The Ochsner Journal 15:241–247, 2015

Hydroxyurea and Zileuton Differentially Modulate Cell Proliferation and Interleukin-2 Secretion by Murine Spleen Cells: Possible Implication on the Immune Function and Risk of Pain Crisis in Patients with Sickle Cell Disease

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Background: Hydroxyurea (HU) reduces major complications associated with sickle cell disease in part because of the induction of fetal hemoglobin. However, because of its antiproliferative property, its long-term use may impair immunity. Zileuton, a derivative of HU, also induces fetal hemoglobin and has antiinflammatory properties, a feature that can reduce the risk of sickling. Our goal was to investigate the capacity of both drugs to modulate the secretion of interleukin-2 (IL-2), a regulatory cytokine for immune responses.

Methods: Spleen cells obtained from 11 4-month-old C57BL/6 female mice were incubated without and with 10 μg/mL HU or zileuton, 2.5 μg/mL concanavalin A (ConA), 20 μg/mL phytohemagglutinin (PHA), and 50 ng/mL anti-CD3 antibody for 12-48 h. IL-2 was measured in the supernatant by enzyme-linked immunosorbent assay and cell proliferation by ^{3}H-thymidine uptake.

Results: While HU reduced lymphocyte proliferation in response to mitogens ($P<0.05$), zileuton did not. Baseline IL-2 concentration and PHA-induced IL-2 were not significantly affected by either drug. Contrary to what we expected, while HU increased IL-2 supernatant levels 1.17-fold to 6.5-fold in anti-CD3 antibody–treated cells ($P<0.05$), zileuton decreased them 35%-65% ($P<0.05$). Zileuton likely reduced IL-2 levels by inhibiting 5-lipoxygenase, hence leukotriene B4 production, an IL-2 inducer. HU did not decrease IL-2 secretion likely because of its lack of effect on mRNA and protein synthesis.

Conclusion: Modulation of IL-2 secretion by zileuton and/or reduced lymphocyte proliferation by HU may impair the immune response of patients with sickle cell disease but may also be beneficial by attenuating inflammation independently of fetal hemoglobin induction.

Keywords: *Anemia–sickle cell, hydroxyurea, interleukin-2, mice, zileuton*

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INTRODUCTION

Sickle cell anemia or sickle cell disease (SCD) is the most common genetic disorder diagnosed in individuals of African ancestry and a subgroup of people from India, the Mediterranean region, and the Middle East.[1,2] The disease is caused by a point mutation (GTG→GAG) in the gene that codes the β-globin chain of hemoglobin, leading to a substitution of glutamic acid by valine at the sixth position. This simple single amino acid change causes hemoglobin to polymerize upon loss of oxygen in the tissue, leading to distortion in the shape of red blood cells (RBCs). The disease is characterized by frequent episodes of vaso-occlusive pain crisis because of increased adhesion of the sickle-shaped RBCs along with white blood cells and platelets to endothelial cells.[1] Other complications include RBC hemolysis, a defective spleen, impaired cell-mediated and natural immunity, increased risk of pneumococcal infection, acute chest syndrome, physical growth retardation in children, and multiorgan failure (bones, lungs, brain, and kidneys).[2-4] Disease severity is variable and depends in part on fetal hemoglobin levels.[5] SCD management often involves frequent blood transfusions that unfortunately induce iron overload and penicillin prophylaxis.[6]

In the early 1990s, hydroxyurea (HU), a drug often used to treat chronic myelogenous leukemia,[7] was shown to reduce the frequency of pain crises and associated complications in patients with SDC in part because of the induction of fetal hemoglobin through nitric oxide production.[8-11] Specifically, patients treated with HU required less frequent hospitalization because of vasoocclusive pain crises and fewer blood transfusions.[9] They also had fewer episodes of new and recurrent neurologic disorders, lower white blood cell counts, fewer cell adhesion molecules, and lower mortality.[9] However, as an antiproliferative agent, HU may induce neutropenia, anemia, and thrombocytopenia because of bone marrow suppression, delayed wound healing, diarrhea, and growth retardation, side effects that are undesirable, especially in young children.[8] The mechanism of HU's antiproliferative property relies on its capacity to inactivate ribonucleotide reductase by binding to subunit B2 of the enzyme.[12] Ribonucleotide reductase is a key enzyme in the pathway of DNA synthesis and cell proliferation because it is required for the biosynthesis of deoxyribonucleotides from ribonucleosides.[12] Its long-term use may also impair immune responses because of the rapid proliferation of the immune cells, an effect that can further compromise the immune response of patients with SCD and potentially increase the risk of infection.[13,14] In fact, a 2014 study conducted by Lederman et al suggested that HU in children with SCD decreased the absolute number of leukocytes, CD4+ cells, and memory CD4+ cells and delayed the production of protective levels of measles antibodies following vaccination.[15] Moreover, HU has also been linked to leukemia and mutations such as those with 17p deletion and may induce male infertility.[16-18]

Zileuton (sold as Zyflo), an HU derivative antiinflammatory drug used for the treatment of asthma, induces fetal hemoglobin synthesis as efficiently as HU.[19-21] Zileuton's mechanism of action in asthma relies on its capacity to inhibit 5-lipoxygenase (in various cell types, specifically macrophages, neutrophils, and eosinophils), the key enzyme that leads to the production of leukotriene A4 and subsequently leukotriene B4 (LTB4), a mediator of inflammation.[19,20] LTB4 is an inducer of interleukin-2 (IL-2) and therefore modulates lymphocyte proliferation in response to mitogens.[22] As with any drug, zileuton also induces certain side effects including nausea, skin rash, diarrhea, insomnia, and elevated liver transaminases but has not been shown to decrease total white blood cell counts (it does not induce bone marrow suppression).[20]

The structures of HU, zileuton, and the parent compound urea are shown in Figure 1. Based on the charts, HU is nothing more than a urea molecule with a hydroxyl (OH) group attached to one of the nitrogens. Zileuton differs from HU by having a molecule N-(1-Benzo[b]thien-2-ylethyl) with 2 rings and a sulfur molecule attached to the same nitrogen as OH. Therefore, it is also called N-(1-Benzo[b]thien-2-ylethyl)-1-hydroxyurea. The hydroxyl group in HU is very likely responsible for its inhibitory effect (inactivation of the subunits) of ribonucleotide reductase, whereas the N-(1-Benzo[b]thien-2-ylethyl) group is likely responsible for zileuton's effect on 5-lipoxygenase.

IL-2, a cytokine secreted by activated CD4+ T lymphocytes, naive CD8+ cells, and dendritic cells, plays crucial roles in several immune functions, including regulation of delayed-type hypersensitivity and cell proliferation, activation of CD8+ T cells and natural killer cells, and induction of other cytokines such as interferon-gamma (IFN-γ).[23,24] Moreover, IL-2 induces the differentiation of monocytes to dendritic cells, the most potent antigen-presenting cells, and is a source of proinflammatory cytokines including IL-1β, tumor necrosis factor-alpha (TNF-α), and IL-12.[25] Reduced IL-2 secretion may therefore impair the immune response by various mechanisms and increase the risk of certain types of infections.

Although HU and zileuton induce fetal hemoglobin synthesis, little is known about their possible differential effect on IL-2 secretion. This question is important in the management of SCD—specifically in children—because of the role of IL-2 in modulating immune function, including the production of inflammatory cytokines such as TNF-α. Although moderate inflammation is required for pathogen killing, chronic inflammation is a risk factor for many chronic diseases, and inflammation appears to either trigger or worsen pain crises.[26,27] Moreover, infections are common in children with SCD, and infections contribute to the risk of pain crisis.[3,4] This study was designed to examine the effects of HU and zileuton on IL-2 secretion and whether the effects, if any, are exerted through modulation of cell proliferation.

METHODS

Reagents were purchased from the following vendors: concanavalin A (ConA) and β-mercaptoethanol from Sigma-Aldrich; RPMI 1640 medium with 25 mmol/L HEPES, fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, penicillin/streptomycin, phytohemagglutinin (PHA), and L-glutamine from GIBCO; ^{3}H-thymidine (6.7 Ci or 247.9 GBq/mmol) from Dupont NEN; CytoScint scintillation cocktail from ICN; anti-CD3 antibody from BD Pharmingen; and zileuton from the University of South Alabama Pharmacy.

Eleven 4-week-old C57BL/6 healthy female mice were purchased from Charles River Laboratories and housed at the Louisiana State University Health Sciences Center (LSUHSC) vivarium for 3 months. They were given a semipurified (AIN93) diet purchased from Research Diets Inc. Mice had free access to their feeds and sterile water 24 h/day. The mice did not receive any drugs. The light-to-dark cycle was 12 h, and the environmental conditions were 22°C with 56% humidity. The study was approved by the LSUHSC Institutional Animal Care and Use Committee.

Mice were subjected to ether inhalation for 60 seconds, and spleens were removed under sterile conditions. Single cell suspensions were prepared from each spleen by standard techniques, and cell viability was assessed by trypan blue exclusion test. Viable cells (2×10^6) were mixed with either HU or zileuton (10 μg/mL) without and with 2.5 μg/mL ConA, 50 ng/mL anti-CD3 antibody, and 20 μg/mL PHA in 12×75 mm sterile culture tubes. These doses were used in a pretest and were found optimal for modulating spleen cell proliferation. The culture medium was composed of 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 50 μmol/L β-mercaptoethanol, 50,000 units penicillin/L, 50 mg streptomycin/L, and 2 mmol/L L-glutamine in RPMI 1640. The culture medium was also supplemented with either 2% or 5% of FCS. The purpose of the low FCS (2%) was to limit the concentration of growth

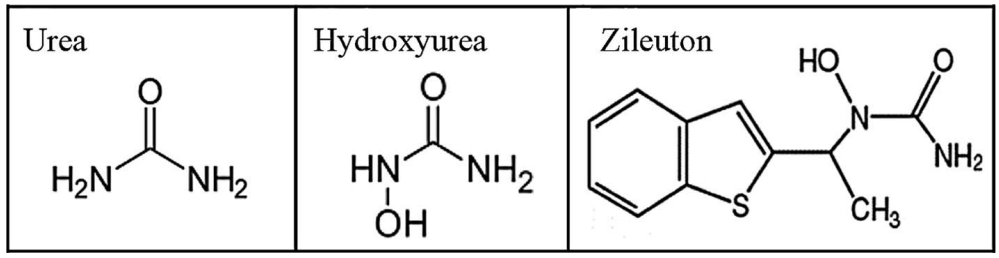

Figure 1. Structures of hydroxyurea, zileuton, and the parent compound urea.

factors that could mask the immunomodulatory effects of HU and zileuton. For baseline (no drug) IL-2 secretion and cell proliferation, culture medium was added to bring the total volume per tube to 1 mL.

Macrocultures were incubated at 37°C, 5% CO_2 in a humidified atmosphere for 12 h, 24 h, and 48 h. At the end of the incubation period, tubes were centrifuged at 2,000 rpm and 4°C for 10 min. The supernatant was collected and immediately frozen at −70°C until used for measurement of IL-2 by enzyme immunoassay with kits purchased from R&D Systems. The methods described in the brochure supplied with the reagents were carefully followed, and all tests were performed in duplicate.

To rule out the possibility that changes, if any, in IL-2 secretion in cultures incubated with either drug were because of an effect on cell proliferation (the rate of DNA synthesis), microcultures were also prepared in culture medium supplemented with 10 μg/mL HU or zileuton in the presence and absence of the same concentrations of mitogens as for macroculture (see above). We transferred 2 $\times 10^5$ cells/200 μL in triplicate to 96-well plates followed by a 48 h incubation at 37°C, 5% CO_2 in a humidified atmosphere, and then pulsed with 1 μCi (37 Bq) per well for an additional 24 h. Cultures were harvested onto filters using a PHD Cell Harvester (Cambridge Technology), and the radioactivity incorporated into DNA was measured by counting each filter dissolved in 2 mL CytoScint scintillation cocktail for 1 min in an LKB Wallac model 1219 liquid scintillation counter.

Descriptive statistics (mean ± SEM of 11 mice used individually) and analysis of variance (ANOVA) were calculated by a microstatistical program (MYSTAT v.2). When ANOVA detected significant differences among the 3 types of cultures, a Tukey test was performed to identify test cultures that were significantly different ($P<0.05$) from baseline cultures.

RESULTS

IL-2 Secretion

In nonactivated cells, neither zileuton nor HU had any effect on IL-2 secretion at any time point studied (Figures 2A and 2B). When cells were incubated in 2% FCS-supplemented medium for 24 h and 48 h, HU did not significantly affect ConA-induced IL-2 secretion compared to nonactivated cultures (Figure 3A). When cells were incubated in 5% FCS for 24 h and 48 h, HU also did not significantly affect IL-2 levels. However, when cells were incubated for 12 h in the same medium, HU slightly, although significantly, decreased ConA-induced IL-2 secretion ($P<0.05$) (Figure 3B). In contrast to HU, zileuton reduced ConA-induced IL-2 secretion 45%-62% ($P<0.05$) (Figures 3A and 3B). The only exception was cells that were incubated in 5% FCS-supplemented medium for 12 h. In this case, zileuton did not affect ConA-induced IL-2 secretion (Figure 3B).

When cells were activated by anti-CD3 antibody, HU increased IL-2 secretion 1.17-fold to 6.5-fold ($P<0.05$), but zileuton reduced it 35%-65% ($P<0.05$) (Figures 3C and 3D). The only exception was the lack of IL-2 inhibition by zileuton in cells incubated with anti-CD3 antibody in 5% FCS for 12 h. Also, HU slightly decreased IL-2 in cells incubated with anti-CD3 antibody in 5% FCS for 12 h. In contrast to ConA- and anti-CD3–treated cells, neither drug had a significant modulatory effect on PHA-induced IL-2 secretion (data not shown).

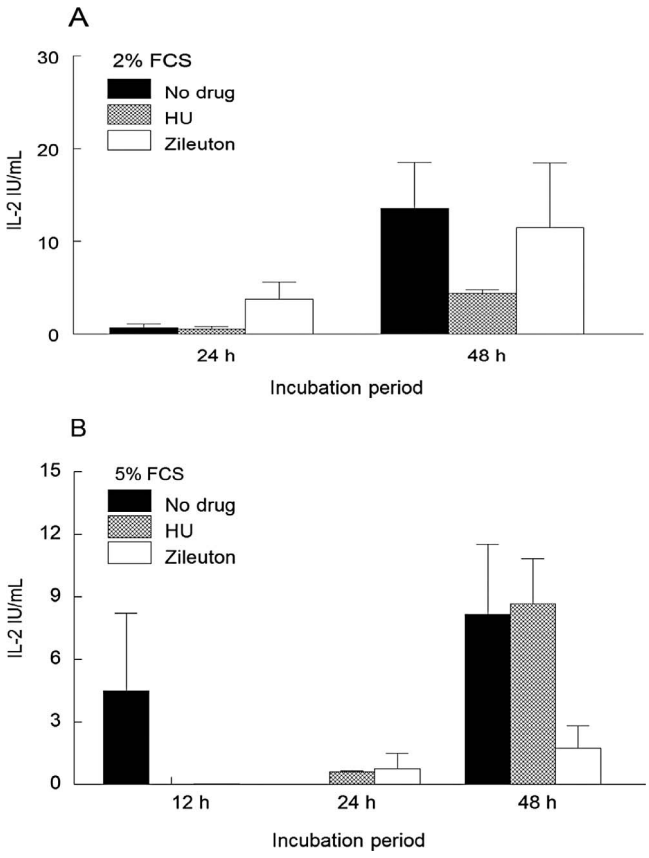

Figure 2. Effect of hydroxyurea (HU) and zileuton on baseline interleukin-2 (IL-2) secretion in cells incubated in (A) 2% fetal calf serum (FCS) and (B) 5% FCS for 12-48 h. Values are means ± SEM, n=11. No significant difference was observed between drugs.

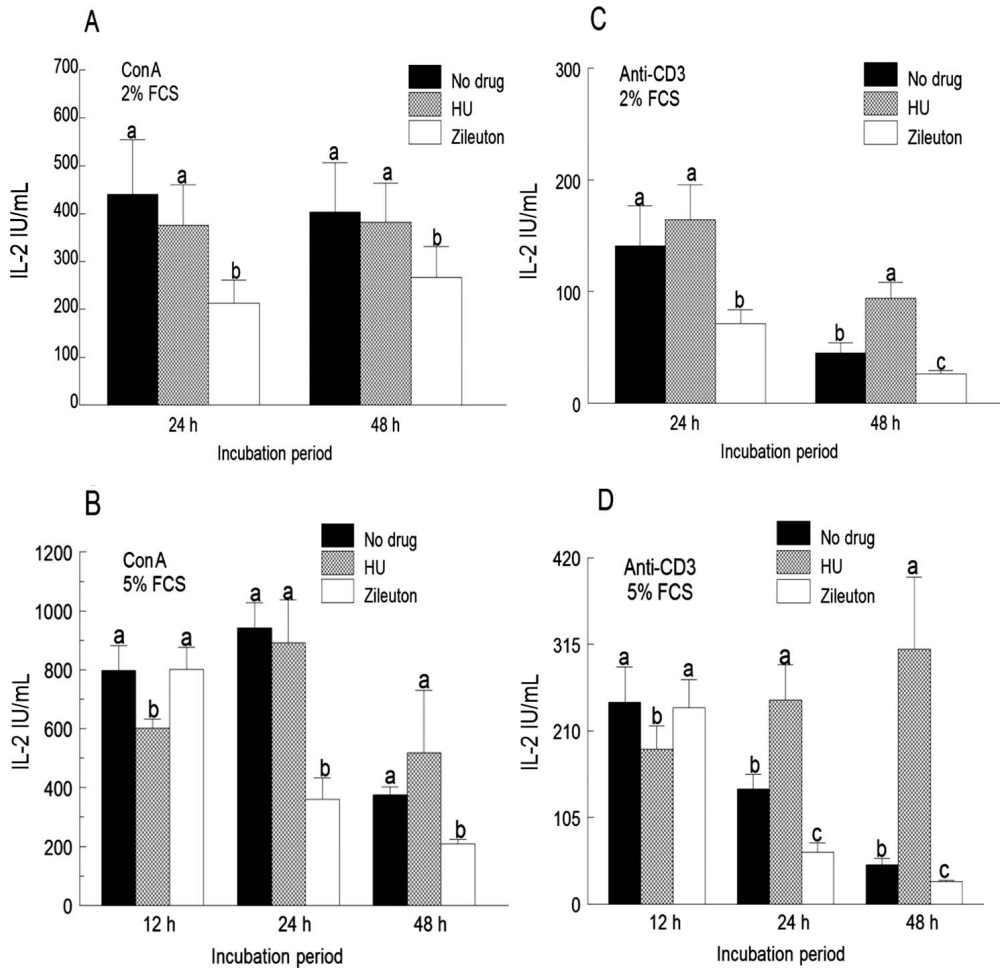

Figure 3. Concentration of interleukin-2 (IL-2) in the supernatant of (A and B) concanavalin A (ConA)-treated and (C and D) anti-CD3 antibody–treated murine spleen cells incubated without and with either hydroxyurea (HU) or zileuton for 12-48 h. Values are means ± SEM, n=11. With each mitogen and incubation period, bars followed by different letters are significantly different: a>b>c, $P<0.05$. FCS, fetal calf serum.

IL-2 data for cells incubated with various drugs in 5% FCS-supplemented medium were also analyzed as a function of incubation period. In the presence of ConA, IL-2 secretion in HU-treated and untreated cells followed the same pattern: IL-2 levels peaked at 24 h and then decreased below the levels observed at the 12 h time point ($P<0.05$) (Figure 4A). For zileuton-treated cultures, IL-2 levels were highest at the 12 h time point but decreased at the 24 h and 48 h time points ($P<0.05$) (Figure 4A). In anti-CD3–treated cells, IL-2 decreased in zileuton-treated and untreated cells with increasing incubation time ($P<0.05$) (Figure 4B). In HU-treated cells, anti-CD3–induced IL-2 secretion increased with time, being lowest at 12 h and highest at 48 h ($P<0.05$).

Cell Proliferation

As shown in Figure 5 and as expected, HU significantly inhibited cell proliferation in all T cell mitogen-treated cultures 54%-79% ($P<0.05$), while zileuton had very little effect. In ConA- and anti-CD3–treated spleen cells, the concentration of FCS (2% in Figure 5A and 5% in Figure 5B) did not modify the proliferative responses to HU and zileuton.

DISCUSSION

In the current study, we investigated the possible differential effects of HU and zileuton on in vitro immune function assessed by cell proliferation and IL-2 secretion by spleen cells obtained from normal young adult mice. The main reasons for conducting the study were to explore the role of IL-2 in modulating both adaptive (T cell and B cell) and natural immunities, the role of IL-2 in monocyte/macrophage activation and subsequent induction of proinflammatory cytokines that are required for pathogen killing, the requirement of cell proliferation for amplification of the immune response once the host is exposed to pathogens or foreign compounds, and the correlation between in vitro immune responses assessed by lymphocyte proliferation and in vivo immunity as in the case of protein-energy malnutrition.[27-29]

Our results suggest that HU does not inhibit ConA- or anti-CD3–induced IL-2 secretion by murine spleen cells; in fact, it tended to upregulate it. This finding was unexpected

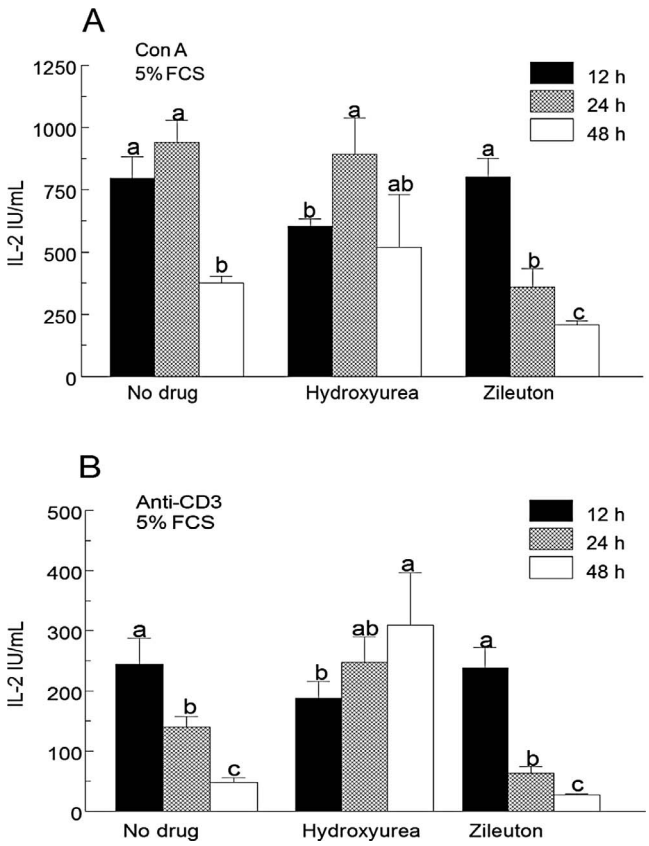

Figure 4. Effects of incubation time and treatment of hydroxyurea and zileuton on interleukin-2 (IL-2) secretion by (A) concanavalin A (ConA)-treated and (B) anti-CD3 antibody–treated murine spleen cells. Values are means ± SEM, n=11. With each drug, bars with different letters are significantly different: a>b>c, $P<0.05$. FCS, fetal calf serum.

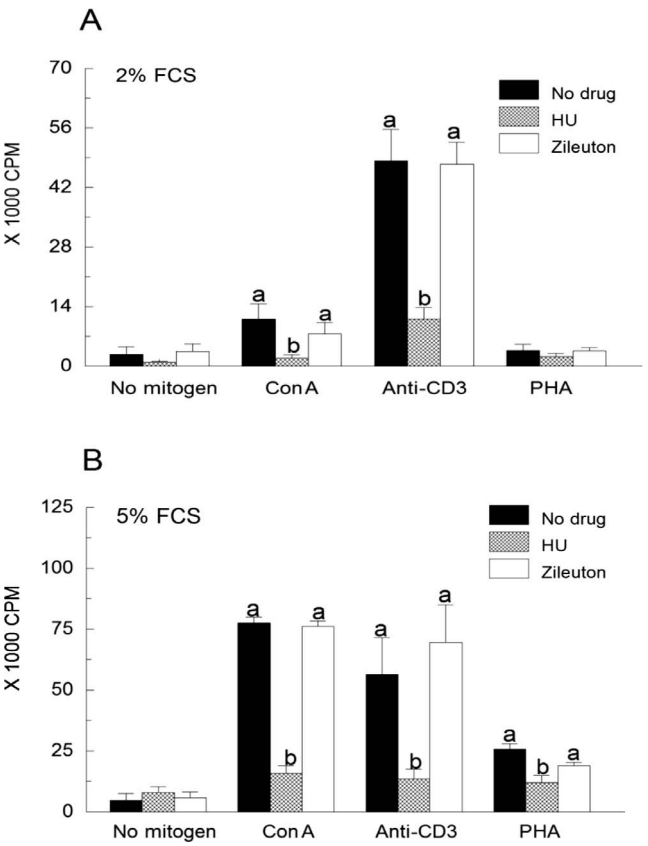

Figure 5. ^{3}H-thymidine incorporation into DNA in mitogen-treated and untreated murine spleen cells incubated without and with hydroxyurea (HU) or zileuton in (A) 2% fetal calf serum (FCS)- and (B) 5% FCS-supplemented culture medium. Values are means ± SEM, n=11. Bars with different letters are significantly different: a>b, $P<0.05$. ConA, concanavalin A; PHA, phytohemagglutinin.

considering that HU is an antiproliferative agent as previously reported and confirmed in our study.[9,12,13] However, the results make sense because HU does not interfere with mRNA and/or protein synthesis but rather with DNA synthesis through inactivation of ribonucleotide reductase.[12] In fact, a similar study conducted in 13 subjects infected with human immunodeficiency virus and 10 controls also did not report any alteration of IL-2 secretion by HU but reported a significant reduction in IFN-γ secretion.[14] The decreased IFN-γ was probably because this cytokine is secreted mostly by CD8+ T cells in addition to CD4+ T cells, natural killer cells, and dendritic cells; therefore, the decrease could be related to non-CD4+ T cells.[30,31] In any case, normal or adequate induction of IL-2 is not sufficient to overcome the antiproliferative property of HU because of continuous block of lymphocytes in the early S phase of the cell cycle. Assuming that we can extrapolate our data to an in vivo scenario, the lack of negative effect of HU on IL-2 secretion may modulate the function of cells involved in innate immunity such as natural killer cells, monocytes, and macrophages in the early phase of an infection. On the other hand, the continuous presence of HU in blood may also compromise the adaptive immune function because of impaired lymphocyte proliferation.

Despite the lack of inhibitory effect on cell proliferation, zileuton significantly reduced IL-2 secretion in ConA- and anti-CD3–treated cells. This observation may be explained by the fact that this drug inhibits 5-lipoxygenase and hence LTB4 production.[20,21] LTB4 is known to upregulate IL-2 secretion and induce lymphocyte proliferation in response to mitogens.[23] Inhibition of LTB4 would reduce IL-2 secretion and may compromise adaptive immunity in zileuton-treated subjects. However, decreased IL-2 may also result in lower macrophage activation and hence in reduced inflammatory cytokines. Additionally, reduced inflammatory cytokines may attenuate the expression of adhesion molecules such as CD36 and VLA-4 on blood cells and thus on lung injury.

Interesting to note are the differences for HU and zileuton in time-dependent changes in IL-2 secretion. In zileuton-treated cells, IL-2 decreased with time, being lowest at 48 h, suggesting that zileuton's inhibitory effect was expressed early during incubation. This finding is not surprising because in the absence of drugs such as HU or zileuton, IL-2 secretion is induced by mitogens within the first 24 h of lymphocyte activation and then decreases during the following 48-72 h.[32]

Despite the low IL-2 levels in the supernatant, zileuton did not inhibit cell proliferation, suggesting that the amounts of IL-2 in the supernatant were high enough to promote cell proliferation. We do not believe that the low IL-2 levels in zileuton-treated cells were the result of cell death because of lack of inhibition of ^{3}H-thymidine uptake (Figures 5A and 5B). Additionally, zileuton does not interfere with ribonucleotide reductase activity.

In summary, our data suggest that zileuton does not inhibit cell proliferation but significantly inhibits IL-2 secretion. In contrast, HU does the opposite. It appears that zileuton may be beneficial in the management of SCD by 3 mechanisms: induction of fetal hemoglobin, reduction of inflammation through low IL-2 secretion, and lack of bone marrow suppression. In contrast, HU may modulate the risk of SCD pain crisis through fetal hemoglobin production and maintenance of IL-2 secretion, and hence adaptive immunity. To our knowledge, this study is the first to show different behaviors of normal murine splenic lymphocytes to HU and zileuton with relation to IL-2 secretion and in parallel cell proliferation.

Our study has the following limitations: the use of normal rather than sickle cell mice, the in vitro rather than in vivo study design, the lack of information on the effects of zileuton on the management of SCD, and the lack of information on the effects of both drugs on the risk of sepsis. These issues are the focus of our next studies.

CONCLUSION

In conclusion, modulation of IL-2 secretion by zileuton and/or reduced lymphocyte proliferation by HU may impair the immune response and increase the risk of or the time required to clear an infection. However, both may also be beneficial in the management of SDC through modulation of inflammation independently of fetal hemoglobin induction. The lack of bone marrow suppression of zileuton gives it an additional advantage over HU in the management of SCD.

ACKNOWLEDGMENTS

Funding for this study was provided from general research funds from the Department of Pediatrics and the Divisions of Research and Hematology/Oncology, LSUHSC. Other than the funding mentioned above, the authors have no financial or proprietary interest in the subject matter of this article.

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This article meets the Accreditation Council for Graduate Medical Education and the American Board of Medical Specialties Maintenance of Certification competencies for Patient Care, Medical Knowledge, and Practice-Based Learning and Improvement.