, and free phenolphthalein were obtained from Worthington. All other chemicals were from Sigma. Solvents were reagent grade.

Retinoids. All-*trans*-retinoic acid was obtained from Eastman. Radioinert all-*trans*-retinoyl glucuronide and all-*trans*-[11-^{3}H]retinoyl glucuronide (0.7 mCi/mmol) were prepared chemically (22). The following radioinert retinoid standards were gifts from Hoffmann–La Roche: all-*trans*-retinol, all-*trans*-retinyl acetate, all-*trans*-retinyl palmitate, all-*trans*-4-oxoretinoic acid, all-*trans*-5,6-epoxyretinoic acid, and 13-*cis*-retinoic acid; 13-demethylretinoic acid was a gift from W. Lambert. All retinoids were stored under nitrogen at −70°C. Prior to use all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide were purified by HPLC on a semi-preparative

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Abbreviation: [^{3}H]dThd, [*methyl*-^{3}H]thymidine.
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reversed-phase column, using solvent mixtures containing methanol/water, 60:40 (vol/vol) (10 min), and methanol/water, 80:20 (vol/vol) (10 min).

Cells and Cell Culture Conditions. HL-60, a continuous human myeloid cell line, was a gift from Beverly Mitchell (University of Michigan, Ann Arbor, MI). It had been propagated for 30 passages in our (R.U.S.) laboratory for 3 years. The cells (2×10^5 cells per ml) were cultured in Corning polystyrene flasks in suspension containing RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated (56°C, 30 min) horse serum (GIBCO). Substitution with 10% (vol/vol) heat-inactivated calf serum gave identical results. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Growth medium was replaced every 4 days, except where otherwise indicated. Experiments were conducted with cells in their midlogarithmic phase of growth. After reaching confluency, cells were passaged at a split ratio of 1:4 with fresh medium containing serum.

HPLC of Retinoids. HPLC was conducted essentially as described earlier (23), except that we used a C_{18} reversed-phase semi-preparative column (Whatman ODS-2 M9, 25 × 0.98 cm) connected to a precolumn (7 × 0.46 cm) containing Whatman Co:Pell ODS pellicular support. The solvent sequence was as follows: H_2O/MeOH, 40:60 (vol/vol), 5 min; H_2O/MeOH, 25:75 (vol/vol), 13 min; H_2O/MeOH, 20:80 (vol/vol), 12 min; and MeOH/$CHCl_3$, 83:17 (vol/vol), 20 min. Aqueous mixtures contained 0.01 M ammonium acetate. UV absorbance was monitored at 340 nm with a Waters model 440 detector (Millipore Waters). Eluates were collected in 3.0-ml fractions, air dried, and redissolved in 0.1 ml of methanol. Radioactivity was measured in an ACS scintillation fluid (Amersham), using Packard 4430 liquid scintillation spectrophotometer. Radioactivity was plotted as a histogram and integrated under the peak; the identity of radioactive metabolites was established by coelution with radioinert retinoid standards. Quantitation of retinoids was by external and internal standardization (24).

Preparation of Biological Materials for Analysis by HPLC. Cells were incubated with retinoids for either 1 hr, 24 hr, 48 hr, or 5 days. After incubation, cells and medium were treated with an equal volume of methanol, containing *N*-propyl gallate (50 ng/ml) and butylated hydroxytoluene (50 ng/ml), and then stored at −70°C. Samples were centrifuged to remove protein; aliquots of supernatants were analyzed for retinoids by HPLC and radioactivity. Recovery of radioactivity from all-*trans*-retinoyl glucuronide in the supernatant was 75–80%; extraction of the protein pellet with methanol resulted in 20–25% additional radioactivity; total radioactivity recovered was 100–105%.

Determination of Cell Proliferation and DNA Synthesis. The number of cells in suspension was determined with Coulter Counter Model F or by standard leukocyte counting. Viability of cells was assessed by dye-exclusion method after an exposure of cells to 0.02% trypan blue and counting 300 cells. DNA synthesis was assessed by measuring incorporation of [^{3}H]dThd into DNA (25). At designated times, 2–5 × 10^5 cells were removed from the suspension culture, harvested by centrifuging, washed twice with 2 ml of Dulbecco's phosphate-buffered saline (PBS), resuspended in 1 ml of medium, and incubated for 1 hr at 37°C with 1 μCi of [^{3}H]dThd (specific activity, 25 Ci/mmol). Following incubation, the cells were harvested and washed twice with 2 ml of PBS. The cell pellet was solubilized with 0.6 ml of 1 M NaOH, then neutralized with 1 M HCl. Acid-soluble material was precipitated with 2 ml of ice-cold 10% (wt/vol) trichloroacetic acid, and the precipitates were collected on glass-fiber filters and washed twice with 2 ml of ice-cold trichloroacetic acid and once with 1 ml of ice-cold ethanol. Radioactivity incorporated into DNA and collected on filter papers was measured in 10 ml of ACS scintillation cocktail by liquid scintillation spectrometry. Results were expressed as dpm/mg of protein.

Evaluation of HL-60 Cell Differentiation. HL-60 cell differentiation was confirmed by assessing cell functional capacity to reduce nitroblue tetrazolium dye in response to phorbol 12-myristate 13-acetate, as described by Collins *et al.* (26). Approximately 2×10^5 cells were harvested by centrifugation, suspended in 0.2 ml of PBS containing 0.1% nitroblue tetrazolium and 40 ng of phorbol 12-myristate 13-acetate, and incubated for 40 min at 37°C. The reaction was stopped by chilling on ice, and 200 cells were examined in a hemocytometer by light microscopy at ×10 or ×45 magnification. Cells containing blue deposits of formazan were scored as positive for HL-60 cell differentiation. The results were expressed in percentage of formazan-positive cells.

Other Procedures. Protein was measured by the method of Bradford (27). β-D-Glucuronidase was assayed as described by Dutton (28). In some experiments 0.7 mM saccharo-1,4-lactone, an inhibitor of β-D-glucuronidase, was included. The cytotoxic effects of all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide were determined by exposing HL-60 cells for 1 hr to the retinoids at 0.1 mM, testing viability by trypan blue dye exclusion at that time and after incubating the cells in the absence of the retinoids for 24 and 48 hr. Cytotoxicity was also assessed after a continuous 24-hr and 48-hr exposure of the cells to various retinoids at 0.2 mM.

RESULTS

Effect of Retinoic Acid and Retinoyl Glucuronide on the Growth of HL-60 Cells. HL-60 cells grown in the continued presence of 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide exhibited growth inhibition (Figs. 1 and 2). While after 2 days in culture there was no significant difference in the number of cells between all-*trans*-retinoic acid- or all-*trans*-retinoyl glucuronide-treated and untreated control cells, after 4 days of growth in the presence of 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide, cell growth was inhibited by 69.6 and 54.7%, respectively. The treatment of cells with 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide continuously for 6 days resulted in 80.3 and 74.9% growth inhibition, respectively. The effect on growth of HL-60 cells by a short-term exposure to all-*trans*-retinoyl glucuronide is illustrated in Fig. 2. HL-60 cells were grown in the presence of 1 μM all-*trans*-retinoyl glucuronide for either 1 hr or 24 hr; thereafter, the all-*trans*-retinoyl glucuronide-containing medium was removed and replaced by fresh medium lacking all-*trans*-retinoyl glucuronide. The

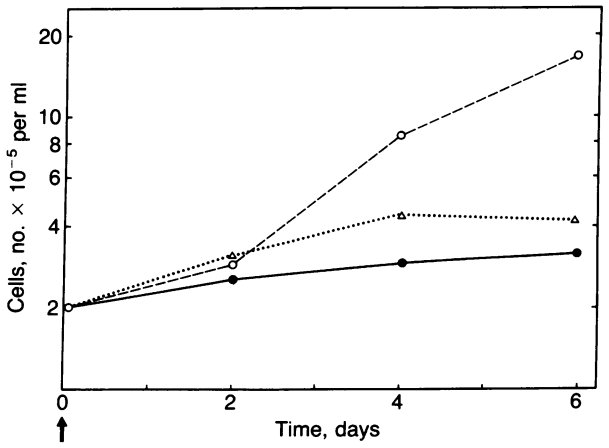

FIG. 1. Growth of HL-60 cells during continuous exposure to 1 μM all-*trans*-retinoic acid (●), to 1 μM all-*trans*-retinoyl glucuronide (△), and in the presence of 0.1% ethanol vehicle (○). Arrow, beginning of treatment.

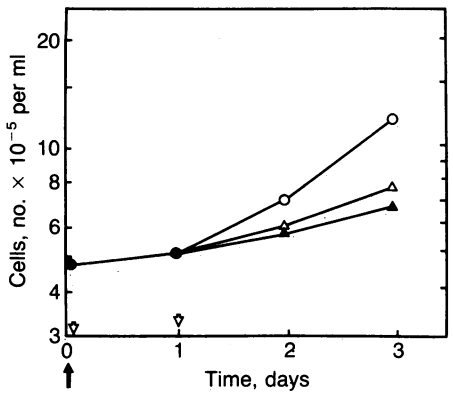

FIG. 2. Growth of HL-60 cells after an exposure to 1 μM all-*trans*-retinoyl glucuronide for 1 hr (△) and 24 hr (▲); 0.1% ethanol vehicle, continuous treatment (○). Solid arrow, beginning of treatment; first open arrow, end of 1-hr treatment; second open arrow, end of 24-hr treatment.

cells were allowed to grow for 3 days. The 24-hr treatment with all-*trans*-retinoyl glucuronide resulted in 37.9% inhibition of growth of HL-60 cells at 3 days; this degree of inhibition was similar to that obtained after only a 1-hr treatment with all-*trans*-retinoyl glucuronide (39.7%).

Effect of All-*trans*-Retinoic Acid and All-*trans*-Retinoyl Glucuronide on the Incorporation of [^{3}H]dThd into DNA. The incorporation of [^{3}H]dThd into DNA of HL-60 cells treated with all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide is shown in Fig. 3*A*. A 1-hr treatment of cells with all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide (1 μM) greatly (72 and 63%, respectively) reduced the incorporation of

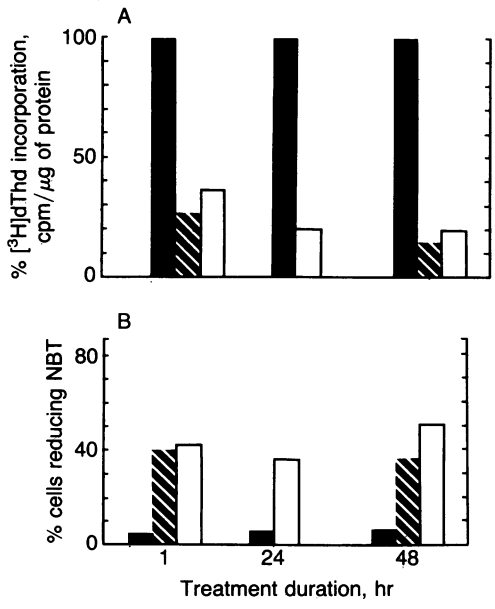

FIG. 3. Effect of all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide on the incorporation of [^{3}H]dThd into DNA of HL-60 cells and on differentiation of HL-60 cells. HL-60 cells were grown in the presence of 1 μM all-*trans*-retinoic acid, 1 μM all-*trans*-retinoyl glucuronide, or 0.1% ethanol vehicle for 1, 24, or 48 hr; thereafter, the medium was replaced with fresh medium lacking all-*trans*-retinoic acid, all-*trans*-retinoyl glucuronide, and ethanol. The cells were allowed to grow for a total time of 48 hr (including duration of treatment). At this time the ability of cells to synthesize DNA was assessed by the incorporation of [^{3}H]dThd into DNA (*A*), and the induction of HL-60 cell differentiation into mature granulocytes was assessed by their ability to reduce nitroblue tetrazolium (NBT) (*B*). Solid bars, control; hatched bars, all-*trans*-retinoic acid; open bars, retinoyl glucuronide.

[^{3}H]dThd into DNA 48 hr after treatment, when compared to the controls. Increasing the length of exposure of cells to all-*trans*-retinoyl glucuronide to 24 hr decreased the [^{3}H]-dThd incorporation into DNA to 20% that of control. A 48-hr continuous exposure to 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide did not further reduce the [^{3}H]dThd incorporation into the DNA of HL-60 cells. All cells were assayed for [^{3}H]dThd incorporation 48 hr after the initial exposure to inducer.

Effect of All-*trans*-Retinoic Acid and All-*trans*-Retinoyl Glucuronide on Differentiation of HL-60 Cells. Induction of HL-60 cell differentiation into mature granulocytes by all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide was assessed by the ability of cells to reduce nitroblue tetrazolium (Fig. 3*B*). The continuous exposure of cells to 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide for 48 hr resulted in the generation of 37 and 50% nitroblue tetrazolium-positive cells, respectively, compared to 7% nitroblue tetrazolium-positive cells present in untreated (control) culture. Only a 1-hr exposure to 1 μM all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide was necessary to induce 40 and 43% differentiation, respectively, measured 48 hr after treatment. Longer treatment times were not used because control cultures were in plateau phase of growth and cell viability was decreased. No additional increase in nitroblue tetrazolium-positive cells occurred if HL-60 cells were incubated with all-*trans*-retinoyl glucuronide for 24 hr, and the cells were grown for an additional 24 hr in medium lacking all-*trans*-retinoyl glucuronide. There was a 10% increase in nitroblue tetrazolium-reducing cells when HL-60 cells were treated with 1 μM all-*trans*-retinoyl glucuronide continuously for 48 hr.

Metabolism of Retinoyl Glucuronide. The metabolite profile of 1 μM all-*trans*-[^{3}H]retinoyl glucuronide in medium and cells, obtained after a 1-hr incubation with HL-60 cells and corrected for artifactual metabolites formed during extraction, is shown in Fig. 4. The majority of radioactivity (95%) was associated with all-*trans*-[^{3}H]retinoyl glucuronide; the remaining radioactivity was distributed among several minor peaks; all-*trans*- and 13-*cis*-retinoic acid were not formed during incubation. A similar profile (data not shown) was obtained after a 24-hr incubation of cells with medium containing 1 μM all-*trans*-[^{3}H]retinoyl glucuronide. Control experiments were conducted by adding 1 μM all-*trans*-[^{3}H]retinoyl glucuronide to either boiled cells and medium or to live cells and medium that had been treated with methanol; the samples were immediately extracted; and the extracts were analyzed by HPLC. A small amount of radioactive products, including all-*trans*-retinoic acid (0.3–0.4%), was generated during the extraction and evaporation procedures; the artifactually generated radioactivity was subtracted from that obtained from incubation samples. The incubation of medium containing unlabeled all-*trans*-retinoyl glucuronide with cells for 48 hr to 5 days resulted in a gradual degradation of all-*trans*-retinoyl glucuronide to various unidentified polar products; all-*trans*-retinoic acid was not detected under these conditions. When medium containing all-*trans*-retinoyl glucuronide and 10 or 15% (vol/vol) calf serum was extracted and the extract was reduced to dryness by evaporation under reduced pressure or by a stream of N_2 and reconstituted with a small volume of methanol, a product was generated from all-*trans*-retinoyl glucuronide that comigrated with methyl retinoate on HPLC (data not shown). This artifact could also be generated from 1 μM all-*trans*-retinoyl glucuronide in boiled RPMI 1640 containing 15% (vol/vol) calf serum, when treated and reconstituted as described above. Only a trace of unaltered all-*trans*-retinoyl glucuronide was recovered from samples treated by this method.

β-Glucuronidase Activity. β-Glucuronidase activity, measured at its optimum at pH 4.5 (29), was not detected in the

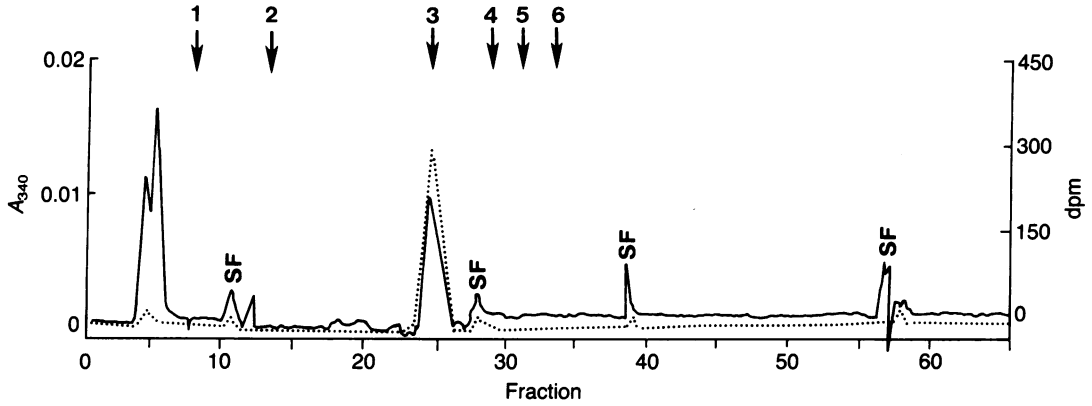

FIG. 4. Reversed-phase HPLC profile of all-*trans*-[11-^{3}H]retinoyl glucuronide metabolism in HL-60 cells. HL-60 cells were incubated for 1 hr with medium containing 1 μM all-*trans*-[11-^{3}H]retinoyl glucuronide. The medium was examined for metabolites of all-*trans*-retinoyl glucuronide by HPLC and by assessment of radioactivity. The elution positions of radioinert internal retinoid standards are indicated by the following arrows: 1, 4-oxo-all-*trans*-retinoic acid; 2, 5,6-epoxyretinoic acid; 3, all-*trans*-retinoyl glucuronide; 4, 13-demethyl-retinoic acid; 5, 13-*cis*-retinoic acid; and 6, all-*trans*-retinoic acid. Solid line, A_{340}; dotted line, dpm; SF, solvent front. Data were plotted as radioactivity per 3-ml fraction.

medium from 1 hr to 6 days of incubation with HL-60 cells. β-Glucuronidase activity was present in HL-60 cells during different stages of growth. When β-glucuronidase was assayed at a time when HL-60 cells were in their late-logarithmic phase of growth, the enzyme activity at pH 4.5 was found to be 3.3×10^{-3} unit/mg of protein (1 unit = 1 μmol of phenolphthalein liberated per min at 37°C) (30); at pH 7.0, the activity was 0.4×10^{-3} unit/mg of protein or 0.12 that of the activity at pH 4.5.

Cytotoxicity. When HL-60 cells were exposed to 0.1 mM all-*trans*-retinoyl glucuronide or all-*trans*-retinoic acid for 1 hr and examined at 24 and 48 hr, all-*trans*-retinoyl glucuronide was found to be 50% less cytotoxic than all-*trans*-retinoic acid; a similar effect was observed after continuous 24- and 48-hr exposures of cells to 0.2 mM of either of these retinoids.

DISCUSSION

Retinoyl glucuronide is a biologically active metabolite of vitamin A (31, 32). While this metabolite had been thought to be solely a product of vitamin A catabolism (33), its discovery in vitamin A target tissues (23, 34) suggests that this very polar vitamin A active compound, because of its amphipathic properties, may also serve in other, physiologically important roles. Our present studies show that all-*trans*-retinoyl glucuronide inhibits HL-60 cell proliferation and induces HL-60 cell differentiation into functionally mature granulocytes to an extent equivalent to that of equimolar amounts of all-*trans*-retinoic acid. HPLC analysis of the retinoid metabolite profile during incubation of HL-60 cells with ^{3}H-labeled all-*trans*-retinoyl glucuronide for 1 hr or for 24 hr revealed that the major (95%) labeled retinoid at all times was all-*trans*-retinoyl glucuronide; retinoic acid was not detected among the minor (5%) radioactive products; some retinoic acid, however, was formed artifactually during the extraction and evaporation procedures. Since we also observed β-D-glucuronidase activity in HL-60 cells, it is possible that some free all-*trans*-retinoic acid could be eventually generated from all-*trans*-retinoyl glucuronide, particularly during longer incubation periods. However, the activity of this enzyme in HL-60 cells was very low at the pH maintained during culture conditions. The presence of saccharo-1,4-lactone, an inhibitor of β-D-glucuronidase, during the incubation did not alter metabolism of all-*trans*-retinoyl glucuronide. β-D-Glucuronidase activity was not detected in the medium. Clearly, during our incubation conditions all-*trans*-retinoyl glucuronide was not hydrolyzed to a measurable amount of all-*trans*-retinoic acid. Although it appears that the observed biological activity can be ascribed solely to all-*trans*-retinoyl glucuronide, we cannot rule out the possibility that the activity of retinoyl glucuronide is the result of it being slowly hydrolyzed to retinoic acid, which is then immediately utilized and metabolized.

Previous studies have indicated that a continuous 48-hr exposure to all-*trans*-retinoic acid is required for onset of growth inhibition or for induction of optimal differentiation (7). We, however, observed a maximum differentiation of 50% of HL-60 cells to granulocytes at 48 hr whether the cells had been exposed to the retinoids for only 1 hr or continuously for 24 or 48 hr. Similarly, either a 1-hr or a 24-hr exposure of HL-60 cells to all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide resulted in 60–70% arrest of cell proliferation measured at 48 hr; this was similar to that observed after the cells had been exposed to the retinoids continuously for 48 hr. These effects were not reversible: the removal of all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide from the medium after a 1-hr treatment did not result in resumption

Table 1. Effect of retinoyl glucuronide on the growth of HL-60 cells

Duration of treatment, days	Duration of experiment, days	Control: Cells, no. × 10^{-5} per ml	Control: % growth inhibition	All-*trans*-retinoyl glucuronide: Cells, no. × 10^{-5} per ml	All-*trans*-retinoyl glucuronide: % growth inhibition
2	2	2.8	0	3.2	(−)*
2	4	9.1	0	3.3	64
2	6	16.3	0	4.1	75
4	2	2.8	0	3.3	(−)*
4	4	9.1	0	4.7	56
4	6	16.3	0	5.9	64
6	2	2.8	0	2.8	0
6	4	9.1	0	4.3	53
6	6	16.3	0	2.9	69

Cells were exposed to 1 μM all-*trans*-retinoyl glucuronide for 2, 4, or 6 days during normal culture conditions. Following treatment, the medium containing all-*trans*-retinoyl glucuronide was replaced with fresh medium lacking all-*trans*-retinoyl glucuronide, and the cells were allowed to continue to grow for a total of 6 days (including growth during treatment). The number of cells was counted at 2, 4, and 6 days of incubation.

*Growth stimulation, 3–18%.

of growth nor did this alter the differentiation pattern of HL-60 cells. From a clinical standpoint, such a pulse–dose treatment may offer a distinct advantage, since a high single dose of a retinoid is better tolerated than high chronic doses (20, 21).

Others have reported a maximum of 90% induction of differentiation of HL-60 cells by 1 μM all-*trans*-retinoic acid (7), while we obtained a maximum of 75% differentiation with a similar concentration of all-*trans*-retinoic acid or all-*trans*-retinoyl glucuronide. The discrepancy in the extent of differentiation is most likely due to a subpopulation in our HL-60 cell culture that is resistant to all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide. Karyotype analysis (data not shown) of the HL-60 cells currently in use in our laboratory revealed the presence of a subclass cell population.

Our studies also briefly addressed the clinically important question of retinoid toxicity. We examined the viability of HL-60 cells in the presence of high (0.1 mM) equimolar concentrations of all-*trans*-retinoic acid and all-*trans*-retinoyl glucuronide. Our data demonstrate that all-*trans*-retinoyl glucuronide is 50% less cytotoxic to HL-60 cells than all-*trans*-retinoic acid (data not shown). Clinically, all-*trans*-retinoyl glucuronide may be a more useful retinoid than free all-*trans*-retinoic acid because of the greater amphipathic potential of the glucuronide and because of its lower toxicity.

The induction of differentiation in HL-60 cells by a brief exposure to all-*trans*-retinoyl glucuronide should facilitate the elucidation of the initial biochemical events that affect the genome and that subsequently result in phenotypic and morphological changes. It remains to be determined whether the expression of the tissue transglutaminase gene, which dramatically increased within 30 min of all-*trans*-retinoic acid administration (13), is linked to the all-*trans*-retinoic acid-(and all-*trans*-retinoyl glucuronide)-induced arrest of HL-60 cell proliferation and induction of differentiation. The mechanism of action of retinoids on growth and differentiation is not understood. The absence in the HL-60 cells of the cellular all-*trans*-retinoic acid binding protein that has been demonstrated in many cells (11, 12) implies that retinoids in these cells act either by a different mechanism or that the all-*trans*-retinoic acid receptor has not yet been identified. This latter view appears to be more in harmony with the present concepts of growth regulation.

While the present work was in progress, we learned that similar studies were being conducted by J. M. Gallup, A.B.B., H. C. Furr, and J. A. Olson; their results appear to be in general agreement with the results reported here.

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1. Wolf, G. (1984) *Physiol. Rev.* **64,** 873–938.
2. Lotan, R. (1980) *Biochim. Biophys. Acta* **605,** 33–91.
3. Roberts, A. B. & Sporn, M. B. (1984) in *The Retinoids*, eds. Sporn, M. B., Roberts, A. B. & Goodman, D. S. (Academic, Orlando, FL), Vol. 2, pp. 209–286.
4. Jetten, A. M. (1985) in *Growth and Maturation Factors*, ed. Guroff, G. (Wiley, New York), Vol. 3, pp. 252–293.
5. Moon, R. C. & Itri, L. M. (1984) in *The Retinoids*, eds. Sporn, M. B., Roberts, A. B. & Goodman, D. S. (Academic, New York), Vol. 2, pp. 327–371.
6. Collins, S. J., Gallo, R. C. & Gallagher, R. E. (1977) *Nature (London)* **270,** 347–349.
7. Breitman, T. R., Selonick, S. E. & Collins, S. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2936–2940.
8. Breitman, T. R., Collins, S. J. & Keene, B. R. (1981) *Blood* **57,** 1000–1004.
9. Koeffler, H. P. (1983) *Blood* **62,** 709–721.
10. Strickland, S., Breitman, T. R., Frickel, F., Nurrenbach, A., Hadicke, E. & Sporn, M. B. (1983) *Cancer Res.* **43,** 5268–5272.
11. Douer, D. & Koeffler, H. P. (1982) *J. Clin. Invest.* **69,** 277–283.
12. Koeffler, H. P. & Amatruda, T. T. (1985) in *Retinoids, Differentiation and Disease*: Ciba Foundation Symposium 113, eds. Nugent, J. & Clark, S. (Pitman, London), pp. 252–267.
13. Murtaugh, M. P., Dennison, O., Stein, J. P. & Davies, P. J. A. (1986) *J. Exp. Med.* **163,** 1325–1330.
14. Yen, A., Powers, V. & Fishbaugh, J. (1986) *Leukemia Res.* **10,** 619–629.
15. Meyskens, F. L., Jr. (1985) in *Retinoids: New Trends in Research and Therapy*, ed. Saurat, J. H. (Karger, Basel), pp. 371–374.
16. Meyskens, F. L., Jr., Goodman, G. E. & Alberts, D. S. (1985) *CRC Crit. Rev. Oncol. Hematol.* **3,** 75–101.
17. Clark, R. E., Smith, S. A., Masters, G., Baileywo, R. & Jacobs, A. (1986) *Br. J. Haematol.* **63,** 200–201.
18. Fontana, J. A., Rogers, J. S. & Durham, J. P. (1986) *Cancer* **57,** 209–217.
19. Daenen, S., Vellenga, E., Dobbenburgh, O. A. & Halie, M. R. (1986) *Blood* **67,** 559–561.
20. Vahlquist, A. (1985) *Clin. Exp. Dermatol.* **10,** 133–143.
21. Mahrle, G. (1985) *Curr. Probl. Dermatol.* **13,** 128–163.
22. Barua, A. B. & Olson, J. A. (1985) *J. Lipid Res.* **26,** 1277–1282.
23. Cullum, M. E. & Zile, M. H. (1985) *J. Biol. Chem.* **260,** 10590–10596.
24. Cullum, M. E. & Zile, M. H. (1986) *Anal. Biochem.* **153,** 23–32.
25. Thomas, G. A. & Simpson, R. U. (1986) *Cancer Biochem. Biophys.* **45,** 221–224.
26. Collins, S. J., Ruscetti, R. W., Gallagher, R. E. & Gallo, R. C. (1975) *J. Exp. Med.* **149,** 969–974.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
28. Dutton, G. J. (1980) *Glucuronidation of Drugs and Other Compounds* (CRC, Boca Raton, FL).
29. Levvy, G. A. (1952) *Biochem. J.* **52,** 464–472.
30. Fishman, W. H., Springer, B. & Brunetti, R. (1948) *J. Biol. Chem.* **173,** 449–456.
31. Sietsema, W. K. & DeLuca, H. F. (1982) *J. Nutr.* **112,** 1481–1489.
32. Stephens-Jarnagin, A., Sietsema, W. K., Miller, D. A. & DeLuca, H. F. (1983) *Arch. Biochem. Biophys.* **220,** 502–508.
33. Lippel, K. & Olson, J. A. (1968) *J. Lipid Res.* **9,** 580–586.
34. Zile, M. H., Inhorn, R. C. & DeLuca, H. F. (1982) *J. Biol. Chem.* **257,** 3544–3550.