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In Vivo and *in Vitro* Effects of Hydralazine on Cellular Growth, Differentiation, and Chromatin Structure

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In Vivo and *in Vitro* Effects of Hydralazine on Cellular Growth, Differentiation, and Chromatin Structure. EVENSON, D. P., AND FASBENDER, A. J. (1988). *Toxicol. Appl. Pharmacol.* **93,** 339–350. The effects of hydralazine (1-hydrazinophthalazine), an antihypertensive drug, on mammalian cell growth, viability, and differentiation were assessed using Friend leukemia cells, Chinese hamster ovary cells, human lymphocytes, and rat lymphocytes, testicular germ cells, and epididymal sperm. Cultured cells in exponential phase growth were more susceptible to hydralazine cytotoxicity than stationary phase (G_0) cells. Growth inhibition was associated with a dose-related slowdown of cell progression through S phase and was observed prior to a decrease of cell viability. At high drug concentrations, progression in all phases of the cell cycle was partially or totally inhibited. Hydralazine did not have an effect on the proliferation and differentiation of testicular germ cells in spontaneously hypertensive rats receiving 0–90 mg/kg/day (up to 20 times the dose used in humans) of hydralazine for a 12-week period. Hydralazine-exposed, histone-containing somatic cells and protamine-containing sperm cells failed to show any alterations in stainability with a DNA-intercalating dye nor in the susceptibility of nuclear DNA to undergo acid-induced denaturation *in situ.* The data suggest that hydralazine causes a dose-related suppression of mammalian cell growth with S phase appearing to be the most susceptible to hydralazine cytotoxicity. Furthermore, the interaction of hydralazine with chromatin at concentrations leading to antigenicity did not inhibit DNA staining with the intercalating dye acridine orange, suggesting that the drug does not competitively intercalate at a detectable level. Association of hydralazine with chromatin did not cause a detectable level of stabilization or destabilization of the DNA to denaturation *in situ.*

The ability of drugs to produce undesirable effects has been apparent since the first medicinal preparations were compounded and administered. Unique among nontherapeutic responses are those in which the drug produces an entire disease state, clinically indistinguishable from the spontaneous disease that it mimics (Kale, 1985). Hydralazine (1-hydrazinophthalazine) was introduced for the treatment of hypertension in 1951 and within 2 years a syndrome resembling systemic lupus erythematosus (SLE) was reported (Morrow *et al.,* 1953). SLE has been regarded as a prototype autoimmune disease even though the etiology remains unknown. Recognition of a lupus-like syndrome related to the use of certain drugs has allowed insight into the mechanisms involved in idiopathic SLE (Totoritis and Rubin, 1985).

Theories proposed to explain the autoimmune effects associated with hydralazine administration have included possible chemical interactions between hydralazine and cellular constituents, including modification of chromatin structure which may trigger an immu-

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nologic response (Dubroff and Reid, 1980). A number of studies have shown that hydralazine can bind to DNA or nucleoprotein and cause an alteration in physical properties (Eldredge *et al.*, 1974; Dubroff and Reid, 1980). Examples of altered DNA causing immunogenic reactions include ultraviolet-irradiated DNA, photooxidized DNA, and carcinogen-modified DNA (Leng *et al.*, 1978; Levine and Seaman, 1967). An association may exist between drug-induced DNA modification and drug-induced autoimmune disease.

Much attention has been focused on the interaction of hydralazine with the immune system. Denatured DNA and nucleohistones are major targets of autoreactivity in hydralazine-related autoimmune disease (Stollar, 1981). A summary of proposed mechanisms include the following: (1) the drug may bind to a macromolecule and serve as a specificity-determining hapten for antibodies that then cross-react with nuclear antigens, (2) the drug may bind to a usually nonantigenic molecule of nuclear origin and render it immunogenic, with the major specificity determinants being those of the macromolecule itself, (3) the drug may cause cell damage and release of contents in a form that is immunogenic, (4) the drug may combine with selected cell surface receptors and stimulate B cells that are normally silent, or (5) the drug may eliminate populations of suppressor cells, leaving unregulated B cells free to produce autoreactive products (Stollar, 1981). In both idiopathic and drug-related lupus the basic question is whether the primary autoimmune process results from an unusual presentation of antigen or from an abnormality of the immune system.

In addition, a genetic predilection related to the metabolism of hydralazine has been considered (Blair *et al.*, 1985; Timbrell *et al.*, 1984). Hydralazine is quickly and extensively metabolized and attempts have been made to correlate metabolites and autoimmune toxicity (Blair *et al.*, 1985; Timbrell *et al.*, 1984). Interest in a toxic metabolic compound or pathway has been stimulated because people with a low capacity for N-acetylation, such as phenotypic slow acetylators, are more prone to develop drug-induced lupus or its manifestations than are rapid acetylators (Weber and Tannen, 1981; Perry, 1973).

Flow cytometry has been used to simultaneously measure cellular DNA and RNA contents and changes associated with progression through the cell cycle (Darzynkiewicz *et al.*, 1976). In addition, flow cytometry has been used to assess the effects of chemical exposure on cellular DNA and RNA contents and chromatin structure (Darzynkiewicz *et al.*, 1981; Traganos *et al.*, 1980a; Evenson *et al.*, 1979, 1985). Simultaneous quantification of cellular DNA and RNA by flow cytometry utilizes a metachromatic dye, acridine orange (AO), which intercalates into double-stranded nucleic acid and produces green fluorescence (530 nm) when excited with blue 488-nm laser light (Lerman, 1963). Electrostatic interaction and stacking of the AO dye molecules with single-stranded nucleic acid produces red fluorescence at 640 nm (Kapuscinski *et al.*, 1982). It is therefore possible to measure the intercellular variability of DNA and RNA contents in large cell populations and to relate the variability to nucleic acid synthesis, chromatin structure, and progression through the cell cycle. The same flow cytometric method can be used to measure the ratio of double-stranded to single-stranded DNA in cells devoid of RNA (Darzynkiewicz *et al.*, 1977).

The objective of this study was to determine the dose–response relationship between hydralazine exposure and alterations in cell growth and differentiation both *in vivo* and *in vitro*. Furthermore, we measured *in situ*, the susceptibility of histone- and protamine-complexed DNA to acid-induced denaturation in hydralazine-exposed cells to further our understanding of why hydralazine-treated patients develop antibodies against DNA/chromatin with lupus-like symptoms.

METHODS

Cells and Media

All tissue culture media and supplements were obtained from Gibco Laboratories, Life Technologies, Inc. (Grand Island, NY). Friend leukemia (FL) cells, which grow as a suspension culture, were routinely passed twice weekly by splitting at a ratio of 1:20 and were grown in a humidified atmosphere of 5% CO_2 at 37°C. Growth medium consisted of RPMI 1640, supplemented to 16% (v/v) with heat-inactivated fetal calf serum, 80 U/ml penicillin, 80 μg/ml streptomycin, and 2 mM L-glutamine. For studies on log-phase FL cells, cultures were split 1:3 daily for 3 days prior to the experiment. This cell line has been used extensively in similar studies (Evenson *et al.*, 1979; Traganos *et al.*, 1980a,b).

Chinese hamster ovary (CHO) cells were maintained as growing monolayer cultures in F-12 (HAM) nutrient medium, supplemented to 16% (v/v) with heat-inactivated fetal calf serum, 80 U/ml penicillin, 80 μg/ml streptomycin, and 2 mM L-glutamine. Incubation conditions were the same as above. These cells were used for survival studies since they grow as a monolayer and can effectively be used for detecting clones of surviving cells (Evenson *et al.*, 1979). The cultures were routinely passed twice weekly by splitting at a ratio of 1:20. For survival studies on cycling cells, 10^4, 10^3, and 10^2 asynchronous, log-phase-growing cells were seeded in 35-mm diameter multiwell plates (Falcon 3046, Becton–Dickinson, & Co., Oxnard, CA) for each drug concentration. Conditioned medium (medium in which log-phase cells were grown for 6–12 hr) was used when initially setting up the cultures for a 2- to 4-hr period during which the cells attached to the culture vessel. Hydralazine was added to the conditioned medium and at the end of a 20-hr period, the cells were washed twice with Hanks' balanced salt solution (HBSS) and refed with 5 ml of fresh medium. Following cell growth for 7 days, the cultures were washed twice with HBSS, fixed for 15 min with Carnoy's fixative (3:1 methanol:glacial acetic acid), and stained with 0.1% crystal violet solution in 0.1 M citric acid. Survival studies on noncycling cells were performed as above with the following exceptions: Noncycling cells (in confluency for about 48 hr) were treated with various concentrations of drug for 20 hr, washed with HBSS, trypsinized with 0.4% trypsin–EDTA, and replated in conditioned medium. The conditioned medium was left on for 20 hr, during which the cells attached to the culture vessel. The conditioned medium was then replaced with fresh medium and the cells cultured for 7 days and stained as above (Evenson *et al.*, 1979).

Hydralazine

Hydralazine (Sigma Chemical Co., St. Louis, MO) solutions were freshly prepared for all experiments by dissolving the hydrochloride salt in appropriate medium at room temperature, filter sterilizing, and serially diluting. Hydralazine in solution is stable for at least 8 hr.

Isolation and Culture of Human Lymphocytes

Peripheral blood from healthy volunteers was collected into heparinized tubes and diluted with 2 vol of HBSS. Aliquots (10–12 ml) of this mixture were layered onto 5 ml of Lymphoprep (Nyegaard & Co., Oslo, Norway) and centrifuged at 400*g* for 20 min at room temperature. The white blood cells (WBC) present at the interface between the serum and HBSS were aspirated off with a Pasteur pipet, washed once in HBSS, and centrifuged at 225*g* for 5 min. The supernatant was aspirated off, the pellet was resuspended, and the cells were cultured in growth medium described for FL cells. Purified phytohemagglutinin (PHA, Wellcome Research Lab., Beckenham, England) was used as the mitogen at 3 μg/ml culture medium (Darzynkiewicz *et al.*, 1976). Replicate cultures of lymphocytes were set up to contain 0.5–1.0 $\times 10^6$ cells/ml and 1.5–4.0 ml per culture, depending on the experiment.

Spontaneously Hypertensive Rats (SHR)

Sixty spontaneously hypertensive male rats were obtained from Taconic Farms (Germantown, NY) at 8 weeks of age and allowed to acclimate for 4 weeks prior to hydralazine exposure. Animals were housed individually in wire cages and allowed free access to Rodent Blox (Wayne Pet Food Division, Chicago, IL) and deionized drinking water. The rats were maintained on a 12-hr light:dark cycle, with the temperature held at 21 ± 2°C. The animals were separated into 11 dose groups, with at least 5 animals in each group. The rats were weighed every 3–4 weeks and dosage calculations were adjusted. The hydralazine was freshly prepared and administered daily at 1700 hr for 12 weeks. The rats received approximately the same volume of drinking water (25–35 ml per day) per kilogram of body weight in order to minimize hydration differences (Greenberg, 1980) and to provide a dosage ranging from 0 to 90 mg/kg body wt per day.

Rat lymphocytes. Rats were anesthesized with ether and weighed. The chest cavity was surgically opened and approximately 10 ml of blood was collected from each rat by intracardiac puncture. Blood was drawn into a syringe rinsed with a heparin solution (150 U/ml) and then transferred to a heparin-rinsed culture tube. The WBCs were isolated as described above and an aliquot was diluted in HBSS for subsequent acridine orange staining and flow cytometric analysis.

Testicular germ cells. The testes were surgically removed from the killed rats and one testis was wrapped

in cellophane and frozen at −95°C. The other testis was weighed and then a section was removed with a razor blade and in a 60-mm petri dish containing HBSS at 4°C minced with curved scissors. The minced tissue was transferred to a 12 × 75-mm test tube. After several minutes to allow for settling of testicular fragments, the supernatant fraction was filtered through a 53-μm-pore nylon mesh (Tetko, Inc., New York, NY) and was then ready for AO staining and flow cytometric analysis (Evenson *et al.*, 1985, 1986).

Epididymal sperm. A caudal epididymus was removed from each animal, sliced open with a razor blade in a petri dish with 4 ml of glycerated (10%) TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM Na_2EDTA, at pH 7.4), minced with curved scissors, and transferred to a tissue culture tube. Tissue fragments were allowed to settle in the tube and the supernatant was filtered through a 153-μm-pore nylon mesh (Tetko, Inc.). The filtrate was initially frozen at −20°C and then stored at −95°C. Samples were prepared for flow cytometric analysis by first thawing in a 37°C water bath, immersing the sample tube in an ice-water slurry, and sonicating a 2-ml aliquot in Falcon 3033 tissue culture tubes using a Biosonik IV (VRW Scientific, San Francisco, CA) at a power setting of 50 for 30 sec. Samples were allowed to cool for 30 sec and sonicated again for 30 sec. One milliliter of sonicated suspension was diluted to approximately 2 ml with TNE buffer and used for AO staining and flow cytometric analysis (Evenson *et al.*, 1985).

Sonication-resistant spermatids. The frozen testes were thawed and weighed, and each testis was homogenized in 2 ml of double-distilled water for 20 sec using a Virtis Model 45 homogenizer operating at a power setting of 55. The homogenizer container was rinsed and the total volume of homogenate was measured. The homogenate was immersed in an ice slurry and sonicated for 60 sec at a power setting of 50 using a Biosonik IV to disrupt tissue debris, sperm tails, and spermatogenic cells sensitive to sonication, leaving only the sonication-resistant sperm heads intact. The suspension was further diluted with double-distilled water and the total volume was recorded. Sperm head concentrations were determined using a hemocytometer (Cassidy *et al.*, 1983).

Acridine Orange Staining Procedures and Fluorescent Measurements

Method I: Low pH pretreatment and neutral pH acridine orange staining. As previously described (Darzynkiewicz *et al.*, 1976), 0.2-ml aliquots of cell suspension containing approximately 3–5 × 10^5 cells were admixed with 0.4 ml of a solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl at pH 1.4. After 30 sec the cells were stained with 1.2 ml staining buffer (1 mM Na_2EDTA, 0.15 M NaCl in 0.2 M Na_2HPO_4/0.1 M citric acid buffer at pH 6.0) containing 6 μg/ml of chromatographically purified AO (Polysciences, Warrington, PA). The stained samples were immediately placed into the flow cytometer sample chamber at 4°C and measured 3 min after staining. The first step of the AO staining procedure utilizes Triton X-100 to permealize the cell membranes, allowing access of AO to the cellular matrix. The pH of 1.4 dissociates histones from somatic cells and histone-containing testicular cells, thereby increasing the accessibility of DNA for AO staining (Darzynkiewicz *et al.*, 1976). Briefly, under these staining conditions, AO intercalates into double-helical nucleic acids (predominantly DNA in this case), fluorescing green (530 nm) upon laser excitation (488 nm), while it "stacks" in polymeric form on single-stranded nucleic acids with a metachromatic shift in maximum emission to red fluorescence (640 nm). For somatic cells or histone-containing germ cells, these staining conditions permit a stoichiometric determination of DNA and RNA content (Kapuscinski *et al.*, 1982). Previous experiments using RNase and DNase controls have shown that the fluorescence at 530 and 640 nm represents cellular DNA and RNA, respectively (Darzynkiewicz *et al.*, 1976, 1977) and that the integrated values of the measured fluorescence pulses remain in proportion to the content of nucleic acid per cell (Traganos *et al.*, 1977). Although this procedure does not denature somatic cell DNA *in situ*, it will denature DNA of sperm with abnormal chromatin structure (Evenson *et al.*, 1985). The procedure used for sperm has been termed the sperm chromatin structure assay (Evenson, 1986).

Method II: Low pH pretreatment and low pH AO staining. This procedure partially denatures somatic cell DNA and was used here to distinguish cells in different phases of the cell cycle and to determine whether hydralazine had any stabilizing or destabilizing effect on DNA denaturation *in situ*. FL cells and human lymphocytes were diluted in 1 ml of HBSS (no phenol red), rapidly admixed with 10 ml of fixative (1:1 70% ethanol:acetone) at 4°C in glass tubes, and then stored at −20°C. Cells may remain fixed in this solution for many months at −20°C without change (Darzynkiewicz *et al.*, 1977). For analysis, the glass tubes were centrifuged at 225*g* for 5 min, the fixative was aspirated off, and the pellet was resuspended in 1 ml of HBSS. RNase A (Worthington Biomedical Corp., Freehold, NJ) was added to a final concentration of 1–2 × 10^3 units/ml, and samples were incubated at 37°C for 1 hr. A 0.2-ml aliquot of cell suspension was transferred to a 12 × 75-mm glass test tube, admixed with 0.5 ml of KCl/HCl buffer (1:1 0.2 M KCl:0.2 M HCl, pH 1.4), and stained 30 sec later with 2 ml of a solution containing 6 μg/ml of AO in 0.2 M Na_2HPO_4/0.1 M citric acid buffer at pH 2.6 (Darzynkiewicz *et al.*, 1977).

Fluorescent measurements. Acridine orange-stained cells were passed at a rate of 30–150/sec through the quartz flow cell in a Cytofluorograf II (Ortho Diagnostics,

Inc., Westwood, MA) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW and interfaced to an Ortho 2150 data handler. Green and red fluorescence were measured for each cell and the ratio of red/(red + green fluorescence), known as α_t (Darzynkiewicz *et al.*, 1975), was determined by software protocols written for that purpose. α_t is a measure of the extent of DNA denaturation. The data were based on 3–5 $\times 10^3$ cells analyzed per sample.

Viability Assessment

Viability was assessed by trypan blue exclusion. Aliquots of suspensions containing FL cells or human lymphocytes were mixed with 0.4% trypan blue in a cell medium:stain ratio of 5:1. Cell concentrations and viability were determined with a hemocytometer between 5 and 15 min after staining with trypan blue.

Cell Counting Using Fluorescent Beads

FL cell concentrations were determined by adding 20 μl of a known concentration of "Full-Bright" fluorescent beads (Coulter Corp., Hialeah, FL) to the AO-stained cell suspension. A working suspension of beads was prepared by diluting the stock fluorescent beads 1:5 in HBSS and then admixing 20 μl with the AO-stained cell mixture. The bead concentration was determined using a hemocytometer and the FL cell concentration per ml of culture was determined comparing the recorded fluorescent events due to beads versus the recorded fluorescent events due to FL cells (Stewart and Steinkamp, 1982).

Stathmokinetic (Terminal Point of Drug Action) Procedure

Vinblastine (Sigma Chemical Co.) was added to asynchronous, exponentially growing FL cells, at a concentration of 0.5 μg/ml, to induce an M phase block (Traganos *et al.*, 1980a). Three or four different concentrations of hydralazine were added 1 hr later. Aliquots of cultures were removed at hourly intervals for 8 to 10 hr after the addition of vinblastine; the cells were fixed in ethanol/acetone and later prepared for flow cytometric analysis by AO staining Method II.

RESULTS

Growth and Viability of FL Cells

Figure 1 shows the effects of 20 hr hydralazine exposure on viability of exponentially growing FL cells as determined by trypan blue dye exclusion. Note that the viability remained above 90% for concentrations up to 80 μg/ml and decreased to 77% at a hydralazine concentration of 320 μg/ml. Figure 1 also shows that the critical concentration of hydralazine for inhibition of cell growth over a 20-hr exposure period ranged between 20 and 40 μg/ml.

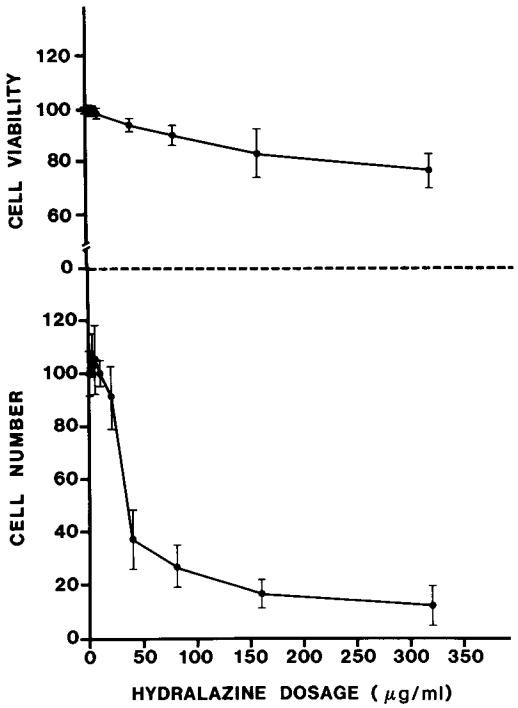

FIG. 1. Effects of hydralazine exposure on the viability and growth (expressed as a percentage of control) of exponentially growing FL cells. Hydralazine concentrations included 0, 2.5, 5, 10, 20, 40, 80, 160, and 320 μg/ml. Each point represents the mean and standard deviation of data from two independent experiments; 500 cells were assayed for viability and 5000 cells and beads for cell concentration.

Terminal Point of Drug Action in FL Cells

Samples from control-vinblastine and hydralazine-vinblastine treated cultures were measured by flow cytometry to compare the

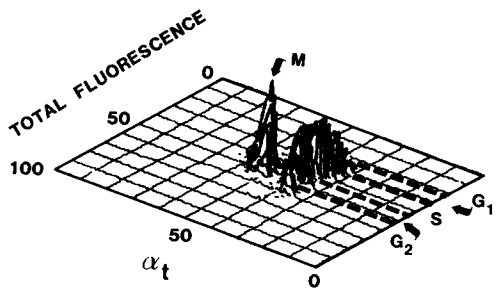

FIG. 2. Isometric display of acid-treated, AO-stained exponentially growing FL cells after a 3-hr vinblastine-induced mitotic phase block. Based on total fluorescence and α_t the cell population has been subdivided into mitotic (M), G_1, S, and G_2 phase subpopulations.

effects of hydralazine on the rate of mitotic cell accumulation with respect to time. Figure 2 shows an isometric display of cell populations obtained by flow cytometric measurements of a control culture exposed to a 3-hr vinblastine block. Cells were subjected to acid denaturation, stained with AO at low pH (Method II), and measured by flow cytometry. Figure 3 shows the effects of varying hydralazine concentrations on the G_1 population kinetics after vinblastine treatment to induce an M phase block. Note that 20 μg/ml caused no significant change in progression through G_1 phase of the cell cycle relative to control. The 40 μg/ml concentration caused a relative increase in the G_1 population and the 80 μg/ml concentration appeared to maintain the same percentage of G_1 cells as that observed at 1 hr. The S phase population, on the other hand, showed a more prominent relative increase in population percentage when 20 μg/ml of hydralazine was present (Fig. 3). In addition, the 40 and 80 μg/ml concentrations maintained the same percentage of S phase cells over the 9 hr measured. Figure 3 also shows the relationship between the percentage of mitotic cells and time after vinblastine addition. The control curve is nearly linear, as would be expected for exponentially growing cells, indicating a time-related accumulation of mitotic cells at the point of the vinblastine block. Exposure to hydralazine (20–80 μg/ml) had no apparent effect on cell progression to mitosis for the first 1.5–2.0 hr after the addition of hydralazine, indicating that cells progressed through the G_2 phase in the presence of hydralazine. At that time a dose-dependent change in the rate of mitotic cell accumulation occurred for all exposure levels, with 80 μg/ml causing a total blockage and 20 and 40 μg/ml doses causing delayed entry into mitosis. Since the delay time is approximately the length of the G_2 period, the evidence suggests that the terminal point of drug action is approximately at the S–G_2 phase transition and that hydralazine affects a block or inhibition of cell progression through S phase. This experiment was repeated three times with nearly identical results. The lower panel in Fig. 3 shows the results for accumulation of mitotic cells in a repeat experiment. Although the cell generation time was slightly longer in this repeat experiment, the percentage decrease in mitotic cell accumulation was very similar.

CHO Cell Colony Formation

Figure 4 shows the effect of 20 hr exposure of hydralazine on stationary phase cells or exponentially growing cells and subsequent colony formation. Each point on the plot represents the colony count expressed as a percentage of control. The viability of exponentially growing CHO cells exposed to hydralazine was concentration dependent. Hydralazine exposure up to 20 μg/ml had no effect on the ability of CHO cells to form colonies; however, colony formation was gradually reduced to 40% as hydralazine concentrations increased to 160 μg/ml. Hydralazine exposure of stationary phase CHO cells (Fig. 4) had no significant effect on subsequent clonability.

Human Peripheral Blood Lymphocytes

Effect of hydralazine exposure on log-phase and stationary (G_0) phase lymphocytes. Since

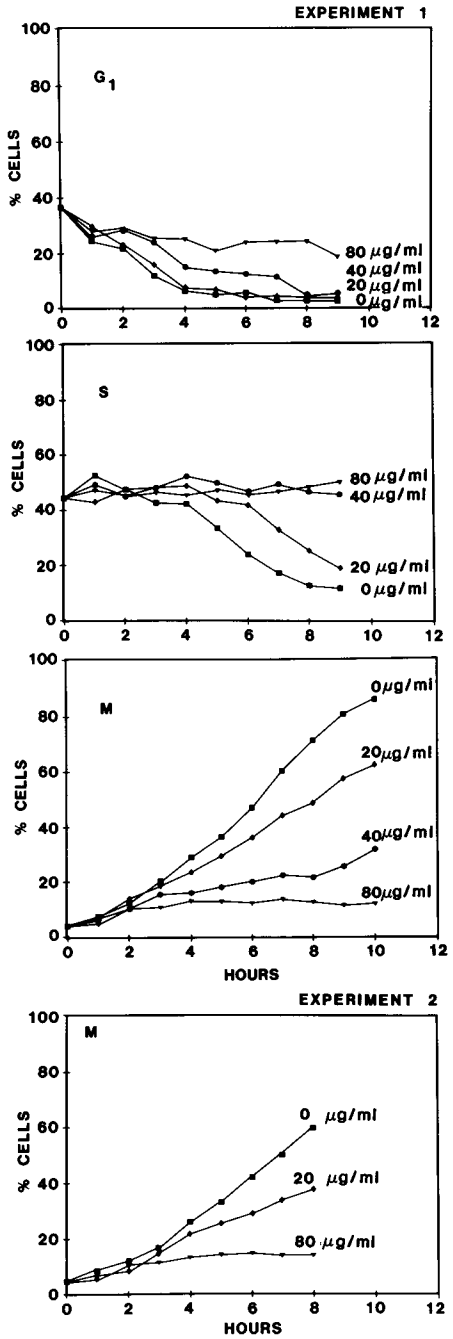

FIG. 3. Effect of 0 (■), 20 (♦), 40 (●), and 80 (▼) μg/ml of hydralazine on percentage of cells in G_1, S, and M phases of exponentially growing FL cells continually exposed to hydralazine and 0.5 μg/ml of vinblastine. The upper three panels represent data from one experiment. The lower panel is derived from an independent experiment showing the effect of hydralazine on the M phase.

our goal is to understand the mechanism of interaction between hydralazine and human lymphocytes, we studied the effect of hydralazine exposure on freshly isolated, cultured lymphocytes. Freshly isolated lymphocytes were treated with 0–80 μg/ml of hydralazine for 24 hr, washed in HBSS, and recultured in the presence of PHA. After 24 hr of PHA stimulation, the viability (as assessed by trypan blue dye exclusion) remained greater than 95% for all hydralazine concentrations (Fig. 5). After 96 hr PHA stimulation, the viability of control cells had dropped to about 80%. There was a gradual reduction in viability down to 62% as the hydralazine concentration increased to 80 μg/ml (Fig. 5). The cell cycle population kinetics were analyzed by flow cytometry at the end of 96 hr of PHA stimulation. Hydralazine exposure of stationary phase (G_0) lymphocytes and subsequent PHA stimulation had no effect on the percentage of cycling cells and the relative distribution of G_1, S, and G_2M phase cells remained constant.

Figure 6 shows the effect of 24 hr hydralazine exposure when added to exponentially growing lymphocytes following 72 hr PHA stimulation. A 5–10% decrease in the percentage of cycling cells occurred with hydralazine exposure at concentrations of 10–20 μg/ml. The variation in distribution of cells in different phases of the cell cycle, again, showed an effect starting at 10–20 μg/ml. Note that the decrease (5–10%) in the percentage of cycling cells is reflected by a corresponding near doubling in the relative percentage of G_0 cells. Also, at 10–20 μg/ml a near doubling in the relative population of S phase cells occurred. This decreased to near control levels as the 40 μg/ml concentration was reached. These data suggest that between 10 and 40 μg/ml hydralazine, there was a progressive slowing through the cell cycle and an accumulation of cells in S phase. This is reflected by a large decrease in G_1 cell population and a slight decrease in G_2M population. Data obtained from measurements made af-

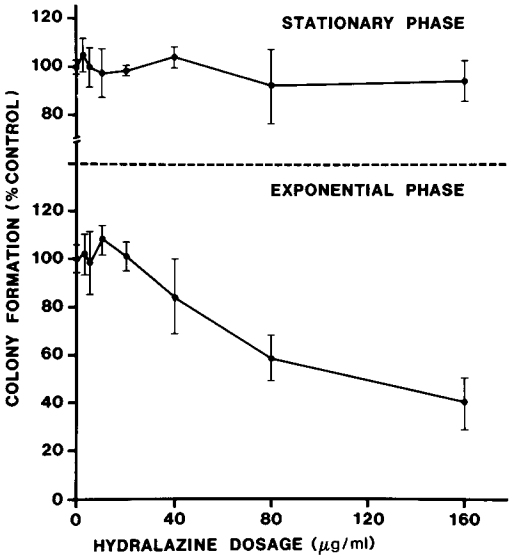

FIG. 4. Effect of 20 hr hydralazine exposure on stationary phase (G_0) and exponential phase CHO cells expressed as a percentage of crystal violet-stained colonies present 7 days after exposure relative to control, nonexposed cultures. Each point represents the mean value of triplicate experiments; the bars represent the standard deviations.

ter 48 hr of hydralazine (not shown) showed the same trends as data obtained after 24 hr.

The same experiment above was repeated using lymphocytes isolated from a normal

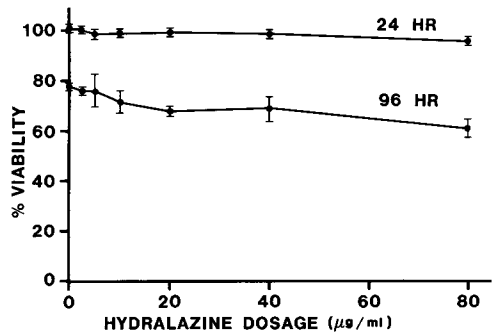

FIG. 5. Effect of pretreatment of human peripheral blood lymphocytes with hydralazine on cell viability following 24 or 96 hr PHA stimulation. Trypan blue dye exclusion was used to assess viability expressed as a percentage of total cells. Hydralazine concentrations included 0, 2.5, 5, 10, 20, 40, and 80 μg/ml.

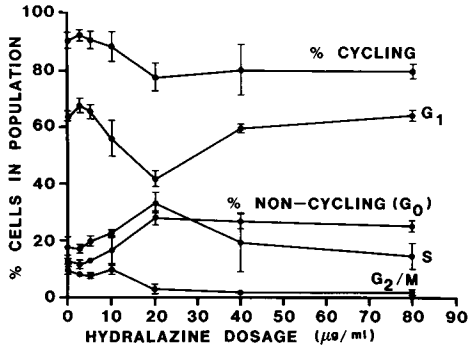

FIG. 6. Effect of 24 hr hydralazine exposure on different phases of the cell cycle when added to human lymphocytes after 72 hr PHA stimulation. Each point represents the mean of relative percentage of cells in each population for triplicate cultures; the bars show the standard deviations.

donor and a patient with idiopathic SLE. The data, not presented here, were essentially the same as those shown in Fig. 6.

Effect of hydralazine on chromatin structure of lymphocytes. Stationary phase (G_0) lymphocytes were treated with hydralazine at concentrations of 0, 2.5, 5, 10, 20, 40, and 80 μg/ml for 24 hr, washed, and fixed in ethanol/acetone for subsequent acid-induced denaturation of DNA *in situ.* Mean red fluorescence (denatured DNA), mean green fluorescence (native DNA), α_t, and the standard deviation of α_t were determined by flow cytometric analysis of AO-stained lymphocytes (Method II). Hydralazine exerted no effect on any of these parameters.

Spontaneously Hypertensive Rats

Body and testicular weights. Hydralazine doses of 0–90 mg/kg/day had no significant effect on body or testicular weights.

Lymphocytes. Twelve weeks of exposure to hydralazine demonstrated no effect on the mean green (DNA content) and mean red (RNA content) fluorescence of rat lymphocytes, stained with AO (Method I) and analyzed by flow cytometry.

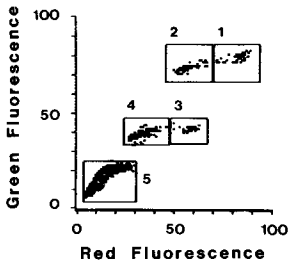

FIG. 7. Cytogram of green versus red fluorescence distribution of AO-stained (Method I) testicular cells isolated from a control rat. Green and red fluorescence values correspond to DNA and RNA stainability, respectively.

Flow cytometric analysis of testicular cells. Figure 7 shows a cytogram of green fluorescence (*Y* axis) versus red fluorescence (*X* axis) of AO-stained (Method I) testicular cells analyzed by flow cytometry. Five clusters of cell types, each boxed off and numbered, are resolved, and correspond to: (1, 2) tetraploid cells; (3, 4) diploid cells; (5) haploid cells. The haploid population can be further resolved into three subpopulations by computer-assisted enhancement of box 5. Green and red fluorescence values correspond to DNA and RNA stainability, respectively (Evenson *et al.,* 1985). Cell counts per box were determined with computer assistance. Hydralazine exerted no effect on the distribution of AO-stained testicular cells.

Flow cytometric analysis of caudal epididymal sperm. Figure 8 shows a cytogram of green (*Y* axis) versus red fluorescence (*X* axis) of normal mature sperm isolated from the caudal epididymis, stained with AO (Method I), and analyzed by flow cytometry. The α_t (red/(red + green) fluorescence) and standard deviation of α_t were determined for the epididymal sperm with computer assistance. Hydralazine exerted no effect on these parameters.

Sonication-resistant spermatids. The number of sonication-resistant sperm heads per gram of testis tissue was calculated. Twelve weeks of hydralazine exposure exerted no effects on sperm head count, indicating that the drug did not inhibit sperm cell production.

DISCUSSION

The effects of hydralazine on the growth, viability, and proliferation potential of mammalian cells were studied in order to elucidate the mechanism of interaction with cellular components that may relate to hydralazine induction of systemic lupus erythematosus.

Hydralazine interacted with cultured cells, causing a perturbation of growth prior to loss of cell viability as determined by trypan blue dye exclusion (Fig. 1). Exposure of G_0 phase CHO cells (Fig. 4) or freshly isolated peripheral blood lymphocytes to hydralazine for 20 hr and at concentrations up to 160 μg/ml had little effect on immediate cell survivability or subsequent cell growth and proliferation as determined by cell colony formation (Fig. 4), cell numbers (Fig. 1), or cell cycle kinetics (Fig. 6). Exposure of rapidly growing cells including Friend leukemia cells (Fig. 1), CHO cells (Fig. 4), or PHA-stimulated human lymphocytes (Fig. 6) caused a marked dose response in reduced cell growth. A stathmokinetic experiment indicated that the terminal point of drug action was near the G_2–S phase transition. Thus, cells at even high exposure levels were able to traverse G_2 phase, but cells were slowed in progression to mitosis in a

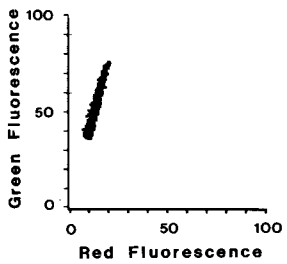

FIG. 8. Cytogram of green versus red fluorescence of AO-stained (Method I) caudal epididymal sperm isolated from a control, nonexposed adult rat.

dose-related manner with exposure to 20 and 40 μg/ml. Exposure at 80 μg/ml resulted in cells not progressing beyond the approximate S–G_2 boundary (Fig. 3). Further analysis of cell cycle kinetics (Fig. 3) indicated that FL cells exposed to 20–40 μg/ml could also traverse G_1 phase but were blocked in G_1 phase by 80 μg/ml. Therefore, hydralazine exerted its effect at concentrations ≤ 40 μg/ml by inhibiting S phase and causing an increased percentage of cells to be blocked in S phase (Figs. 3 and 6). The maximum concentration that resulted in selective S phase block was 20 μg/ml, i.e., a concentration that apparently allowed progression through all phases of the cycle but caused a slowing in S phase.

Inhibition of S phase progression is likely related to previous observations that hydralazine causes DNA damage (Eldredge *et al.,* 1974) and alterations in nuclear proteins (Tan and Portanova, 1981). Thymine bases in DNA are modified by hydralazine at therapeutic concentrations and subsequently excised, the extent of both processes being dependent on the concentration and time of exposure to the drug. Modified DNA can be highly immunogenic (Stollar, 1973); thus, it is possible that the reaction of hydralazine with DNA can lead to a marked enhancement of the immunogenicity of nucleosides (Shapiro, 1967). In addition, such modification of DNA may also result in an alteration of the secondary and tertiary structure of proteins associated with DNA, thereby causing the histones to become immunogenic as seen in hydralazine-induced lupus (Fritzler and Tan, 1978; Tan and Portanova, 1981). Additionally or alternatively, antihistone antibodies could be generated by direct modification of histones via reaction of the hydrazine group of hydralazine at specific peptide bonds of histones.

Considerable evidence in this study with four different *in vivo* or *in vitro* derived cell types showed that hydralazine-exposed cells had stainability of DNA/RNA with the intercalating dye acridine orange equal to that of nonexposed cells. Thus, hydralazine apparently does not compete for AO-binding sites either by intercalation with double-stranded DNA or by association with single-stranded RNA.

This study also addressed the question of whether hydralazine caused changes in chromatin structure as defined by an increased susceptibility to DNA denaturation *in situ.* As seen in the stathmokinetic experiment (Figs. 2 and 3), DNA in untreated cells has a differential sensitivity to denaturation *in situ* related to cell cycle phase (Darzynkiewicz *et al.,* 1977; Traganos *et al.,* 1980a). The most dramatic difference is in the mitotic phase in which the chromatin structure is apparently altered significantly, resulting in increased susceptibility to DNA denaturation. Exposure to drugs may result in increased (Traganos *et al.,* 1980b) or decreased (Darzynkiewicz *et al.,* 1981; Evenson, 1985) susceptibility to DNA denaturation. Recent studies have shown marked changes in susceptibility to DNA denaturation in sperm chromatin in response to chemical exposure (Evenson *et al.,* 1986). Because the primary interest in this study was to determine whether changes in chromatin structure could be detected in hydralazine-exposed cells, the sensitivity of DNA to acid-induced denaturation was measured in G_0 phase and rapidly growing cultured cells and sperm cells from treated rats. Because no differences were found, hydralazine apparently does not interact with histone- or protamine-complexed DNA in a manner that causes a detectable alteration of susceptibility to DNA denaturation *in situ.* Because hydralazine at concentrations more than one log above human therapeutic doses had no effect on testicular germ cell proliferation and differentiation, it is possible that it was a true no-effect phenomenon or due to poor penetration across the blood–testis barrier. However, the molecular size and charge of hydralazine would not suggest a penetration problem across this barrier.

In conclusion, hydralazine caused a dose-related perturbation of DNA synthesis, but at doses ≤ 40 μg/ml it had little effect on progression through G_1 and G_2 phases. Because hydralazine induces the formation of antibodies against DNA and associated proteins, it is very likely that hydralazine causes changes in chromatin structure. However, these changes do not competitively inhibit DNA staining with an intercalating dye nor do they stabilize or destabilize the DNA to acid-induced DNA denaturation *in situ*.

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