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IMMUNOTOXICITY OF 2',3'-DIDEOXYINOSINE IN FEMALE B6C3F1 MICE

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ABSTRACT

2',3'-dideoxyinosine (ddI) is one of several purine analogues used for the treatment of HIV and the acquired immunodeficiency syndrome (AIDS). These nucleoside analogues are promising in their inhibition of viral reverse transcriptase and termination of DNA synthesis. However, each of these drugs has toxicity associated with its use. A previous immunotoxicological evaluation of 2',3'-dideoxyadenosine (ddA), the parent compound of ddI, showed that ddA suppresses humoral immunity. These studies were undertaken to determine the potential for immunotoxicity due to treatment with ddI. This evaluation included an assessment of innate and acquired immunity after exposure to ddI

(100, 250, 500, and 1000 mg/kg/day) for 14, 28 or 180 days. There were no overt signs of toxicity related to treatment with ddI except for a decrease in body weight in the group treated with the highest dose of ddI for 180 days. Overall, 6 months of treatment with ddI showed minimal effects on specific organs with the exception of the spleen and thymus. ddI selectively targets the immune system, with assays that challenge humoral immunity being more affected than those testing cell-mediated immunity. Innate immunity was unaffected by ddI treatment. Cell-mediated immunity, as measured by proliferative response to allogeneic cells (MLR) and the T cell mitogen (Concanavalin A), was moderately suppressed. There were no ddI associated effects on NK function or macrophage function as measured by the vascular clearance rate and phagocytic uptake of the tissue macrophages. The most sensitive indicator of ddI-induced immunotoxicity is suppression of the response to the T-dependent antigen, sheep red blood cells (sRBC). The No Observable Adverse Effect Level (NOAEL) for toxicity to the immune system following 14 days of exposure to ddI is 250 mg/kg. A suppression of the humoral immune response was seen at the lowest dose tested after treatment for 28 and 180 days. Thus, the NOAEL for both of these treatment periods is below 100 mg/kg/day.

INTRODUCTION

ddI is one of several purine and pyrimidine 2',3'-dideoxynucleosides that have been investigated for their antiviral activity against the human immunodeficiency virus (HIV)[1]. ddI is a purine analogue that is active against HIV viruses in cell cultures. It has been proposed that after anabolic phosphorylation ddI is converted to a 2',3'-dideoxyadenosine triphosphate (ddATP), which preferentially inhibits HIV reverse transcriptase. After incorporation of ddAMP residues during chain elongation, the process is terminated[2]. The specificity of ddI is due to its selective inhibition of DNA polymerase β and γ, as well as reverse transcriptase, but not mammalian DNA polymerase α[3].

ddI is presently being used for the treatment of HIV positive individuals and is also used for the treatment of AIDS patients[4]. Previous studies by Luster *et al.*[5] showed that some of the anti-HIV drugs had adverse effects on the immune system by targeting rapidly cycling cell populations. Previous studies have shown that ddA has suppressive action on the humoral response in the mouse and the B lymphocyte is selectively targeted[6]. These subchronic studies were undertaken to determine the potential risks to the immune system caused by ddI.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice (pathogen free) were obtained from Taconic Quality Laboratory Animals, Germantown, NY and housed in animal facilities at Virginia Commonwealth University. Animals arrived at 4 to 6 weeks of age and had body weights of 16 to 20 grams. Mice were housed 5 per cage in filter bonneted plastic cages with sawdust (hardwood) bedding. Mice were maintained on Agway Rat and Mouse rations and tap water *ad libitum* from water bottles. A light/dark cycle of 12 hours, temperature of 72-78°F and a relative humidity of 40-70% were maintained. Mice were identified by tattoo and cage cards marked to identify treatment groups. Mouse cages were cleaned and sanitized three times per week. The mice (sentinels) were also monitored for Sendai, hepatitis and murine pneumonia virus.

Test Article

ddI (M.W. 236.23) was obtained from the Research Triangle Institute and administered twice daily in Maalox, Therapeutic Concentrate (Maalox T.C.). This vehicle allows for the suspension of ddI, as well as an increased bioavailability by the oral route. ddI is unstable in the acidic environment of the stomach and is therefore commonly administered with an antacid to increase the amount of ddI absorbed from the stomach. The concentrations of the solutions were 5.0, 12.5, 25.0, and 50.0 mg/ml. ddI

was prepared weekly and stored at 4°C. The negative controls were sterile distilled water and Maalox T.C. The distilled water was administered to a separate group of mice as a control for the Maalox group to ensure that the Maalox itself was not altering immune function. All values were compared to the Maalox control as this was the vehicle in which ddI was suspended. The doses of ddI administered were equivalent to 100, 250, 500 and 1000 mg/kg/day. The dose was divided (50, 125, 250 and 500 mg/kg) between the morning treatment and again in the afternoon, approximately 6 to 10 hours apart. Cyclophosphamide (CPS) was administered as an assay positive control for suppression of the immune system. Positive control animals received 25 mg/kg cyclophosphamide on the last 4 days of the treatment period by i.p. injection. Injections were administered in a concentration of 0.1 ml/10 grams of body weight. The positive control for the natural killer cell assay was rabbit anti-Asialo GM1 antibody (Wako BioProducts). The positive control animals received 0.2 ml of a 1:10 dilution by intravenous injection 24 hours prior to evaluation of the NK. Methylvinylether copolymer (MVE), provided by Hercules Inc., was used as the positive control for modulation of the functional activity of the reticuloendothelial system (RES). MVE was prepared in phosphate buffered saline at a concentration of 5.0 mg/ml. MVE was given by i.v. injection 24 hours prior to evaluation of the RES.

Experimental Protocol

The test article was administered by gastric intubation using an 18 gauge gavage tube twice a day, seven days a week. Animals received 0.1 ml/10 grams body weight. All animals received the test article for 14, 28 or 180 days. The animals were evaluated for the appropriate immunological and toxicological parameters on day 15, day 29 or day 181 (181 ± 7 days) The variability around the 180 day treatment period provide flexibility in the schedule.

Serum Levels

Levels of ddI were obtained after one day of treatment and after 183 days of treatment. A separate group of mice were used for the one day of treatment. Mice were

gavaged with ddI in two daily divided doses separated by 10-11 hours. One hour after the morning dose, the animals were anesthetized with CO_2, blood was collected by cardiac puncture, and plasma was harvested to be submitted to Research Triangle Park Institute for analysis.

Body Weights

The body weights of the animals were taken on the first day of treatment, bimonthly, and on the day of sacrifice. Animals were observed at the time of dosing for any toxicological signs. Throughout the treatment period, necropsies were conducted on all moribund animals and on all animals that died. On the day of sacrifice, one day after the last exposure to the test article, the animals were weighed, sacrificed by CO_2 anesthesia, and a pathological examination was conducted.

Hematology and Serum Chemistries

Blood was collected by a retro-orbital bleed into EDTA treated tubes. A blood smear was prepared at the time of blood collection, dehydrated with methanol, and stained with Wright-Giemsa. The following parameters were assessed: erythrocyte and leukocyte number, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and reticulocytes. Leukocytes were differentiated into lymphocytes, neutrophils, monocytes and eosinophils. Total differential counts were calculated by multiplying the leukocyte count by the percentage of each cell type.

Serum chemistries were determined. On the day of sacrifice, blood was collected via cardiac puncture into a 1 ml syringe and dispensed into a glass tube for clotting. Serum was harvested after 2 hours of clotting and the following serum chemistries were measured using an Abbott Bichromatic Analyzer (ABA-100): SGPT, BUN, glucose, albumin, globulin and total protein.

Bone Marrow

On day 169, 12 hours after the last drug treatment, bone marrow cells were obtained from mice by washing the medullary cavities of femurs with *alpha*-MEM

medium. Nucleated cell numbers were enumerated with a Coulter counter. The bone marrow cells were adjusted to a concentration of $3x10^6$ cells/ml. A sample was removed to monitor DNA synthesis by assaying the incorporation of ^{3}H-thymidine into $3x10^5$ bone marrow cells over a 3 hour period. An additional aliquot of bone marrow cells was cultured in MethoCult™ M3430 (Stem Cell Techonologies, Inc.) for the colony assay. MethoCult™ M3430 is a methylcellulose medium containing 30% fetal bovine serum, 1% pokeweed mitogen-stimulated murine spleen cell conditioned medium (PWM-SCCM), 1% bovine serum albumin, 0.9% methylcellulose prepared in Alpha MEM, 10^{-4} M 2-mercaptoethanol and 3 units/ml erythropoietin. The PWM-SCCM in this formulation is the source of colony stimulating factors (CSF). A 0.3 ml ($3x10^5$ cells/0.3ml) aliquot of bone marrow cells was mixed with 3 mls of the MethoCult™ M3430. Duplicate cultures (1.1 ml per dish) were plated in 35 mm culture dishes and incubated at 37°C, 5% CO2 and 95% humidity for 14 days. Duplicate cultures were counted for each mouse. CFU-C (CFU-GM + CFU-M + CFU-G) and CFU-E were enumerated in the same dish. Colonies per femur and per 10^5 bone marrow cells were calculated and used as a criterion for comparison between treatment groups.[7]

Lymphocyte Phenotyping

To enumerate B cells, T cells and T cell subsets ($CD4^+$ helper and $CD8^+$ suppressor T-cells), each of the respective cell types was labeled with an appropriate monoclonal antibody, conjugated to a fluorescent probe for visualization. Single cell suspensions were prepared from excised spleens. The cells were collected by centrifugation and suspended in phosphate buffered saline, pH 7.4, containing 1% bovine serum albumin and 0.1% sodium azide to a cell density of $1.5x10^6$/ml. For T cell enumeration, 100 µl anti-mouse CD3-ε monoclonal antibody conjugated to phycoerythrin (PE) was used. For B cell enumeration, 100 µl goat anti-mouse IgG (heavy and light chain specific) conjugated to fluorescein isothiocyanate (FITC) was used. Spleen cells were also incubated with 100 µl anti-mouse CD4 monoclonal antibody conjugated to PE and 100 µl anti-mouse CD8 monoclonal antibody conjugated to FITC. Following a 5 minute

incubation with propidium iodide as a viability stain, the wells were washed once with staining buffer and then enumerated on a Becton Dickinson FACScan Flow Cytometer. Fluorescence was gated on propidium iodide to eliminate dead cells. The threshold for forward light scatter was set to eliminate red blood cells. The values are expressed as the percent of gated (live) cells and the absolute number of cells staining positive for each cell surface marker[8].

Spleen IgM Antibody Response to the T-Dependent Antigen, sRBC

The primary IgM response to sRBC was determined using a modified hemolytic plaque assay[9]. Mice were sensitized with $7.5x10^7$ sRBC by i.v. injection four days prior to sacrifice for each study. Day 4 after antigen is the peak day for the IgM response. One day after the last day of treatment, animals were sacrificed and spleen cells prepared into a single cell suspension in Hanks' balanced salt solution. The IgM response was measured as described previously[10]. The resulting plaques were counted using a Bellco plaque viewer. Cell counts were performed and the number of cells/spleen, AFC/spleen (total spleen activity) and AFC/10^6 spleen cells (specific activity) were determined.

An ELISA assay system was used to determine the serum titers of the primary IgM response to sRBC[11]. The ELISA lends support to the plaque assay in that both measure the primary IgM response to a T-dependent antigen; however, the plaque assay measures the number of antibody forming cells (plasma cells) while the ELISA measures the amount of total antibody secreted.

Spleen Cell Proliferative Response to the Mitogens Con A and LPS

The spleen cell mitogenicity assay is an *in vitro* assay which evaluates the ability of B cells and T cells to undergo blastogenesis and proliferation following stimulation with a cell-type specific mitogen. On day 183, one day after the last exposure, mice were sacrificed and spleen cells prepared. Spleen cells were counted and adjusted to a concentration of $1x10^6$/ml in complete medium (RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin (GIBCO) and 10% fetal bovine serum (Hyclone, Logan, UT). Mitogenicity assays were conducted as described

previously[12]. One hundred µl of spleen cells were added to each well of a microtiter plate, containing four wells of each mitogen at its optimal concentration. The T cell mitogen was concanavalin A (Con A) at a concentration of 2.5 µg/ml and the B cell mitogen was lipopolysaccharide (LPS) at 100 µg/ml. The plates were incubated for 3 days. During the last 18 hours of incubation, 1 µCi ^{3}H-thymidine was present in the culture. Cells were harvested with a PHD Harvester and counted in an LKB liquid scintillation counter. The incorporation of ^{3}H-thymidine into the proliferating cells was used as the endpoint of the assay, and the data are expressed as cpm/culture.

Mixed Leukocyte Response (MLR) to DBA/2 Spleen Cells

The one way mixed leukocyte response (MLR) is an *in vitro* assay which determines the ability of spleen T cells from a treated mouse to recognize allogeneic cells as "non-self" and proliferate in response to the presence of the allogeneic cells. On day 178, mice were sacrificed and spleen cells were prepared into single cell suspensions. The cells were counted and suspended in RPMI 1640 media at a concentration of 1 x 10^6 cells/ml. DBA/2 spleen cells were used as the allogeneic cell (stimulator cell) for the spleen cells (responder cells) from B6C3F1 mice. DBA/2 cells were pretreated with Mitomycin C to render them unable to proliferate. The ratio of stimulator cells to responder cells has previously been optimized at 4:1[10]. The cells were cultured for 5 days, and 1 µCi ^{3}H-thymidine was added to each well for the last 18 hours of incubation. The cells were collected onto filter paper with a PHD Harvester and counted in an LKB liquid scintillation counter. The incorporation of ^{3}H-thymidine into the proliferating cells was used as the assay endpoint and the data are expressed as cpm/culture.

Cytotoxic T Lymphocyte (CTL) Activity

The CTL assay measures differentiation of the T cell to the cytotoxic effector cell. This occurs after the proliferative response takes place, as measured in the mixed leukocyte response. On day 178, mice were sacrificed and a single cell suspension of splenocytes from control and treated mice was washed once with phosphate buffered saline. The cell concentration was adjusted to 4x10^7 total cells in 19.5 ml of sensitization

medium, and the suspension was added to a 25 cm^2 tissue culture vessel. The sensitization medium was Eagle's Minimal Essential Medium (E-MEM, Hazelton, Lenexa, KS) supplemented with 10% fetal bovine serum, 25 mM HEPES, 1 mM l-glutamine, 50 μg/ml gentamicin and 10 μM 2-mercaptoethanol. Sensitizer cells, P815 mastocytoma cells treated with 50 μg of mitomycin C per $2x10^7$ cells, were added to each flask in a volume of 0.5 ml. After 5 days incubation, the cultured spleen cells were harvested, washed once with phosphate buffered saline, and suspended in E-MEM for determination of cytotoxic T cell activity. P815 cells, cultivated in Dulbecco's Modified Minimal Essential Medium with 10% fetal bovine serum, were labeled with ^{51}Cr for 60 min. Radiolabeled P815 target cells ($2x10^4$ cells/well) were co-cultured with splenic effector cells at effector:target ratios from 25:1 to 0.75:1. After 4 hours co-culture, the microtiter culture plates were centrifuged and the supernatant fluid assayed in an LKB gamma spectrometer. Labeled target cells in E-MEM served as the control for spontaneous release of radioactivity, and cells lysed with Triton X-100 provided the maximum release data[8].

Natural Killer (NK) Cell Activity

Natural killer cells are large granular lymphocytes possessing anti-tumor activity and the ability to kill viral infected host cells without prior exposure to the foreign cell. The effects of ddI on natural killer cell activity in the spleen were assessed by the lysis of ^{51}Cr-labeled YAC-1 target cells (ATCC TIB160) using methods described previously[12]. The amount of specific Cr released was calculated as follows: % cytotoxicity = $(cpm_e - cpm_{spon})/(cpm_{total} - cpm_{spon}) \times 100$; where cpm_e = counts per minute in experimental wells, cpm_{spon} = spontaneous release, and cpm_{total} = total release upon addition of 0.05% Triton X-100.

Clearance of Sheep Erythrocytes by the Reticuloendothelial System (RES)

The fixed macrophages of the RES provide the first line of defense for blood borne particulates, including pathogens and non-pathogens. These macrophages line the liver endothelium (Kupffer cells), the spleen, the lymph nodes (reticular cells), the lungs

(interstitial macrophages) and other organs such as the thymus. On day 175, the functional activity of the RES was assessed by determining the vascular clearance of ^{51}Cr-labeled sheep erythrocytes, and the uptake of sRBC into the liver, spleen, lungs and thymus by the fixed macrophages. ^{51}Cr-sRBC were adjusted to a 20% hematocrit which was approximately equal to $5x10^9$ cells/ml. Mice were injected with 0.1 ml/10 grams body weight. The clearance of ^{51}Cr-sRBC from the blood was determined over the first 30 minutes and used to calculate the phagocytic index and determine half-life. After 60 minutes, the animals were sacrificed, and the distribution of the labeled sheep erythrocytes into the major organs of the RES was determined[13]. Uptake into the liver, spleen, lungs, thymus, and kidneys was determined by radioassay and the data expressed as percent uptake in the total organ or per milligram organ weight.

Statistical Analysis

For results from non-mortality studies, the Bartlett's Chi Square Test for homogeneity was used to select the type of analysis to be conducted[14]. Homogeneous data were analyzed using a one-way analysis of variance, and the Dunnett's t Test was used to determine differences between experimental and control groups[15]. For non-homogeneous data, a non-parametric analysis of variance was used[16]. When significant differences occurred, treatment groups were compared to the vehicle control groups (Maalox) using the Wilcoxon Rank Test[17]. The Jonckheere's Test was used to detect trends and provide insight into dose dependency[18]. The Student t Test was used to compare the positive/comparative control group and the vehicle group[19].

For mortality data, survival times were recorded and percent survival calculated. The Fisher's Exact Test was used to determine whether or not a specific group was significantly different statistically from the control group. P values of 0.05 or less were considered statistically significant and were indicated with a single asterisk (*) as compared to the Maalox vehicle control group. A double asterisk (**) was used to indicate a $p < 0.01$ as compared to the Maalox vehicle control group. In the text, the word significant indicates that the response was statistically significant at $p < 0.05$ or less.

RESULTS

Serum Levels of ddI

Levels of ddI were obtained after one day of treatment and at 183 days of treatment. Mice treated with ddI for 183 days had slightly lower levels than mice treated for only 1 day (Figure 1).

Terminal Body and Organ Weights

The changes in body weights over the course of the 180 day studies are shown in Figure 2. Mice treated with lower doses of ddI differed only slightly from the Maalox control group in terms of body weight gain. However, at a dose of 1000 mg/kg/day for 14 days, there was a 41% decrease in body weight gain compared to the Maalox group. After treatment with ddI at all doses for 28 days, there was no change in body weight gain. Overall, naive mice gained an average of 21.8 grams over the 180 day period. Mice treated with water, Maalox or ddI had a similar weight gain as naive mice up to 40 days. Treatment with water slowed the growth rate of mice as compared to naive animals. This effect was seen from day 40 to day 180. The difference in the rate of growth between the naive and the water-treated mice could indicate that the daily gavage treatment had a slight effect on the ability of the mice to grow normally. However, mice gavaged with water, Maalox or ddI at 100, 250, and 500 mg/kg/day did show a similar weight gain over the 180 day period. Mice treated with 1000 mg/kg/day ddI did not gain weight after day 40.

The organ weights of mice exposed to ddI for 180 days are shown in Table I. When the organ weight data are expressed in mg, there were no biologically significant changes in the brain, liver, thymus and kidney of mice treated with 100, 250 or 500 mg/kg/day. The 15% decrease in spleen weight of mice treated with 500 mg/kg/day is biologically significant. At a dose of 1000 mg/kg/day, the liver, spleen and thymus weights were all significantly decreased. However, when the data are expressed as percent of body weight, increases in the weight of the brain, liver and kidney of mice

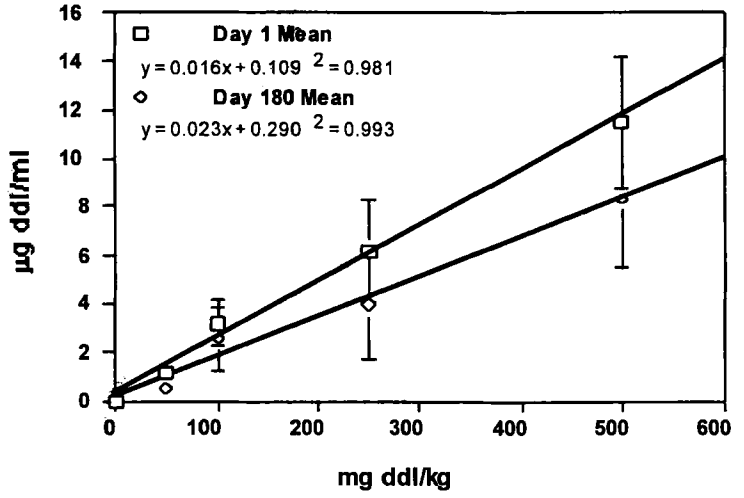

FIGURE 1:

Serum levels of ddI after one day and after 180 days of exposure to ddI.

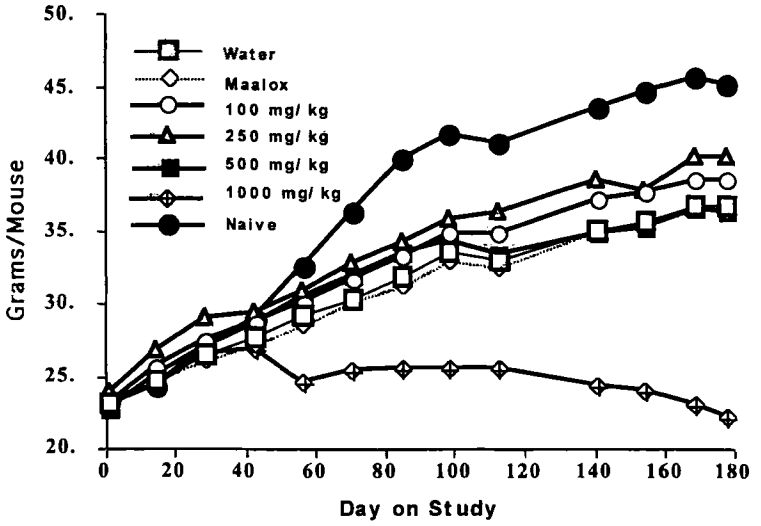

FIGURE 2:

Body weights of female B6C3F1 mice treated with ddI for up to 180 days.

TABLE I

Body Weight (g) and Organ Weights (mg) in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Parameter	Distilled Water (8)	Maalox (10)	2',3-Dideoxyinosine (mg/kg) 100 (10)	250 (10)	500 (10)	1000 (10)	Cyclophosphamide 25 mg/kg (6)	H/ NH	Trend Analysis
Body Weight (g)	36.4 ± 1.5	40.0 ± 1.7	40.4 ± 0.9	41.7 ± 1.0	38.2 ± 1.5	24.0 ± 0.8**	43.7 ± 2.4	H	p < 0.01
Brain (mg)	468 ± 4*	435 ± 11	461 ± 8	455 ± 9	459 ± 8	440 ± 9	400 ± 12	H	NS
% Body Wt	1.30 ± 0.05*	1.11 ± 0.06	1.15 ± 0.03	1.10 ± 0.04	1.22 ± 0.05	1.85 ± 0.06**	0.93 ± 0.05	H	p < 0.01
Liver (mg)	1276 ± 42	1354 ± 48	1371 ± 39	1384 ± 26	1397 ± 51	1070 ± 55**	1490 ± 80	H	p < 0.05
% Body Wt	3.52 ± 0.07	3.40 ± 0.08	3.40 ± 0.07	3.33 ± 0.07	3.66 ± 0.07	4.45 ± 0.12**	3.4 ± 0.2	H	p < 0.01
Organ/Brain Ratio	2.73 ± 0.08*	3.14 ± 0.16	2.98 ± 0.10	3.05 ± 0.08	3.05 ± 0.13	2.43 ± 0.12**	3.73 ± 0.15*	H	p < 0.01
Spleen (mg)	78 ± 3	88 ± 4	85 ± 4	78 ± 2	75 ± 3*	53 ± 3**	63 ± 3**	H	p < 0.01
% Body Wt	0.215 ± 0.006	0.221 ± 0.007	0.209 ± 0.008	0.190 ± 0.008*	0.201 ± 0.008	0.220 ± 0.009	0.145 ± 0.008**	H	NS
Organ/Brain Ratio	0.166 ± 0.007*	0.207 ± 0.013	0.186 ± 0.008	0.173 ± 0.006*	0.166 ± 0.007**	0.121 ± 0.005**	0.160 ± 0.006*	H	p < 0.01
Thymus (mg)	52 ± 5	58 ± 6	63 ± 7	56 ± 6	40 ± 5	21 ± 2**[a]	28 ± 5**	H	p < 0.01
% Body Wt	0.143 ± 0.010	0.146 ± 0.015	0.156 ± 0.016	0.134 ± 0.014	0.106 ± 0.012	0.090 ± 0.011*[a]	0.065 ± 0.012**	H	p < 0.01
Organ/Brain Ratio	0.111 ± 0.011	0.134 ± 0.015	0.136 ± 0.015	0.123 ± 0.012	0.087 ± 0.011*	0.048 ± 0.005**[a]	0.068 ± 0.012**	NH	p < 0.01
Kidney (mg)	355 ± 11*	405 ± 18	435 ± 14	429 ± 10	455 ± 16	365 ± 21	400 ± 14	H	NS
% Body Wt	0.98 ± 0.04	1.03 ± 0.06	1.08 ± 0.03	1.04 ± 0.04	1.20 ± 0.04*	1.52 ± 0.05**	0.93 ± 0.04	H	p < 0.01
Organ/Brain Ratio	0.76 ± 0.02**	0.94 ± 0.05	0.95 ± 0.03	0.94 ± 0.02	0.99 ± 0.04	0.83 ± 0.04	1.00 ± 0.03	H	p < 0.05

Values represent the mean ± SE derived from the number of animals indicated in parentheses.

[a] N = 9

*p < 0.05; **p < 0.01

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

treated with 1000 mg/kg/day can be explained as a calculational response (variable dependent upon the body weight) to the decrease in body weight. The 38% decrease in thymus weight as a percent of total weight in mice treated with 1000 mg/kg/day reflects the drug-induced effects on this lymphoid organ. When the organ weight data are expressed as a function of brain weight, there is a dose-dependent decrease in both spleen and thymus weights emphasizing the selectivity of ddI for the immune system. The decrease in spleen and thymus weights of mice treated with the positive control, CPS, is expected and provides a basis for comparison of ddI. It should be noted that treatment with CPS was for 4 days compared to the 6 months treatment with ddI.

Hematology

Table II shows the effect of ddI on hematological parameters pertaining to erythroid elements. Mice treated with Maalox showed no differences compared to the naive or water-treated groups in the hematological parameters measured, including erythrocyte number, hemoglobin concentration, hematocrit, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), platelets and leukocytes. The mice treated with ddI at doses of 250, 500 and 1000 mg/kg/day showed a decrease in the MCV, MCH and MCHC as compared to the Maalox treatment group. There was no ddI-associated change in the percent of reticulocytes. Table III extends the data on the effect of ddI on myeloid cells. Total leukocyte number was unchanged by 180 day exposure to ddI as was the leukocyte differential. Serum chemistries are shown in Table IV. Mice treated with 1000 mg/kg/day showed significant decreases in BUN (23%), Glucose (26%) and Globulin (26%). There were no significant changes in albumin levels in any of the experimental groups. No biologically significant changes occurred in mice treated with ddI in doses up to and including 500 mg/kg/day.

Bone Marrow

Bone marrow cells were also assessed for their ability to incorporate 3H thymidine, an indicator of ability to synthesize DNA. The data are provided in

TABLE II

Hematology in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Erythrocytes (10^6/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelets (10^5/mm^3)	Leukocytes (10^3/mm^3)
Naive	9.46 ± 0.11	15.7 ± 0.1	47.0 ± 0.5	49.7 ± 0.3	16.56 ± 0.10	33.3 ± 0.1	961 ± 70	3.98 ± 0.63
Distilled Water	9.54 ± 0.11	15.5 ± 0.1	46.5 ± 0.4	48.8 ± 0.2	16.28 ± 0.09	33.4 ± 0.1	1070 ± 20	2.74 ± 0.14
Maalox	9.48 ± 0.08	15.5 ± 0.1	46.6 ± 0.2	49.1 ± 0.3	16.40 ± 0.11	33.4 ± 0.1	1020 ± 42	2.53 ± 0.39
2',3'-Dideoxyinosine								
100 mg/kg	9.48 ± 0.09	15.4 ± 0.1	46.1 ± 0.4	48.7 ± 0.2	16.30 ± 0.10	33.5 ± 0.2	1000 ± 50	4.06 ± 0.90
250 mg/kg	9.24 ± 0.10	14.9 ± 0.2	44.2 ± 0.5**	47.8 ± 0.1**	16.07 ± 0.08*	33.6 ± 0.1	930 ± 67	4.19 ± 0.86
500 mg/kg	9.32 ± 0.08	15.0 ± 0.1	44.7 ± 0.4**	47.9 ± 0.2**	16.16 ± 0.04	33.7 ± 0.1*	938 ± 41	3.14 ± 0.75
1000 mg/kg	10.04 ± 0.10**	15.5 ± 0.2	47.0 ± 0.4	46.8 ± 0.2**	15.48 ± 0.09**	33.1 ± 0.2	925 ± 54	3.88 ± 1.06
Cyclophosphamide								
25 mg/kg	8.86 ± 0.11**	14.8 ± 0.2**	44.1 ± 0.8**	49.8 ± 0.4	16.65 ± 0.11	33.5 ± 0.2	967 ± 133	2.17 ± 0.66
H/NH	H	NH	H	H	H	NH	H	H
Trend Analysis	p < 0.05	NS	NS	p < 0.01	p < 0.01	NS	p < 0.05	NS

Values represent the mean ± SE derived from the number of animals indicated in parentheses.

*p < 0.05; **p < 0.01

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

TABLE III

Leukocyte Cell Differential Blood Counts in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Parameter	Distilled Water (8)	Maalox (10)	2',3'-Dideoxyinosine (mg/kg) 100 (10)	250 (10)	500 (10)	1000 (10)	Cyclophosphamide 25 mg/kg (6)	H/NH	Trend Analysis
Leukocytes (10^3/mm^3)	2.74 ± 0.14	2.53 ± 0.39	4.06 ± 0.90[a]	4.19 ± 0.86	3.14 ± 0.75	3.88 ± 1.06	2.17 ± 0.66	H	NS
% Lymphocytes	88 ± 1	87 ± 2	90 ± 2	86 ± 1	86 ± 1	82 ± 2	87 ± 2	H	$p < 0.01$
% Neutrophils	11 ± 1	12 ± 1	9 ± 1	13 ± 1	12 ± 1	14 ± 1	13 ± 3	H	NS
% Eosinophils	1.38 ± 0.42	1.70 ± 0.82	1.10 ± 0.53	1.40 ± 0.34	2.10 ± 0.69	4.60 ± 1.24	0.17 ± 0.17	NH	NS
				per mm^3					
Lymphocytes	2413 ± 137	2190 ± 339	3581 ± 793[a]	3668 ± 796	2742 ± 689	3105 ± 822	1878 ± 606	H	NS
Neutrophils	287 ± 26	284 ± 44	422 ± 104[a]	469 ± 70	357 ± 70	529 ± 142	287 ± 84	NH	NS
Eosinophils	38 ± 12	56 ± 28	53 ± 24[a]	54 ± 13	41 ± 10	246 ± 118	2 ± 2	NH	NS

Values represent the mean ± SE derived from the number of animals indicated in parentheses.

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

[a]N = 9

TABLE IV

Serum Chemistries in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	SGPT (IU/L)	Albumin (g/dL)	BUN (mg%)	Glucose (mg/dL)	Total Protein (g/dL)	Globulin (g%)	Alb/Glob Ratio
Naive	44 ± 2	4.55 ± 0.13	27.7 ± 0.9	192 ± 5	6.47 ± 0.10	1.9 ± 0.1*	2.5 ± 0.2*
Distilled Water	47 ± 4[a]	4.44 ± 0.16	25.4 ± 0.6**	191 ± 5	6.41 ± 0.06	2.0 ± 0.2	2.6 ± 0.5
Maalox	43 ± 2[b]	4.23 ± 0.16	28.6 ± 0.7	197 ± 6	6.55 ± 0.10	2.3 ± 0.1	1.9 ± 0.1
2',3'-Dideoxyinosine							
100 mg/kg	43 ± 4	4.41 ± 0.06	25.6 ± 1.0	192 ± 4	6.59 ± 0.08	2.2 ± 0.1	2.1 ± 0.1
250 mg/kg	39 ± 2	4.49 ± 0.21	25.6 ± 0.7	198 ± 5	6.52 ± 0.11	2.0 ± 0.2	2.6 ± 0.5
500 mg/kg	35 ± 1[a]	4.23 ± 0.12	26.5 ± 1.3	191 ± 7	6.18 ± 0.08*	2.0 ± 0.1	2.2 ± 0.2
1000 mg/kg	51 ± 7[b]	4.57 ± 0.15	21.9 ± 0.9**	146 ± 3**	6.22 ± 0.09	1.7 ± 0.1**	3.1 ± 0.4
H/NH	NH	NH	H	H	H	H	NH
Trend Analysis	NS	NS	p < 0.01	p < 0.01	p < 0.01	p < 0.01	p < 0.01

Values represent the mean ± SE derived from the number of mice indicated. N = 10 unless otherwise noted and N = 6 for the positive control.

[a]N = 9; [b]N = 8; [c]N = 5

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

SGPT = serum glutamic pyruvic transaminase; BUN = blood urea nitrogen.

Table V. There were no biologically significant changes in the number of cells/femur, number of CFU-E or DNA synthetic ability of the bone marrow cells.

Lymphocyte Phenotyping

The splenocyte surface marker differentials are shown in Table VI. Reduced absolute numbers of both B cells (sIg^+) and T cells ($CD3^+$, $CD4^+$, $CD8^+$) are a reflection of a reduction in the total number of spleen cells. On a percent basis, only B cells (sIg^+) are increased (41%).

Spleen IgM Antibody Response

The spleen IgM antibody responses to the T-dependent antigen, sRBC, following 14 day, 28 day and 180 day exposure to ddI are shown in Tables VII, VIII and IX, respectively. The ability of mice treated with ddI to respond to sRBC is the most sensitive indicator of toxicity by ddI. For the 14 day exposure (Table VII), ddI produced a dose-dependent suppression of the AFC response compared to the Maalox-treated group with a maximum suppression of 27% seen in the IgM AFC/10^6 spleen cells response, 34% when expressed on a per spleen basis, and 32% for the antibody titer (Table X). The no effect level for 14 day exposure is 250 mg/kg/day. CPS, the positive control, produced the expected suppression of the AFC response as well as a decrease in spleen weight and spleen cell number. The AFC/10^6 spleen cells and AFC/spleen responses were suppressed by 99%. There were significant differences in the distilled water and naive controls compared to the Maalox control; however, these values are within our historical range for a normal response. After treatment with ddI for 28 days (Table VIII), there was a dose-dependent suppression of the AFC response with maximum suppression of 91% seen in the IgM AFC/10^6 spleen cells response, 91% when expressed on a per spleen basis and 83% for the antibody titer (Table X). The no effect level for 28 days of exposure is less than 100 mg/kg/day. CPS, the positive control, produced the expected suppression of the AFC response as well as a decrease in spleen weight and spleen cell number. The AFC/10^6 spleen cell and AFC/spleen responses were suppressed by 99%. For the 180 day exposure (Table IX), ddI produced a

TABLE V

Bone Marrow Cell Number, DNA Synthesis, CFU-C and CFU-E in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Cells/Femur ($\times 10^6$)	CFU-C	CFU-C/Femur ($\times 10^3$)	CFU-E	CFU-E/Femur ($\times 10^3$)	DNA Synthesis (CPM $\times 10^3$)
Naive	16.5 ± 0.6	89 ± 5	14.6 ± 0.6	8 ± 1	1.3 ± 0.1	82.2 ± 2.7*
Distilled Water	14.6 ± 1.3[a]	99 ± 5[a]	14.5 ± 1.6[a]	7 ± 1[a]	1.1 ± 0.2[a]	64.0 ± 4.3*[a]
Maalox	15.3 ± 0.4	104 ± 6	15.9 ± 1.1	8 ± 1	1.3 ± 0.1	75.0 ± 1.5
2',3'-Dideoxyinosine						
100 mg/kg	14.4 ± 0.7	102 ± 4	14.7 ± 1.2	9 ± 1	1.2 ± 0.1	73.5 ± 3.2
250 mg/kg	14.5 ± 0.6	95 ± 3	13.7 ± 0.7	8 ± 1	1.2 ± 0.2	78.6 ± 4.7
500 mg/kg	15.0 ± 0.6	85 ± 3**	12.8 ± 0.8	10 ± 1	1.5 ± 0.1	74.7 ± 4.0
1000 mg/kg	16.1 ± 1.1	77 ± 3**	12.3 ± 0.9	10 ± 1	1.6 ± 0.1	67.2 ± 3.4
Cyclophosphamide						
25 mg/kg	9.0 ± 0.4**	163 ± 3**	14.7 ± 0.8	13 ± 1**	1.1 ± 0.1	103.8 ± 3.0**
H/NH	H	H	H	H	H	NH
Trend Analysis	NS	$p < 0.01$	$p < 0.01$	NS	NS	NS

Values represent the mean ± SE derived from the number of animals indicated.

N = 10 unless otherwise noted and N = 6 for the positive control.

[a]N = 8

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

TABLE VI

Splenocyte Surface Marker Differential in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days
(Percent and Absolute Values)

Exposure		Ig^+	$CD3^+$	$CD4^+CD8^-$[a]	$CD4^-CD8^+$[b]	$CD4^+CD8^+$
Naive	percent	54.5 ± 2.3*	24.3 ± 1.5	13.0 ± 0.9**	9.4 ± 0.7	0.64 ± 0.05
	absolute	100.7 ± 5.6	44.8 ± 3.0	23.9 ± 1.7*	17.3 ± 1.4	1.19 ± 0.11
Distilled Water	percent	54.4 ± 1.4*	25.5 ± 1.2*	12.5 ± 0.7**	8.9 ± 0.7	0.79 ± 0.13
	absolute	96.5 ± 5.3	45.0 ± 2.3	21.8 ± 0.9	15.7 ± 1.4	1.45 ± 0.30
Maalox	percent	44.7 ± 3.1	21.2 ± 1.5	9.3 ± 0.8	8.3 ± 1.0	0.76 ± 0.25
	absolute	87.6 ± 4.6	41.9 ± 3.2	18.6 ± 1.6	16.1 ± 1.7	1.41 ± 0.37
2',3'-Dideoxyinosine						
100 mg/kg	percent	44.5 ± 3.2	22.7 ± 1.7	10.9 ± 1.2	8.7 ± 1.0	0.55 ± 0.03
	absolute	100.0 ± 5.0	51.0 ± 2.6	23.8 ± 1.5	19.4 ± 1.7	1.27 ± 0.10
250 mg/kg	percent	43.5 ± 2.1	23.3 ± 1.4	10.0 ± 0.6	10.0 ± 0.9	0.64 ± 0.08
	absolute	96.1 ± 8.4	51.3 ± 4.7	21.9 ± 1.9	22.2 ± 2.7	1.48 ± 0.30
500 mg/kg	percent	46.0 ± 4.6	16.8 ± 2.0	8.3 ± 0.9	7.4 ± 1.2	0.66 ± 0.14
	absolute	71.7 ± 7.5	26.5 ± 3.4**	12.9 ± 1.5*	11.8 ± 1.9	1.00 ± 0.21
1000 mg/kg	percent	63.0 ± 2.8**	16.2 ± 0.8	9.9 ± 0.5	6.5 ± 0.7	0.71 ± 0.08
	absolute	54.0 ± 4.6**	14.0 ± 1.3**	8.6 ± 1.0**	5.7 ± 0.8**	0.59 ± 0.06*
Cyclophosphamide						
25 mg/kg	percent	44.7 ± 2.8	33.4 ± 1.5**	17.5 ± 0.6**	11.0 ± 1.3	0.92 ± 0.13
	absolute	43.2 ± 4.3**	32.6 ± 3.4	17.1 ± 1.5	10.8 ± 1.7	0.90 ± 0.17
H/NH	percent	H	H	H	H	NH
	absolute	H	NH	H	NH	NH
Trend Analysis	percent	$p < 0.01$	$p < 0.01$	NS	$p < 0.05$	NS
	absolute	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values represent the mean ± SE derived from the number of animals indicated.
N = 10 unless otherwise noted and N = 6 for the positive control.
[a]Helper/DTH - T Cell; [b]Suppresser/Cytotoxic T Cell
*$p < 0.05$; **$p < 0.01$
H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.
Absolute = absolute number per spleen x 10^6.

TABLE VII

Spleen Antibody-Forming Cell Response to T-dependent Antigen Sheep Erythrocytes in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 14 Days

Exposure	Body Wt. (g)	Spleen Wt. (mg)	Spleen Cells ($x10^7$)	IgM AFC/ 10^6 Spleen Cells	IgM AFC/Spleen ($x\ 10^3$)
Naive	23.3 ± 0.5	115 ± 3	22.99 ± 0.65	1240 ± 82**	286 ± 21**
Distilled Water	23.4 ± 0.3	108 ± 4	20.92 ± 0.90	1465 ± 98*	305 ± 20**
Maalox	23.2 ± 0.3	114 ± 3	21.80 ± 0.63	1803 ± 93	391 ± 17
2',3'-Dideoxyinosine					
100 mg/kg	23.3 ± 0.4	104 ± 4	20.89 ± 0.77	1561 ± 78	328 ± 22
250 mg/kg	23.1 ± 0.3	112 ± 4	21.59 ± 0.82	1513 ± 116	328 ± 29
500 mg/kg	23.7 ± 0.6	115 ± 5	22.29 ± 0.92	1431 ± 74*	320 ± 22
1000 mg/kg	22.8 ± 0.3	102 ± 3	19.63 ± 0.50	1319 ± 38**	259 ± 11**
Cyclophosphamide					
25 mg/kg	23.4 ± 0.3	91 ± 7**	13.43 ± 0.83**	15 ± 2**	2 ± 0**
H/NH	H	H	H	H	H
Trend Analysis	NS	NS	NS	$p < 0.01$	$p < 0.01$

Values represent the mean ± SE derived from the number of animals indicated. N = 8 unless otherwise noted.
*$p < 0.05$; **$p < 0.01$
H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

TABLE VIII

Spleen Antibody-Forming Cell Response to T-dependent Antigen Sheep Erythrocytes in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 28 Days

Exposure	Body Wt. (g)	Spleen Wt. (mg)	Spleen Cells ($x10^7$)	IgM AFC/ 10^6 Spleen Cells	IgM AFC/Spleen ($x\ 10^3$)
Naive	25.4 ± 0.5	116 ± 5*	20.65 ± 0.84	2436 ± 231	497 ± 40
Distilled Water	24.6 ± 0.5	108 ± 5	21.08 ± 0.68	2599 ± 112	548 ± 29
Maalox	24.4 ± 0.5	99 ± 4	20.57 ± 0.71	2368 ± 69	488 ± 24
2',3'-Dideoxyinosine					
100 mg/kg	24.5 ± 0.6	95 ± 3	19.46 ± 0.98	1346 ± 185**	270 ± 44**
250 mg/kg	25.2 ± 0.6	91 ± 5	19.29 ± 0.79	983 ± 137**	195 ± 33**
500 mg/kg	26.0 ± 0.4	104 ± 5	20.67 ± 0.74	535 ± 75**	113 ± 19**
1000 mg/kg	24.5 ± 0.5	91 ± 4	18.96 ± 1.05	219 ± 29**	43 ± 7**
Cyclophosphamide					
25 mg/kg	24.7 ± 0.4	86 ± 4*	13.61 ± 0.59**	21 ± 4**	3 ± 1**
H/NH	H	H	H	NH	NH
Trend Analysis	NS	NS	NS	$p < 0.01$	$p < 0.01$

Values represent the mean ± SE derived from the number of animals indicated. N = 8 unless otherwise noted.
*$p < 0.05$; **$p < 0.01$
H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

TABLE IX

Spleen Antibody-Forming Cell Response to T-dependent Antigen Sheep Erythrocytes in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Body Wt. (g)	Spleen Wt. (mg)	Spleen Cells ($\times 10^7$)	IgM AFC/ 10^6 Spleen Cells	IgM AFC/Spleen ($\times 10^3$)
Naive	45.1 ± 1.3**[b]	129 ± 9[b]	21.72 ± 1.48[b]	1533 ± 155[b]	338 ± 40[b]
Distilled Water	36.8 ± 1.7	114 ± 4	21.25 ± 1.22	972 ± 69	210 ± 21
Maalox	36.4 ± 1.7[a]	116 ± 3[a]	21.01 ± 0.42[a]	1218 ± 124[a]	258 ± 28[a]
2',3'-Dideoxyinosine					
100 mg/kg	38.5 ± 0.9	117 ± 3	20.30 ± 0.67	688 ± 68**	141 ± 16**
250 mg/kg	40.1 ± 1.5	112 ± 3	19.97 ± 0.65	246 ± 29**	49 ± 6**
500 mg/kg	36.3 ± 1.2	103 ± 5*	16.43 ± 0.94**	156 ± 15**	25 ± 2**
1000 mg/kg	22.0 ± 0.7**[a]	61 ± 2**[a]	9.45 ± 0.46**[a]	158 ± 22**[a]	15 ± 2**[a]
Cyclophosphamide					
25 mg/kg	43.1 ± 1.4*	84 ± 5**	10.45 ± 0.47**	79 ± 23**	9 ± 3**
H/NH	H	NH	H	NH	NH
Trend Analysis	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values represent the mean ± SE derived from the number of animals indicated. N = 10 unless otherwise noted and N = 6 for the positive control.

[a]N = 9; [b]N = 8

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

Table X

Serum IgM Antibody Titers in Female B6C3F1 Mice Treated with 2'3'-Dideoxyinosine Daily for 14, 28 or 180 Days

Exposure	mg/kg	14 Days	28 Days	180 Days
Naive		638 ± 28	185 ± 12	343 ± 41
Distilled Water		598 ± 74	244 ± 15*	263 ± 27
Maalox		619 ± 35	187 ± 17	351 ± 49
ddI	100	614 ± 50	148 ± 19	202 ± 23*
	250	494 ± 46	84 ± 10**	121 ± 18**
	500	463 ± 54	68 ± 10**	56 ± 11**
	1000	423 ± 39*	32 ± 5**	48 ± 16**
CPS		48 ± 5 **	22 ± 2*	114 ± 21**

Values respresent the mean +/- SE. N = 7-10 animals per group.
* = $p < 0.05$; ** = $p < 0.01$
Using Bartlett's Test for homogeneity, data from the 14 day study was homogeneous and data from the 28 and 180 day studies were non-homogeneous.

dose-dependent suppression of the AFC response with a maximum suppression of 94% seen in the IgM AFC/spleen response. At the lowest dose (100 mg/kg/day) tested, the immune response was suppressed by 44% and 45% for AFC/10^6 spleen cells and AFC/spleen respectively. Spleen weight and spleen cell number were also decreased dose dependently with a NOAEL of 250 mg/kg/day. CPS, the positive control, produced the expected suppression of the AFC response as well as a decrease in spleen weight and spleen cell number. The AFC/10^6 spleen cell response was suppressed 94% and 97% by the positive control. The antibody titer data from the 180 day study are shown in Table X. In each of the studies (14, 28 or 180 day), the decrease in serum titer to sRBC is similar to the suppression of the spleen AFC response. Note that there is large interexperiment variability between the control serum titers from 14, 28 and 180 days of treatment. The variability may be related to the preparation of the sRBC membranes used to coat the microtiter plates and capture the antigen-specific antibodies. Alternatively, the variability may be due to the time-related differences in specific activity of the anti-IgM

antibody used in the different experiments. However, comparisons are made within an experiment to determine the effects of treatment on the serum titers.

Spleen Cell Proliferative Response to Mitogens

The proliferative response of lymphocytes after treatment with ddI for 182 days is shown in Table XI. There was no change in the B cell responsiveness to LPS at any dose of ddI, but ddI treatment with the high dose (1000 mg/kg/day) caused a 65% decrease in lymphocyte proliferation in response to Con A. There was a significant decrease in the proliferative response of the mice treated with distilled water; however, the value is still within the limits of our historical data.

Mixed Leukocyte Response to DBA/2 Spleen Cells

On day 178, one day following the last treatment with ddI, the response to allogeneic lymphocytes was assessed in the one-way mixed leukocyte reaction (MLR). The results are presented in Table XII. The MLR was suppressed dose dependently in mice treated with ddI. The NOAEL for the MLR is 250 mg/kg/day. The maximum suppression was 70% in mice treated with 1000 mg/kg/day. There was no suppression in the basal response level of spleen cells in ddI-treated mice as seen in the responder cells only column. The MLR is a T cell proliferative response and the suppression of this response is in concordance with the Con A response. Taken together, the data are consistent with a moderate suppression of T cell proliferation.

Cytotoxic T Lymphocyte Activity

The response to allogeneic lymphocytes for cytotoxic T cell activity was assessed after exposure to ddI for 180 days. As can be seen in Table XIII, exposure to 1000 mg/kg/day ddI produced a significant decrease in the cytotoxic T lymphocyte response at 0.75/1, 1.5/1, 3/1 and 6/1 effector/target ratios. In addition, there were significant decreases in the responses at 1.5/1, 3/1, 6/1 and 25/1 after a dose of 250 mg/kg/day. At an effector/target ratio of 1.5/1 there was also a significant decrease after 500 mg/kg/day.

TABLE XI

Lymphocyte Proliferative Response in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Con A 2.5 µg/ml	LPS 100 µg/ml	Media[a]	Spleen Cells x 10^7
Naive	116454 ± 4839 (8)	84050 ± 2875** (8)	1740 ± 12 (8)	18.48 ± 0.58 (10)
Distilled Water	112603 ± 8756 (8)	78052 ± 4278 (9)	955 ± 60** (9)	17.84 ± 1.01 (10)
Maalox	101666 ± 9928 (6)	66046 ± 3600 (6)	1640 ± 21 (6)	20.40 ± 1.72 (10)
2',3'-Dideoxyinosine				
100 mg/kg	94392 ± 12380 (8)	64236 ± 4235 (8)	1883 ± 181 (8)	23.59 ± 2.24 (10)
250 mg/kg	98760 ± 13583 (9)	59712 ± 2547 (9)	1388 ± 55 (9)	22.06 ± 1.44 (10)
500 mg/kg	62147 ± 9699 (7)	60005 ± 6581 (7)	1350 ± 46 (7)	15.59 ± 0.79* (10)
1000 mg/kg	35922 ± 4621** (6)	64249 ± 3000 (6)	1569 ± 101 (6)	8.54 ± 0.56** (10)
Cyclophosphamide				
25 mg/kg	52441 ± 8434** (6)	33008 ± 7052** (6)	1282 ± 141 (6)	9.74 ± 0.84** (6)
H/NH	H	H	NH	NH
Trend Analysis	$p < 0.01$	NS	NS	$p < 0.01$

Values represent the mean ± SE derived from the number of animals indicated in parentheses.

[a] = Media + 2-ME

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

TABLE XII

Mixed Leukocyte Response in Female B6C3F1 Mice Exposed to 2-3'-Dideoxyinosine Daily for 180 Days

Exposure	Responders	Responders + Stimulators	Total Cells x 10^7
Distilled Water	1650 ± 258	69483 ± 7658	17.66 ± 0.72
Maalox	1754 ± 180	69444 ± 6216	17.54 ± 0.88
2',3'-Dideoxyinosine			
100 mg/kg	1402 ± 175[a]	68149 ± 10817[a]	17.11 ± 0.47[a]
250 mg/kg	1191 ± 140[b]	68770 ± 9827[b]	15.14 ± 0.39[b]
500 mg/kg	1145 ± 133	34424 ± 4863**	13.71 ± 0.34**
1000 mg/kg	1732 ± 534	20729 ± 6000**	9.70 ± 0.53**
Naive	1589 ± 176[b]	45591 ± 6004[b]	17.89 ± 1.54[b]
Cyclophosphamide			
25 mg/kg	1111 ± 122	14337 ± 2591**	10.60 ± 0.75**
H/NH	NH	NH	NH
Trend Analysis	p < 0.05	p < 0.01	p < 0.01

The data are presented as mean ± SE from 4 replicate cultures. Values represent the mean ± SE derived from the number of animals indicated. N = 10 unless otherwise noted and N = 5 for the positive control.

[a]N = 11; [b]N = 9

*p < 0.05; **p < 0.01

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

TABLE XIII

Cytotoxic T Cell Activity in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Effector/Target Ratio						LU/10^7 Cells	LU/ Spleen	Spleen Weight (mg)
	0.75/1	1.5/1	3/1	6/1	12.5/1	25/1			
Naive	36.1 ± 3.7 (7)	52.2 ± 7.0 (7)	66.7 ± 8.3 (7)	77.7 ± 8.3 (7)	82.7 ± 7.2 (7)	87.4 ± 5.2** (7)	128 ± 25 (7)	905 ± 191 (7)	129 ± 9 (8)
Distilled Water	45.5 ± 5.1 (10)	60.6 ± 7.0 (10)	75.8 ± 7.8 (10)	83.2 ± 6.9 (10)	94.9 ± 5.5 (10)	96.1 ± 3.9 (10)	241 ± 58 (10)	1654 ± 375 (10)	114 ± 4 (10)
Maalox	43.4 ± 4.0 (8)	66.2 ± 4.8 (8)	79.5 ± 4.8 (8)	88.8 ± 2.9 (8)	96.0 ± 1.5 (8)	97.8 ± 0.7 (8)	225 ± 37 (8)	1564 ± 257 (8)	116 ± 3 (9)
2',3'-Dideoxyinosine									
100 mg/kg	45.4 ± 2.1 (10)	62.5 ± 2.6 (10)	79.8 ± 1.9 (10)	87.6 ± 1.6 (10)	94.1 ± 1.4 (10)	95.3 ± 1.0 (10)	202 ± 18 (10)	1384 ± 145 (10)	117 ± 3 (10)
250 mg/kg	33.7 ± 2.8 (9)	49.5 ± 3.8* (9)	65.6 ± 3.7* (9)	78.2 ± 3.6* (9)	88.0 ± 1.9 (9)	89.1 ± 1.3** (9)	109 ± 15* (9)	741 ± 115** (9)	112 ± 3 (10)
500 mg/kg	37.3 ± 4.4 (10)	47.3 ± 5.2* (10)	63.6 ± 7.0 (10)	76.6 ± 7.7 (10)	85.9 ± 7.7 (10)	91.8 ± 7.5 (10)	131 ± 30 (10)	674 ± 140** (10)	103 ± 5* (10)
1000 mg/kg	30.2 ± 2.5* (8)	41.3 ± 4.1** (8)	56.8 ± 5.9** (8)	71.8 ± 5.9** (8)	86.2 ± 4.9 (8)	94.6 ± 2.3 (8)	207 ± 132* (8)	263 ± 49** (8)	61 ± 2** (9)
Cyclophosphamide									
25 mg/kg	13.7 ± 1.4** (6)	14.9 ± 1.7** (6)	15.2 ± 2.0** (6)	17.2 ± 2.8** (6)	22.0 ± 4.3** (6)	30.1 ± 6.6** (6)	1 ± 0** (6)	4 ± 1** (6)	84 ± 5** (6)
H/NH	NH	NH	NH	NH	NH	NH	NH	NH	NH
Trend Analysis	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.05$	NS	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values represent the mean ● SE derived from the number of animals indicated in parentheses.

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

Lytic Unit (LU) is defined as the number of splenocytes required to kill 75% of the target cells.

Spontaneous release over the 4-hour incubation period was 13.8% of maximum release.

Natural Killer Cell Activity

On day 178 NK cell activity was assessed. Six ratios of effector-to-target cells were used. This data is shown in Table XIV. Natural Killer cell activity was unaltered in ddI-treated mice when compared to the Maalox control. Although some statistical differences occurred for selected effector/target ratios, overall, no ddI treatment-related effect occurred.

Clearance of Sheep Erythrocytes by the Reticuloendothelial System

The functional activity of the reticuloendothelial system was measured on day 175, one day after the last treatment with ddI, by determining the vascular clearance rate of ^{51}Cr sRBC and the uptake of the sheep erythrocytes into the liver, spleen, lungs and thymus. Kidney localization of sheep erythrocytes was also determined as a representative organ where phagocytosis does not occur. The data are shown in Table XV. RES function, as manifested in vascular clearance rate and phagocytic uptake of ^{51}Cr sRBC in the liver, spleen, lungs and thymus, was not affected by ddI in doses of 100 and 250 mg/kg/day. The positive control, MVE (50 mg/kg), was used as a positive control for suppression of phagocytosis. The mice treated with MVE showed increased half-life of sRBC in the blood and decreased uptake in the organs. Mice treated with 1000 mg/kg/day ddI showed a decrease in vascular clearance rate and decreased hepatic, splenic and thymic uptake of sRBC. The decreased functioning of fixed macrophages in the high dose animals is not due to the size of the animal because the number of sRBC is given on a weight basis. The vascular clearance rate and hepatic uptake of sRBC are lower than that seen for younger animals. For comparison, the RES data on a group of eight week old animals is provided in the last column. Hepatic phagocytosis of the 8 week old mice is higher than that of the control animals for the 180 day study (data not shown). Similarly, spleen uptake in the 8 week old mice is also increased compared to the control groups.

TABLE XIV

Natural Killer Cell Activity in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 Days

Exposure	Effector/Target Ratio						Spleen Weight
	6.25/1	12.5/1	25/1	50/1	100/1	200/1	(mg)
Naive	0.4 ± 0.1**	0.9 ± 0.2*	1.6 ± 0.2*	2.8 ± 0.4**	4.6 ± 0.7	6.7 ± 0.8	102 ± 5*
Distilled Water	0.2 ± 0.1*	0.8 ± 0.2*	1.6 ± 0.2*	2.8 ± 0.4**	4.5 ± 0.7	7.4 ± 1.0	102 ± 6*
Maalox	-0.3 ± 0.1	0.1 ± 0.2	0.4 ± 0.2	0.9 ± 0.3	2.3 ± 0.6	4.3 ± 1.1	132 ± 11
2',3'-Dideoxyinosine							
100 mg/kg	0.1 ± 0.1	0.5 ± 0.1	1.1 ± 0.2	2.1 ± 0.3	3.4 ± 0.5	4.8 ± 0.8	137 ± 12
250 mg/kg	0.7 ± 0.2**	1.1 ± 0.3**	1.9 ± 0.4**	3.2 ± 0.5**	4.7 ± 0.8	6.4 ± 1.0	123 ± 4
500 mg/kg	0.2 ± 0.1*	0.6 ± 0.2	1.2 ± 0.3	2.1 ± 0.5	3.0 ± 0.8	5.7 ± 0.9	94 ± 4**
1000 mg/kg	0.2 ± 0.1*	0.6 ± 0.2	1.1 ± 0.3	1.8 ± 0.4	3.0 ± 0.7	5.4 ± 1.0	53 ± 1**
Anti Asialo GM1							
1:10	-0.2 ± 0.1	-0.1 ± 0.1	-0.1 ± 0.1	0.0 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	114 ± 7
H/NH	H	H	H	H	H	H	NH
Trend Analysis	$p < 0.01$	$p < 0.05$	$p < 0.05$	NS	NS	NS	$p < 0.01$

Values represent the mean ± SE derived from the number of mice indicated. N = 10 unless otherwise noted and N = 6 for the positive control.

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

Spontaneous release over the 4-hour incubation period was 5.4% of maximum release.

TABLE XV

Functional Activity of the Reticuloendothelial System in Female B6C3F1 Mice Treated with 2',3'-Dideoxyinosine Daily for 180 D

Parameter	Naive	Distilled Water	Maalox	2',3-Dideoxyinosine (mg/kg)				MVE	H/
				100	250	500	1000	50 mg/kg	NH
	(10)	(10)	(10)	(10)	(9)	(10)	(10)	(6)	
Half-Life (min)	20.5 ± 2.5	19.8 ± 2.0	18.5 ± 1.1	17.8 ± 1.9	18.6 ± 1.4	18.9 ± 2.1[a]	32.2 ± 8.9[b]	311.6 ± 231.1[b]	N
Body Wgt (g)	43.6 ± 1.2*	34.9 ± 1.5	38.9 ± 1.2	37.4 ± 1.7	38.9 ± 1.0	35.5 ± 1.2	23.9 ± 2.0**	40.0 ± 3.7	H
Liver Wgt (mg)	1187 ± 43	1031 ± 34	1090 ± 28	1058 ± 33	1151 ± 28	1113 ± 27	825 ± 44**	1242 ± 76*	H
% Uptake	24.9 ± 1.6[a]	33.6 ± 2.4	29.9 ± 2.1	29.4 ± 1.8	27.0 ± 2.2	23.3 ± 1.5*	16.5 ± 1.2**	3.7 ± 0.5**	H
cpm/mg	164 ± 11[a]	203 ± 13	189 ± 10	185 ± 9	164 ± 14	132 ± 7**	82 ± 7**	22 ± 3**	H
Spleen Wgt (mg)	91 ± 5*	78 ± 2	77 ± 2	79 ± 2	80 ± 4	71 ± 3	48 ± 4**	82 ± 7	H
% Uptake	16.3 ± 0.4	17.6 ± 0.5	16.0 ± 1.1	15.5 ± 0.7	15.7 ± 1.0	14.3 ± 1.0	9.8 ± 1.2**	6.0 ± 0.7**	H
cpm/mg	1415 ± 36	1404 ± 40	1441 ± 91	1309 ± 72	1378 ± 85	1275 ± 81	869 ± 108**	509 ± 54**	H
Lung Wgt (mg)	196 ± 6*	177 ± 7	177 ± 7	181 ± 8	199 ± 8	190 ± 4	182 ± 6	203 ± 13	H
% Uptake	2.3 ± 0.3	1.8 ± 0.2	2.0 ± 0.3	1.9 ± 0.2	2.2 ± 0.3	2.3 ± 0.2	7.4 ± 1.3	3.6 ± 0.7*	N
cpm/mg	92 ± 10	65 ± 6	82 ± 14	72 ± 10	77 ± 9	76 ± 9	159 ± 28	132 ± 23	N
Thymus Wgt (mg)	58 ± 3	48 ± 3	52 ± 4	54 ± 3	53 ± 3	38 ± 3*	21 ± 5**	42 ± 6	H
% Uptake	0.050 ± 0.008	0.045 ± 0.005	0.049 ± 0.011	0.045 ± 0.005	0.045 ± 0.004	0.042 ± 0.007	0.072 ± 0.014	0.167 ± 0.036**	N
cpm/mg	6.8 ± 1.0	5.7 ± 0.5	6.6 ± 1.1	5.7 ± 0.7	5.9 ± 0.4	7.3 ± 1.1	18.6 ± 3.4	26.6 ± 4.1**	N
Kidney Wgt (mg)	417 ± 9	359 ± 14	394 ± 16	394 ± 9	435 ± 7**	448 ± 7**	347 ± 15	474 ± 22*	N
% Uptake	1.9 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.2 ± 0.2	1.9 ± 0.1	2.9 ± 0.3	1.2 ± 0.2**	N
cpm/mg	35 ± 2	39 ± 2	36 ± 2	34 ± 2	35 ± 3	26 ± 1*	33 ± 3	20 ± 3**	H

Values represent the mean ± SE derived from the number of animals indicated in parentheses.

[a]N = 9; [b]N = 5

*$p < 0.05$; **$p < 0.01$

H = homogeneous data and NH = non-homogeneous data using the Bartlett's Test for homogeneity.

DISCUSSION

ddI is one of the more recent additions to the nucleoside analogues used for the treatment of patients with acquired immunodeficiency syndrome. All of these agents work fairly well at inhibiting reverse transcriptase. However, because of the nature of these drugs and their chronic use, dose-limiting toxicities prevent them from having a higher therapeutic index. AZT, the most widely used of the analogues, causes bone marrow toxicity. 2', 3'-dideoxycytidine (ddC) use is associated with peripheral neuropathy, neutropenia and thrombocytopenia[20]. ddA is associated with nephritis. Therefore, it is not surprising that ddI also produces dose-limiting toxic effects of its own. It has generally been thought that ddI is fairly well tolerated, except for the associated pancreatitis and peripheral neuropathy[21,22].

B6C3F1 mice were chosen as the model for this study because of the overall similarities of the immune system between rodents and humans and because of the large database documenting similar immunotoxicological assays performed with this strain. The doses of ddI used to treat the animals were chosen in an attempt to predict the possibility of any adverse effects in humans. The maximal tolerated dose in humans is 12 mg/kg/day[23], and the average dose administered in a clinical setting is approximately 10 mg/kg/day (5 mg/kg given twice daily) as indicated in the Physicians Desk Reference. The conversion factor for surface area to body weight between mouse and man is approximately 12[24]. Therefore 10 mg/kg in man is approximately 120 mg/kg in mouse. The low dose in this study, 100 mg/kg/day, was escalated to 1000 mg/kg/day in an attempt to safely predict the risk of adverse effects and the type of toxicity that may be seen in such a diverse population as those treated with the drug. The endpoints for the studies were 14 days, 28 days and 180 days. The study was carried out for 6 months in order to mimic the long term treatment that many patients would undergo. Longer exposures were not performed because the variability in the baseline immune parameters

increases as a function of aging. Previous studies by Luster *et al.*[25] carried out the treatment with ddI for 28 days. However, many HIV positive patients may be subjected to treatment with ddI on a long term basis and it is necessary that toxic effects be evaluated after an equivalent treatment period.

The major adverse effect is a decrease in body weight gain over the experimental period. This decrease was significant only in mice administered 500 mg/kg twice daily, i.e. 1000 mg/kg/day. It appears that the animals in the high dose group failed to gain weight after about 40 days of treatment with ddI (Figure 1), yet there was no change in the eating patterns of this group. The decrease in body weight was not associated with major kidney or liver damage. The animals appeared normal in all respects except for the lack of body fat. This decrease in body weight is responsible for the increased brain, liver and kidney weight when the data are expressed as percent of body weight. It is also worth noting the difference in the weight gain between the naive mice and the Maalox control or distilled water control mice (Figure 2). The daily gavaging causes a decrease in body weight gain as compared to mice that were not handled twice daily; however, other than the high dose group, the mice treated with ddI were not different from the Maalox control in body weight gain.

Although there are significant differences in several of the hematological parameters, these slight changes in erythrocyte number, hematocrit, MCV, MCH and MCHC are not reflected in an alteration in the erythroid stem cell and may be related to erythrocyte differentiation. Compensation for the decreased MCV, MCH and MCHC may be seen in the 6% increase in the number of erythrocytes in mice treated with the highest dose of ddI. The lack of a change in percent reticulocytes indicates that a feedback mechanism from a peripheral hemolytic event is not occurring to increase erythrocyte production. The changes in the serum chemistries after treatment with the high dose of ddI (1000 mg/kg/day) most likely are related to the altered metabolism in these animals as seen by the lack of body weight gain. Overall, 6 months of treatment with ddI shows minimal effects on a specific target organ with the exception of the spleen and thymus.

Because the use of 3'-azido-3'-deoxythymidine (AZT) is accompanied by bone marrow toxicity characterized by macrocytic anemia and granulocytopenia[26], there was a compelling interest in the possibility of ddI causing similar toxicity. These studies indicate that ddI does not induce bone marrow toxicity like AZT, but instead alters the ability to mount a normal humoral immune response, a process AZT does not affect after 28 days of exposure. This type of suppression was also observed by Luster *et al.*[25]. Previously, Cao *et al.*[6] demonstrated that ddA, the parent compound of ddI, also inhibits the ability to produce antibodies. They observed that the target of this immunosuppression by ddA is the B lymphocyte. This may also be the case for the toxicity associated with ddI. The total number of T and B cells in the spleen decrease proportional to the decrease in spleen cellularity. ddI selectively targets the immune system, with humoral immunity being more sensitive than cell-mediated immunity. Innate, or natural, immunity includes physical barriers, phagocytic cells and eosinophils, Natural Killer (NK) cells, and other blood-borne molecules which participate in the defense against foreign microbes or macromolecules upon initial exposure, without an ability to distinguish between antigens. Innate immunity was, in essence, unaffected by ddI treatment. The effect on humoral immunity was expressed as an inability to produce IgM antibody to the T-dependent antigen sRBC. The antibody response as measured either by the number of spleen antibody forming cells or antibody titer was decreased by as much as 94%, and the lowest dose tested (100 mg/kg/day) produced a 44% suppression after a treatment period of 180 days. ddI produced a slight suppression of humoral immunity with 14 days of treatment and treatment for 28 days produced up to a 91% decrease in antibody production. Thus, the NOAEL is 250 mg/kg for 14 days of exposure and less than 100 mg/kg for 28 days and 180 days of dosing. Cell mediated immunity as measured by proliferative response to allogeneic cells (MLR) and the T cell mitogen, Con A, was significantly suppressed at 1000 mg/kg/day. It is unlikely that the suppression of T cell response is related to the decrease in antibody response because the antibody response was suppressed at the lowest dose, whereas the ability to proliferate in

response to Con A was only suppressed at the highest dose. There were no major biologically relevant ddI-associated effects on NK function or effects on macrophage function as measured by the vascular clearance rate and phagocytic uptake of the fixed macrophages except at the highest dose. Therefore, the effects of ddI on the immune system are best observed by the decrease in plaque forming ability and in IgM antibody formation as measured by ELISA, with less sensitive effects seen with MLR and the cytotoxic T cell activity.

There are several possibilities for the mechanism of toxicity associated with ddI. One finding that was specific only for ddI was a significant increase in cytoplasmic vacuoles in pancreatic cells after treatment with ddI[27]. These vacuoles contain lysosomal hydrolases and other digestive enzymes that may contribute to the degradation of the pancreas[28,29]. Several studies have also implicated a toxic effect on mitochondria by members of this class of drugs[30,31,32]. The potency of mitochondrial toxicity is in the order of ddC > D4C > D4T > AZT > ddI. The effect on mitochondria is most likely due to the nucleoside analogues' selective inhibition of DNA polymerase γ, as this is the polymerase utilized during mitochondrial DNA (mtDNA) synthesis. In addition, the turnover of mitochondrial DNA is much more rapid than nuclear DNA of non-proliferating cells, thus enabling cells that are not in a highly proliferative state to still be targets for mitochondrial toxicity. This depletion of mitochondrial DNA has been proposed as a cause of the delayed toxicity seen with these compounds, including the peripheral neuropathy which does not appear until after 4-8 weeks of continuous treatment[30]. ddI may target the lymphoid cells while they are in a non-proliferative state, and the effects would not be observed until the cells are called upon to differentiate into effector cells. Because the nucleoside analogues are dependent on the metabolism of cells, it is possible that a toxic effect on mitochondria could be the underlying cause of any of the toxic effects of these drugs, including the immunosuppression seen with ddI. The B lymphocyte might be a specific target then due to its enormous energy requirement during differentiation to a plasma cell and antibody secretion. The patients using ddI are

immunosuppressed; therefore it is especially important that the drugs used for their treatment do not exacerbate their susceptibility to opportunistic infections and other illnesses related to AIDS.

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