TOXICOLOGY AND APPLIED PHARMACOLOGY **145,** 260–267 (1997)
ARTICLE NO. TO978150

2′,3′-Dideoxyinosine Inhibits the Humoral Immune Response in Female B6C3F1 Mice by Targeting the B Lymphocyte

Kathleen E. Phillips* and Albert E. Munson*,†,[1]

Department of Pharmacology and Toxicology, Medical College of Virginia/Virginia Commonwealth University, Box 980613, Richmond, Virginia 23298-0613; and †National Institute for Occupational Safety and Health (NIOSH), 1095 Willowdale Road, Morgantown, West Virginia 26505

Received October 21, 1996; accepted February 25, 1997

2′,3′-Dideoxyinosine Inhibits the Humoral Immune Response in Female B6C3F1 Mice by Targeting the B Lymphocyte. Phillips, K. E., and Munson, A. E. (1997). *Toxicol. Appl. Pharmacol.* **145, 260–267.**

2′,3′-Dideoxyinosine (ddI) is a purine nucleoside analog currently being used for the treatment of HIV-positive individuals and patients with AIDS. Preliminary immunotoxicity studies have shown that a consequence of ddI treatment in female B6C3F1 mice is the inhibition of the humoral immune response. This effect was dose dependent in a range of 100 to 1000 mg/kg with a no observed adverse effect level of less than 100 mg/kg for a 28-day treatment period. These studies were undertaken to investigate the immune cell target of ddI and to determine the mechanism of this toxicity. B6C3F1 mice were treated with 1000 mg/kg/day by oral gavage for 28 days. The B lymphocyte was identified as the cellular target of ddI through separation–reconstitution experiments of the adherent and nonadherent cell populations and of the T and B lymphocyte populations. These studies revealed a deficit in the ability of the nonadherent cells from ddI-treated mice to mount a normal antibody response to sRBC. A further separation of the nonadherent cells into T and B cells revealed a decreased ability of ddI-treated B cells to develop specific humoral immunity. Additional studies were undertaken to determine the mechanism by which ddI is affecting the B cell. Surface marker analysis of splenocytes revealed no difference in the cell populations between vehicle- and ddI-treated mice. B cell proliferation was also unaffected as shown by incubation with either a polyclonal stimulator, lipopolysaccharide, or anti-IgM plus IL-4. These results indicate that the primary cellular target of ddI is the B lymphocyte.

2′,3′-Dideoxyinosine (ddI) is a member of the class of drugs that closely resemble endogenous nucleosides. Several of these nucleoside analogs are currently used for the treatment of HIV-positive individuals and patients with AIDS. The mechanism by which these drugs inhibit viral replication appears to be by competition with the natural substrate for the viral reverse transcriptase (McGowan *et al.*, 1990). The drugs are selective against the virus because the phosphorylated dideoxynucleosides have a higher affinity for the reverse transcriptase and DNA polymerase β and γ than for mammalian DNA polymerase α (Waqar *et al.*, 1984). In addition, the active forms of the drugs compete with normal nucleotides and are incorporated into the growing DNA chain. The lack of a hydroxyl at the 3′ position on the ribose moiety inhibits chain elongation through the 5′ to 3′ phosphodiester linkage (Mitsuya *et al.*, 1987). The differences among the nucleoside analogs lie in their metabolism and toxicity profiles.

Originally, ddI was thought to have a better toxicological profile than other nucleoside analogs. Each has its own dose-limiting toxicity, but the most severe toxicity, as seen with AZT and 2′,3′-dideoxycytidene (ddC), is the inhibition of hematopoiesis in murine and human primary progenitor cells, especially the erythroid burst-forming units and granulocyte-macrophage colony-forming units (Du *et al.*, 1989). Fortunately, ddI was not found to cause bone marrow suppression. However, other dose-limiting toxicities were identified, including pancreatitis and peripheral neuropathy (Yarchoan *et al.*, 1989; Cooley *et al.*, 1990).

The immunosuppression caused by nucleoside analogs was initially examined by Luster *et al.* (1991). In addition, 2′,3′-dideoxyadenosine (ddA), the parent compound of ddI, was specifically found to inhibit humoral immunity in mice exposed to the drug by oral gavage (Cao *et al.*, 1990). Since ddA is metabolized to ddI, these studies warranted a further investigation of ddI in terms of immunotoxicity. A thorough examination of ddI revealed that the most sensitive indicator of immunotoxicity is suppression of humoral immunity as measured by the *in vivo* antibody forming cell (AFC) Response and ELISA quantification of IgM antibody (Phillips *et al.*, in progress). These dideoxynucleosides are able to inhibit selectively the activity of viral reverse transcriptase. However, the drugs also cause toxicity to cells that do not contain this particular enzyme. This implies that they may be toxic by a mechanism other than that by which they inhibit HIV. Therefore these studies were undertaken to identify the

[1] To whom correspondence should be addressed at National Institute for Occupational Safety and Health (NIOSH), 1095 Willowdale Road, Morgantown, WV 26505. Fax: (304) 285-6126.

0041-008X/97 $25.00

immune cell target of ddI and to determine the mechanism by which ddI exerts its toxic effect on this cell. Patients using ddI are already severely 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.

METHODS

Animals. Female B6C3F1 mice (pathogen free) were obtained from Taconic Quality Laboratory Animals (Germantown, NY). Animals arrived at 4 to 6 weeks of age and had body weights of 16 to 20 g. Mice were housed 4 per cage in 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 hr, a temperature between 72 and 78°F, and a relative humidity of 40–70% were maintained. Mice were identified by ear punch and cage cards marked to identify treatment groups. Mouse cages were cleaned and sanitized twice per week. The mice (sentinels) were also monitored for Sendai and murine pneumonia virus.

Chemicals. 2′,3′-Dideoxyinosine (MW 236.23) was obtained from the Research Triangle Institute. ddI was prepared every 2 weeks in Maalox Therapeutic Concentrate. The concentration of the solution was 100 mg/ml. The dose administered was 1000 mg/kg/day. The ddI was stored at 4°C. The vehicle was Maalox Therapeutic Concentrate (Maalox TC) high potency antacid.

Route of administration and duration of exposure. The test article was administered by gastric intubation using an 18-gauge gavage tube once daily. Animals received 0.1 ml/10 g body weight. All animals received the test article for 28 days. The animals were evaluated for the appropriate immunological parameters on Day 29.

In vitro IgM AFC assay. The methods presented here are a modification of the procedure originally developed with sRBC as the T-dependent antigen (Mishell and Dutton, 1967). For *in vitro* studies, spleens from vehicle or ddI-treated mice were aseptically removed 1 day after the last day of ddI exposure. Single cell suspensions were prepared. At this point, separations of the various cell populations were performed as described below. After the appropriate reconstitutions were made with the separated cells, 5×10^6 cells were placed in each well of a 48-well plate. In addition, the same number of sRBC were placed in the wells for a total volume of 0.5 ml per well. Culture plates were placed in metal chambers and the pressure was raised to 6 p.s.i. with a gas mixture of 10% O_2, 7% CO_2, and balanced N_2. Plates were rocked in a 37°C incubator for 5 days. On the day of the assay, 100 μl of cell culture was used for the plaque assay.

In vivo IgM AFC assay. Mice were sensitized with 7.5×10^7 sRBC iv after 25 days of exposure to ddI. On Day 29, 1 day after the last treatment with ddI, the animals were sacrificed and spleen cells were prepared into a single cell suspension in Hanks' balanced salt solution. After 1:30 or 1:120 dilution of the cells, 100 μl of the cell suspension was used for the plaque assay as discussed below.

Measurement of antibody response. The primary IgM response to sheep erythrocytes (sRBC) was enumerated using a modified hemolytic plaque assay (Jerne and Nordin, 1963). An aliquot of spleen cells was added to a 12 × 75-mm test tube containing 25 μl of guinea pig complement, 25 μl of sRBC, and 350 μl warm agar (0.5% agar and 0.05% DEAE dextran). After mixing, the contents of the tube were plated in a petri dish, covered with a microscope coverslip, and incubated for 3 hr at 37°C. The resulting plaques were counted using a Bellco plaque viewer. Cell counts were performed and the number of cells/spleen, AFC/spleen, and AFC/10^6 splenocytes were determined. An ELISA assay system, developed in our laboratory (Temple *et al.*, 1993), was used to determine the serum titers of the primary IgM responses to sRBC. This assay provides support to the hemolytic plaque assay.

Separation–reconstitution studies. Spleen cell suspensions were prepared from either vehicle- or ddI-treated mice. The splenocytes were washed and resuspended in RPMI-complete. The suspensions from each group were divided in order to obtain nonadherent (NAD), adherent (ADH), T and B cells, and $CD4^+$ and $CD8^+$ cell populations (Cao *et al.*, 1990). To obtain the ADH cells, 0.5 ml of 1×10^7 cells/ml was added to a 48-well Costar tissue culture plate that had been incubated (37°C) overnight with 0.3 ml FBS/well. The cells were allowed to adhere for 3 hr. The nonadhering cells were eliminated from the ADH population by washing the wells with prewarmed RPMI. The NAD cells were obtained by running the splenocytes through a column packed with glass wool, glass beads, and Sephadex G-10 beads. The elutant contained the NAD cell population. The NAD cells were either reconstituted over the ADH cells or were further separated into T and B cells. Purified T cells were obtained by negative selection with a B lymphocyte antibody and immunomagnetic beads to remove the B lymphocytes. The B lymphocytes were obtained through treatment with a T lymphocyte antibody (Thy 1.2) and low-tox rabbit complement to negatively select for the B lymphocytes. T and B lymphocytes prepared in these manners are routinely >90% pure as assessed by flow cytometry. The lymphocytes were then reconstituted over naive ADH cells. The $CD4^+$ and $CD8^+$ populations from the groups were obtained using an anti-$CD4^+$ antibody for the $CD8^+$ cells or an anti-$CD8^+$ antibody for the $CD4^+$ cells during the procedure for the negative selection for the T lymphocytes. All reconstituted cultures were sensitized with sRBC at 5×10^6 cells/well and incubated for 5 days at 37°C according to a modification of a procedure developed by Mishell and Dutton (1967). The modified procedure is described by Cao *et al.* (1990). Enumeration of the IgM AFCs was performed as described above for the measurement of antibody response.

Cell population analysis studies. To enumerate B cells, T cells, and T cell subsets ($CD4^+$ helper and $CD8^+$ suppressor T cells), each of the cell types was labeled with an appropriate monoclonal antibody conjugated to a fluorescent probe (Mishell and Shiigi, 1980). The procedure was followed as described by Cao *et al.* (1990). On the day after the last exposure, a spleen cell suspension was obtained and 1×10^6 cells/well were added to a 96-well microtiter plate. The splenocytes were incubated with Fc Block (Pharmingen, cat. No. 01241) for 5 min to prevent nonspecific binding and then stained for 30 min with either fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45R/B220 (Pharmingen, cat. No. 01124), phycoerythrin (PE)-conjugated rat anti-mouse CD3 (Pharmingen, cat. No. 01084), FITC-conjugated rat anti-mouse CD4 (Pharmingen, cat. No. 09004), or PE-conjugated rat anti-mouse CD8 (Pharmingen, cat. No. 01045). Following the staining with antibodies and washing with buffer, 100 μl of a 1:20 dilution of 0.1 mg/ml propidium iodide (PI) was added to each well as a viability stain. After a wash, the viable splenocytes were then enumerated on a Becton Dickinson FACScan Flow Cytometer. The cell subtypes are expressed as a percentage of cells that are positive for a given antibody.

Proliferation studies. The proliferative response to the T cell mitogen, Concanavalin A (Con A), and the B cell stimulators lipopolysaccharide (LPS) or anti-IgM + IL-4 was evaluated. One day after the last exposure to ddI, the mice were sacrificed and individual spleen cell suspensions were prepared. Spleen cells (2×10^5 cells/well) were added to a 96-well flat bottom microtiter plate (Costar). Each concentration of the mitogens was added in triplicate. The concentrations used for each mitogen were as follows: 10, 5, 2, 1, and 0.5 μg/ml Con A (Pharmacia, Piscataway, NJ); 500, 50, and 10 μg/ml LPS (Difco, Detroit, MI); 2 μg/ml anti-IgM (Accurate, Westbury, NY) + 100 U/ml IL-4 (Genzyme, Cambridge, MA); and 0.5 μg/ml anti-IgM + 50 U/ml IL-4. Controls were culture wells treated with RPMI alone. The plates were incubated for 3 days. During the last 18 hr of incubation, 1 μCi [^{3}H]thymidine was present in the culture. Cells were harvested with a PHD cell harvester (Cambridge Technology, Watertown, MA) and counted in an LKB liquid scintillation counter (Pharmacia LKB Nuclear Inc., Gaithersburg, MD). 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.

Statistical analysis. Data analysis was performed using JMP statistical package version 3.0.2 (Copyright 1989-1994, SAS Institute, Inc.). The Dunnett *t* test (Dunnett, 1955) was used to compare ddI-treated groups to the vehicle control group. If significant differences occurred in the Dunnett's test, the Bartlett's test for homogeneity was employed (Bartlett, 1937). Nonhomogenous groups were then compared using the Tukey–Kramer method of analysis (Tukey, 1977). p values of 0.05 or less are considered statistically significant and are indicated with a single asterisk (*) as compared to the vehicle control group. A double asterisk (**) is used to indicate a $p \leqslant 0.01$ as compared to the vehicle group.

RESULTS

Antibody Response

The preliminary immunotoxicology studies showed a decreased ability to mount a normal humoral immune response to sRBC after treatment with ddI for 28 days (Fig. 1). The number of cells able to secrete antibody in response to sRBC is decreased by 43% at the lowest dose tested (100 mg/kg) and decreases dose dependently with higher doses as compared to the Maalox vehicle control group. The suppression is as great as 91% at the 1000 mg/kg/day dose. This effect is also seen in the measurement of IgM antibody production in response to sRBC with an ELISA. The IgM antibody titer is decreased by 81% after treatment with 1000 mg/kg/day ddI for 28 days. These data confirm the results of the plaque forming cell (PFC) assay. The ability to secrete IgG antibody to sRBC was also tested to ensure that the ddI treatment was not simply causing an increase in the amount of isotype switching to IgG (Fig. 2). These data show that there is a suppression in sRBC-IgG in addition to the decreased sRBC-IgM.

Identification of the Immune Cell Target of ddI

A separation of the adherent, nonadherent, T and B cells, and $CD4^+$ and $CD8^+$ cell populations was performed so that subsequent reconstitutions could be done to identify the cells most affected by ddI treatment. A cross-over reconstitution of the nonadherent splenocytes (predominantly T and B lymphocytes) from the ddI-treated group and the adherent splenocytes (predominantly macrophages) from the Maalox vehicle group showed a significantly decreased plaque forming ability as seen in the Mishell–Dutton assay (Fig. 3). The results from the reconstitution of T and B lymphocytes from the two groups are seen in Fig. 4. The cross-over of B lymphocytes from ddI-exposed mice and T lymphocytes from vehicle-exposed mice represents an impaired function of the B lymphocytes after treatment with ddI for 28 days. The reciprocal reconstitution of B lymphocytes from vehicle mice and T lymphocytes from ddI mice did not result in a significant decrease in the number of plaque forming cells, indicating that the T lymphocyte is unaffected by treatment with ddI. As it has been shown that $CD8^+$ T cells can sometimes compensate for deficient $CD4^+$ cells in their ability to function in a humoral immune response to a T-dependent antigen, a separation–reconstitution of the $CD4^+$ and $CD8^+$ cell populations (Fig. 5) was performed (Mossman, 1995). No deficit in the ability of T cells of either type (helper or suppressor/cytotoxic) to function in the immune response to a T-dependent antigen was observed.

Cell Population Analysis

Because a primary immune response to sRBC requires the cooperation of antigen-presenting cells and T and B lymphocytes, cell population analysis was performed to determine if ddI altered the distribution of T or B cells. Figure 6 shows that there was no difference in the percentages of these cell populations in spleens from ddI-treated mice.

Lymphocyte Proliferative Ability

The proliferative response of the lymphocytes from mice treated with ddI (1000 mg/kg) for 28 days was analyzed using cell-specific mitogens: Con A for T cells and LPS or anti-IgM + IL-4 for B cells. Two concentrations were used for each B cell mitogen and five concentrations were used for Con A. The data are represented in Table 1. After treatment with ddI, there was no significant decrease in the ability of either T cells or B cells to proliferate in response to mitogens. There was a slight increase in the incorporation of [^{3}H]thymidine for splenocytes from the ddI-treated group when stimulated with anti-IgM (2 μg/ml) alone (27% increase), anti-IgM (2 μg/ml) + IL-4 (100 U/ml) (20% increase), or Con A (5 μg/ml–19% increase; 10 μg/ml–39% increase). At only one mitogen concentration (50 U/ml IL-4) was there a slight decrease of 9% in [^{3}H]thymidine uptake. Overall, this assay is indicative of the lack of inhibition of proliferation after treatment with ddI.

DISCUSSION

The dideoxynucleosides that are currently in use for treatment of the HIV virus work well to inhibit viral reverse transcription. However, these benefits are also associated with potential for toxicity. As these drugs are designed to target a virus that suppresses the immune system, it is important to study any potentially harmful effects that the drug itself may have on this system. In an immunotoxicological evaluation, ddI was examined for any adverse immune effects and the results indicated that ddI inhibits the humoral immune response in mice (Phillips *et al.*, in progress). In order to begin to understand the mechanism by which ddI suppresses humoral immunity, it was necessary to determine which immune cell is targeted.

In keeping with the previous work done to characterize the toxicity of ddI, the female B6C3F1 mouse was the model for this study. The original reason for conducting the study in mice was the extensive data base concerning

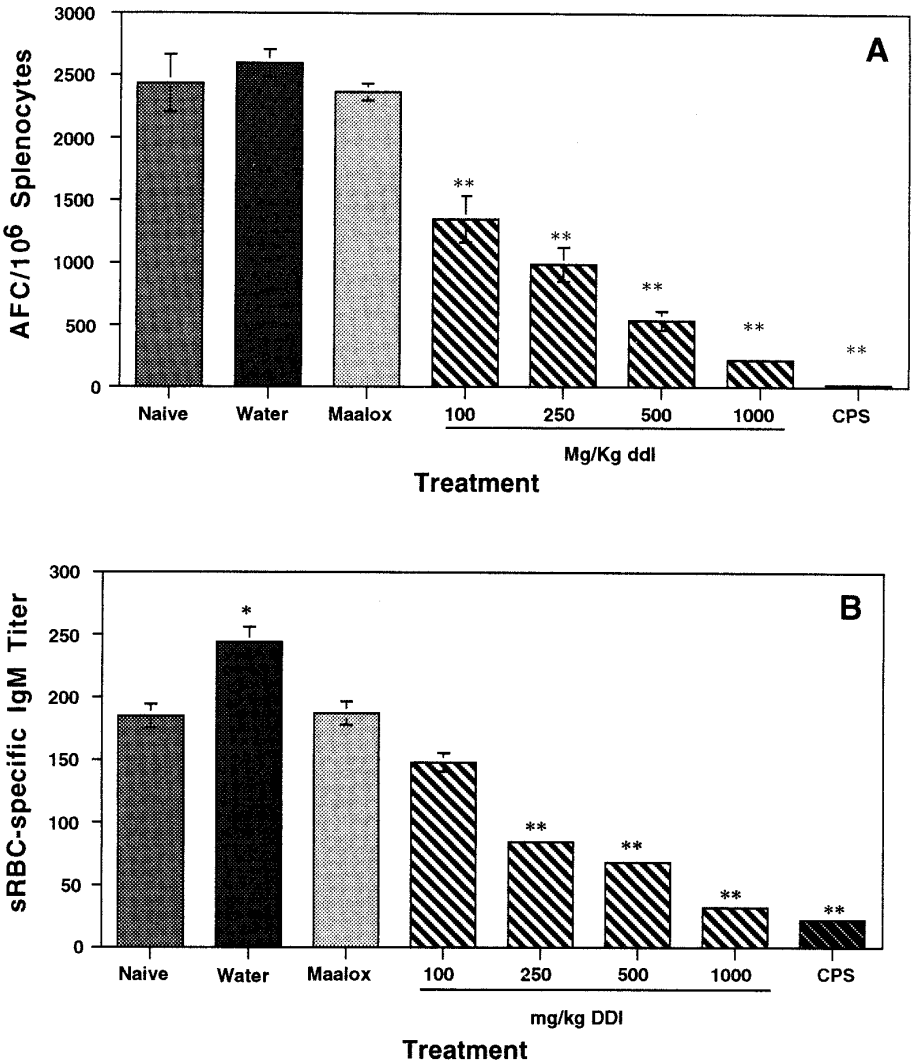

FIG. 1. Dose–response of 28 days of treatment with ddI on the humoral immune response to sheep red blood cells (sRBC), a T-dependent antigen. (A) Antibody forming cell (AFC) response to sRBC after treatment with ddI. Mice were dosed with ddI by oral gavage twice daily for 28 days. The positive control was cyclophosphamide (CPS). Four days prior to sacrifice, mice were sensitized with 7.5×10^7 sRBC by iv injection. Antibody response was measured as described under Materials and Methods. (B) Serum from the same mice in (A) was measured for sRBC-specific IgM by ELISA. Bars in both (A) and (B) represent the mean ± SE of eight mice per group. Values significantly different from the vehicle control (Maalox), $p < 0.05$, are represented by an asterisk. Differences of $p < 0.01$ are expressed with two asterisks.

the overall similarities and differences of the immune system between rodents and humans. The highest dose of ddI from the preliminary studies, 1000 mg/kg/day, was administered for 28 days. Typically, the separation and reconstitution studies require the use of a dose that is at the high end of the dose–response curve. Because several types of cells may contribute to the toxicity, the suppression must be substantial enough to be observable even in the cross-over groups where the degree of suppression may be less than the drug control group. At this time there is no evidence that the suppression seen at this high dose of ddI is caused by a mechanism different than that at the low dose of 100 mg/kg. The doses used are relevant to the amount of ddI administered to human patients, who receive approximately 10 mg/kg/day. Upon conversion based on the difference in surface area to body weight ratios between humans and mice (12), this 10 mg/kg/day is equivalent to 120 mg/kg/day in mice (Voisin *et al.*, 1990). Therefore the doses of ddI that cause suppression of the humoral immune system in mice are pertinent to the administration of ddI to humans. The initial immunotoxicity study included evaluations at various time points (14, 28, and 180 days). After 28 days of dosing with ddI, a suppression of the humoral immune response is seen at all doses.

Previously, Cao *et al.* (1990) demonstrated that ddA,

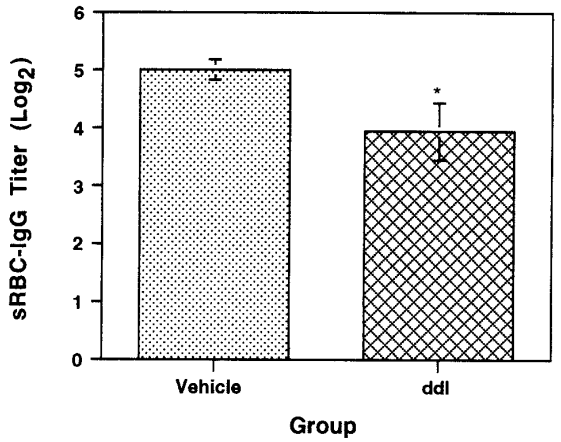

FIG. 2. ELISA measurement of sRBC-specific IgG. Mice were dosed with ddI by oral gavage twice daily for 28 days. Four days prior to sacrifice, mice were sensitized with 7.5×10^7 sRBC by iv injection. Serum was assessed by ELISA for the amount of sRBC-specific IgG produced. Bars represent the mean ± SE of 7 mice per group. Values significantly different from the vehicle control (Maalox), $p < 0.05$, are represented by an asterisk.

the parent compound of ddI, also inhibits the ability of immune cells to produce IgM to the T-dependent antigen, sRBC. It was observed that the target of this immunosuppression by ddA is the B lymphocyte. This is also the case for the toxicity associated with ddI. Separation of the cell populations (nonadherent and adherent, T and B lymphocytes, and $CD4^+$ and $CD8^+$ T cells) and subsequent reconstitution into the Mishell–Dutton assay indicated that the B lymphocytes from mice treated with ddI had an impaired ability to mount a normal immune response to sRBC. From these experiments, it is apparent that adherent cells (mostly macrophages) and T lymphocytes (including $CD4^+$ and $CD8^+$ T cells) are not affected by ddI, and that the toxicity is specific for the B lymphocyte. The total number of T and B cells in the spleen do not decrease after treatment with ddI for 36 days. This suggests that the drug is altering the function of the B lymphocyte, but not the distribution of the cell populations. B lymphocytes, once activated, go through several stages in order to effect their function. They must proliferate and then differentiate into plasma cells. We measured the ability of the lymphocytes to incorporate [^{3}H]-thymidine as a marker of their proliferative capability. However, the ability of these cells to proliferate in response to stimulation with sRBC was not performed. From the studies presented here, it is apparent that the proliferative response to polyclonal stimulators is unaffected by ddI. It would be insightful to examine the splenocytes for their ability to respond to the TD antigen used in the *in vivo* AFC assay, sRBC. At this time, it appears that ddI may be either inhibiting the ability of B cells to differentiate into plasma cells or inhibiting the ability of the plasma cells to secrete their antibodies.

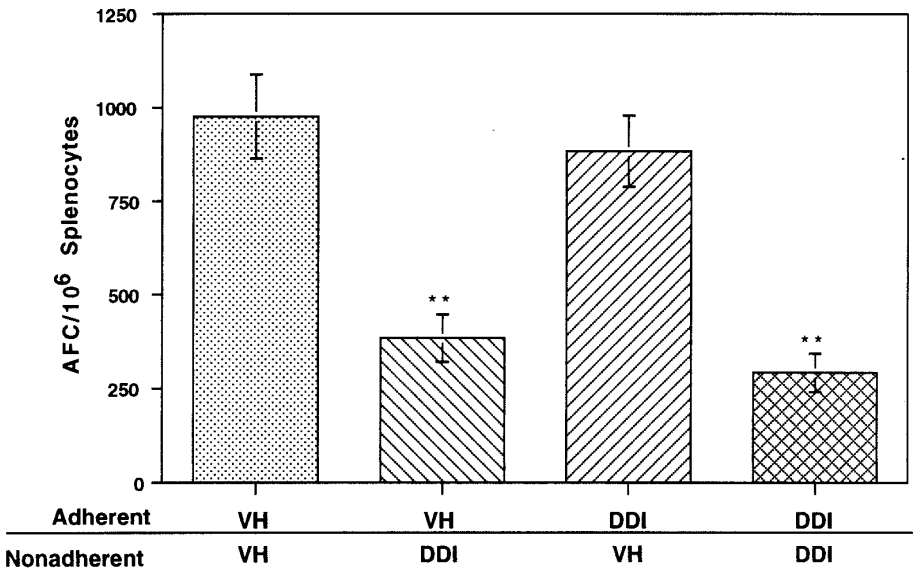

FIG. 3. Splenocytes from ddI-treated and vehicle-treated mice were separated into nonadherent cells (T and B lymphocytes) and adherent cells (mainly macrophages). The cell populations were then recombined into the Mishell–Dutton (*in vitro* AFC Response) assay. Cross-over reconstitutions allow for the identification of the cell population that is causing the suppressed AFC response. The plaques formed are represented as AFC/10^6 splenocytes. Bars are the mean AFC/10^6 splenocytes ± SE of 18 wells per group. Double asterisks represent a significant difference of $p < 0.01$ as compared to the Vehicle Adherent + Vehicle Nonadherent reconstitution.

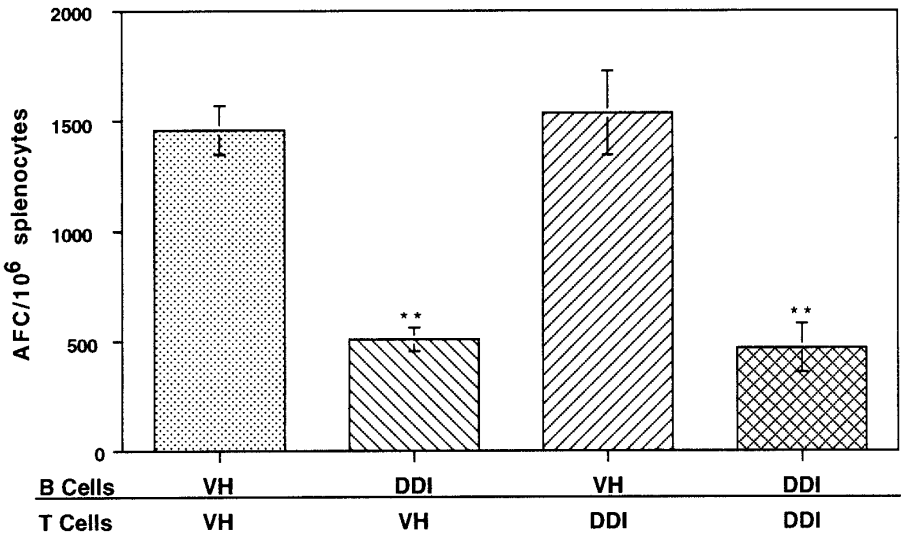

FIG. 4. Nonadherent splenocytes from mice treated with either vehicle (Maalox) or ddI for 28 days were separated into T and B lymphocytes. The T and B cells were reconstituted over naive macrophages in the Mishell–Dutton assay. The plaques formed are represented as AFC/10^6 splenocytes. Bars are the mean AFC/10^6 splenocytes ± SE of 12 wells per group. Double asterisks represent a significant difference of $p < 0.01$ as compared to the Vehicle B + Vehicle T reconstitution.

ddI is one of the more recent additions to the nucleoside analogs used for the treatment of patients with acquired immunodeficiency syndrome. All of these agents work fairly well at inhibiting the activity of reverse transcriptase. However, because of the nature of these drugs and their chronic use, some kind of toxicity prevents them from having a higher therapeutic index. AZT, the most widely used of the analogs, causes bone marrow toxicity. ddC use is associated with peripheral neuropathy, neutropenia, and thrombocytopenia (Yarchoan *et al.*, 1988). 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

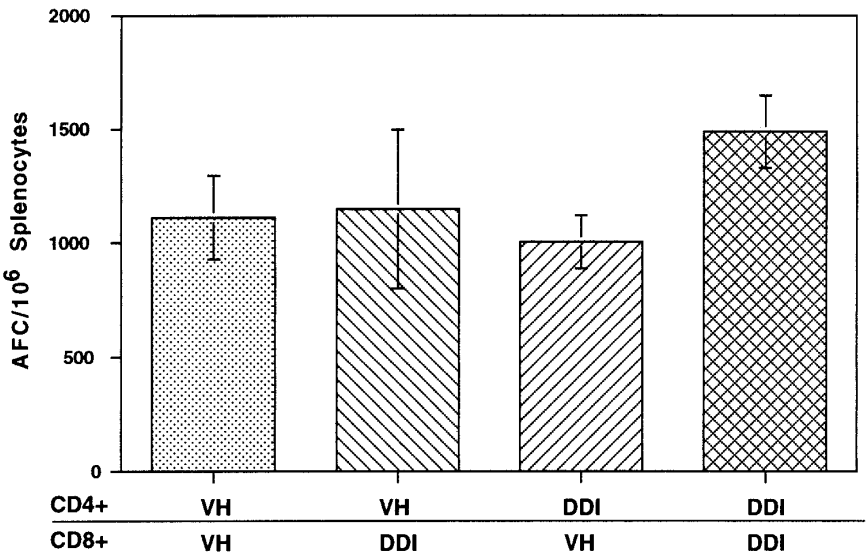

FIG. 5. T lymphocytes from mice treated with either vehicle (Maalox) or ddI for 28 days were separated into $CD4^+$ (T helper) and $CD8^+$ (cytotoxic) T cells. The T_H cells and CTL cells were reconstituted over naive B lymphocytes and macrophages in the Mishell–Dutton assay. The plaques formed are represented as AFC/10^6 splenocytes. Bars are the mean AFC/10^6 splenocytes ± SE of six wells per group. Double asterisks represent a significant difference of $p < 0.01$ as compared to the Vehicle $CD4^+$ + Vehicle $CD8^+$ reconstitution.

peripheral neuropathy (Yarchoan *et al.*, 1989; Cooley *et al.*, 1990). More recently, it has also been reported that these nucleoside analogs all cause varying degrees of mitochondrial toxicity (Chen *et al.*, 1991; Youssef and Badr, 1992; Medina *et al.*, 1994). The effect on mitochondria is most likely due to the nucleoside analogs' selective inhibition of DNA polymerase γ, as this is the polymerase utilized during mtDNA synthesis. In addition, the turnover of mitochondrial DNA is much more rapid than nuclear DNA of nonproliferating 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 (Chen *et al.*, 1991). It is possible that ddI may be targeting the B lymphocytes while they are in a nonproliferative state and the toxicity remains undetected until they are challenged with an antigen such that the B cells must be activated to proliferate and differentiate. Because the nucleoside analogs are dependent on the metabolism of cells, it is very likely 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.

Because the patients using ddI are already severely immunosuppressed, use of a drug that is itself immunosuppressive could exacerbate their susceptibility to infections. ddI has been shown to inhibit the ability to mount a normal humoral immune response to a T-dependent antigen. It is suggested that this inhibition is due to an inability of B lymphocytes to differentiate into plasma cells after exposure to ddI.

ACKNOWLEDGMENT

This work was supported by Grant ES05288 from the National Institute for Environmental Health Sciences.

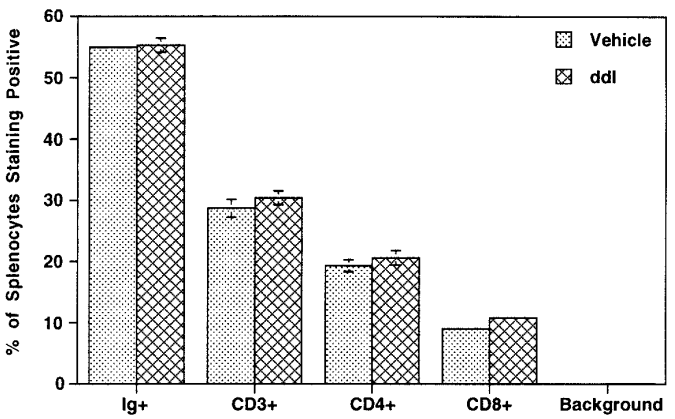

FIG. 6. Splenocytes from mice treated with either vehicle (Maalox) or ddI for 36 days were stained with FITC-conjugated antibodies to Ig (B cells), CD3 (T cells), CD4 (T helpers cells), CD8 (cytotoxic T cells), or staining buffer alone (background). The percentages of splenocytes that stained positive for each of the antibodies are represented by the bars.

TABLE 1

Lymphocyte Proliferative Response in Female B6C3F1 Mice after Exposure to 2′,3′-Dideoxyinosine for 28 Days

Mitogen	Vehicle	2′,3′-Dideoxyinosine
B cell stimulators		
LPS (10 μg/ml)	26,221 ± 457	25,873 ± 291
LPS (50 μg/ml)	27,160 ± 481	27,318 ± 363
Anti-IgM (0.5 μg/ml)[a]	864 ± 45	959 ± 49
Anti-IgM (2 μg/ml)[a]	4,208 ± 247	5,769 ± 324**
IL-4 (50 U/ml)[a]	1,992 ± 52	1,815 ± 58*
IL-4 (100 U/ml)[a]	2,120 ± 49	2,128 ± 42
Anti-IgM (0.5 μg/ml) + IL-4 (50 U/ml)[a]	3,300 ± 231	2,674 ± 303
Anti-IgM (2 μg/ml) + IL-4 (100 U/ml)[a]	13,121 ± 428	16,310 ± 278**
T cell stimulators		
Con A (0.5 μg/ml)	34,277 ± 1250	32,488 ± 1401
Con A (1 μg/ml)	42,921 ± 1071	41,007 ± 1047
Con A (2 μg/ml)	40,943 ± 1001	42,311 ± 867
Con A (5 μg/ml)	23,112 ± 2246	28,703 ± 1769*
Con A (10 μg/ml)	1,652 ± 273	2,687 ± 440*

Note. Spleen cells (2×10^5 cells/well) were stimulated with mitogens for 3 days. [^{3}H]thymidine (1 μCi) was present in the wells for the last 18 hr of incubation. $N = 16$ mice per group (3 replicates of each).

[a] $N = 15$ mice per group (three replicates of each) for anti-IgM + IL-4 study.

* $p < 0.05$.

** $p < 0.01$.

REFERENCES

Bartlett, M. S. (1937). Sub-sampling for attributes. *J. R. Stat. Soc. Suppl.* **4,** 131–135.

Cao, W., Sikorski, E. E., Fuchs, B. A., Stern, M. L., Luster, M. I., and Munson, A. E. (1990). The B lymphocyte is the immune cell target for 2′,3′-dideoxyadenosine. *Toxicol. Appl. Pharmacol.* **105,** 492–502.

Chen, C., Vazquez-Padua, M., and Cheng, Y. (1991). Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. *Mol. Pharmacol.* **39,** 625–628.

Cooley, T. P., Kunches, L. M., Saunders, C. A., Ritter, J. K., Perkins, C. J., McLaren, C., McCafferey, R. P., and Liebman, H. A. (1990). Once-daily administration of 2′,3′-dideoxyinosine (ddI) in patients with the acquired immunodeficiency syndrome or AIDS-related complex. *N. Engl. J. Med.* **322,** 1340–1345.

Du, D. L., Volpe, D. A., Murphy, M. J., Jr., and Grieshaber, C. K. (1989). Comparative myelotoxic effects of two anti-HIV agents on human and murine hematopoietic progenitor cells [abstract 2411]. *Proc. Am. Assoc. Canc. Res.* **30,** 606. [Abstract]

Dunnett, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50,** 1096–1121.

Jerne, N. K., Nordin, A. A., and Henry, C. (1963). The agar plaque technique for recognizing antibody producing cells. In *Cell-Bound Antibody,* pp. 109–120. Wistar Institute Press, Philadelphia.

Luster, M. I., Rosenthal, G. J., Cao, W., Thompson, M. B., Munson, A. E., Prejean, J. D., Shopp, G., Fuchs, B. A., Germolec, D. R., and Tomaszew-

ski, J. E. (1991). Experimental studies of the hematologic and immune system toxicity of nucleoside derivatives used against HIV infection. *Int. J. Immunopharmacol.* **13**(Suppl. 1), 99–107.

McGowan, J. J., Tomaszewski, J. E., Cradock, J., Hoth, D., Grieshaber, C. K., Broder, S., and Mitsuya, H. (1990). Overview of the preclinical development of an antiretroviral drug, 2′,3′-dideoxyinosine. *Rev. Inf. Dis.* **12**(Suppl. 5), S513–S521.

Medina, D. J., Tsai, C., Hsiung, G. D., and Cheng, Y. (1994). Comparison of mitochondrial morphology, mitochondrial DNA content, and cell viability in cultured cells treated with three anti-human immunodeficiency virus dideoxynucleosides. *Antimicrob. Agents Chem.* **38**(8), 1824–1828.

Mishell, B. B., and Shiigi, S. M. (1980). *Selected Methods in Cellular Immunology.* Freeman, San Francisco.

Mishell, R. I., and Dutton, R. W. (1967). Immunization of dissociated mouse spleen cell culture from normal mice. *J. Exp. Med.* **126,** 423–442.

Mitsuya, H., Jarrett, R. F., Matsukura, M., Veronese, F. D., DeVico, A. L., Sarngadharan, M. G., Johns, D. G., Reitz, M. S., and Broder, S. (1987). Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides *in vitro. Proc. Natl. Acad. Sci. USA* **84,** 2033–2037.

Mossman, T. R. (1995). Cytokines, differentiation and functions of subsets of CD4 and CD8 T cells. *Behring Inst. Mitt.* **96,** 1–6.

Phillips, K. E., McCay, J. A., Brown, R. D., Musgrove, D. L., Meade, B. J., Butterworth, L. F., Wilson, S., Luster, M. I., White, K. L., Jr., and Munson, A. E. (1996). Immunotoxicity of 2′,3′-dideoxyinosine in female B6C3F1 mice. Submitted for publication.

Temple, L., Kawabata, T. T., Munson, A. E., and White, K. L., Jr. (1993). Comparison of ELISA and plaque-forming cell assay for measuring the humoral immune response to sRBC in animals treated with benzo[*a*]-pyrene or cyclophosphamide. *Fundam. Appl. Toxicol.* **21,** 412–419.

Tukey, J. W. (1977). *Exploratory Data Analysis.* Addison-Wesley, Reading, PA.

Voisin, E. M., Ruthsatz, M., Collins, J. M., and Hoyle, P. C. (1990). Extrapolation of animal toxicity to humans: Interspecies comparisons in drug development. *Reg. Toxicol. Pharmacol.* **12,** 107–116.

Waqar, M. A., Evans, M. J., Manly, K. F., Hughes, R. G., and Huberman, J. A. (1984). Effects of 2′,3′-dideoxynucleosides on mammalian cells and viruses. *J. Cell. Physiol.* **121,** 402–408.

Yarchoan, R., Mitsuya, H., Thomas, R. V., Pluda, J. M., Hartman, N. R., Perno, C. F., Marczyk, K. S., Allain, J. P., Johns, D. G., and Broder, S. (1989). In vivo activity against HIV and favorable toxicity profile of 2′,3′-dideoxyinosine. *Science* **245,** 412–415.

Yarchoan, R., Thomas, R., Allen, J. P., McAtee, N., Dubinsky, R., Mitsuya, H., Lawley, T., Safai, B., Myers, C., Perno, C., Klecker, R., Wills, R., Fischl, M., McNeely, M., Pluda, J., Leuther, M., Collins, J., and Broder, S. (1988). Phase I studies of 2′,3′-dideoxycytidine in sever human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). *Lancet* **1,** 76–81.

Youssef, J. A., and Mostafa, M. Z. (1992). Disruption of mitochondrial energetics and DNA synthesis by the anti-AIDS drug dideoxyinosine. *Toxicol. Lett.* **60,** 197–202.