Combination Exposure to Zidovudine plus Sulfamethoxazole-Trimethoprim Diminishes B-Lymphocyte Immune Responses to Pneumocystis murina Infection in Healthy Mice

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Received 24 August 2005/Returned for modification 24 October 2005/Accepted 9 November 2005

We have previously shown that zidovudine plus sulfamethoxazole-trimethoprim exposure decreases immune cell populations in the bone marrow of healthy mice by inducing apoptosis. The hypothesis of the current work was that this toxicity would have an adverse impact on the immune response. To determine this, BALB/c mice were treated with zidovudine, sulfamethoxazole-trimethoprim, the combination of both drugs, or vehicle only (control) via oral gavage for 21 days. On day 4 after dosing completion, the mice were infected intratracheally with 1 × 10⁷ Pneumocystis murina organisms. Immune cell populations (in lung digest, bronchoalveolar lavage fluid, tracheobronchial lymph node, and bone marrow samples), the lung Pneumocystis burden, and serum Pneumocystis-specific antibody titers were determined at days 6, 10, and 20 postinfection. While total bone marrow cellularity was recovered by day 6 postinfection in the combination exposure group, B-cell numbers did not recover until 10 days postinfection, primarily due to the persistent depletion of the late pre-B-cell phenotype. The numbers of CD4⁺ and CD8⁺ T cells, as well as the numbers of total B cells and activated B cells in tracheobronchial lymph nodes, were decreased at days 10 and 20 as a result of zidovudine plus sulfamethoxazole-trimethoprim exposure compared to the numbers in the control group. No significant differences in lung lavage or lung digest cell populations were observed. There was a trend of a delay in Pneumocystis clearance in the combination treatment group, and Pneumocystis-specific serum immunoglobulin G titers were reduced at day 20 postinfection. Together, these data indicate that the combination of zidovudine and sulfamethoxazole-trimethoprim adversely affects the humoral immune response to Pneumocystis.

Iatrogenic drug effects associated with the agents used for the treatment of patients infected with human immunodeficiency virus (HIV) have the potential to adversely affect clinical outcomes. Highly active antiretroviral therapy (HAART) controls viral replication in infected patients, thereby increasing CD4⁺ T-lymphocyte counts and improving patient survival (19, 32, 56). However, HAART therapy can also cause a variety of adverse effects that can affect morbidity and quality of life. Bone marrow suppression is associated with the use of many antiretrovirals, with zidovudine (ZDV; also known as AZT) being responsible for the highest incidence (20, 72). ZDV was the first antiretroviral agent on the market in the United States in 1986 and remains the most commonly used agent worldwide. ZDV is a thymidine analog that inhibits the reverse transcriptase in HIV after being transformed into its triphosphorylated form intracellularly. As a component of HAART, it has been shown to control the viral load in infected patients, decrease the incidence of opportunistic infections, and improve patient survival (32, 56).

Pneumocystis jirovecii pneumonia continues to be one of the most common AIDS-defining illnesses (39, 57). Current Centers for Disease Control and Prevention recommendations require clinicians to provide prophylaxis against Pneumocystis jirovecii pneumonia when HIV-infected individuals have peripheral blood CD4⁺ T-lymphocyte counts of less than 200 cells/μl (40). The drug of choice for the prophylaxis and treatment of this fungal infection is sulfamethoxazole-trimethoprim (SMX-TMP), which has been shown to improve survival rates among patients infected with HIV (40). TMP is a dihydrofolate reductase inhibitor that interferes with the conversion of dihydrofolate to tetrahydrofolate, and SMX inhibits folate production by inhibiting the conversion of para-aminobenzoic acid to dihydrofolate. SMX and TMP are used in combination to potentiate their inhibition of folate synthesis to provide increased activity against Pneumocystis, as well as many susceptible bacteria.

The individual adverse effects of both ZDV and SMX-TMP on cells in the bone marrow have been well defined. Although the triphosphorylated form of ZDV is responsible for its antiretroviral activity, the monophosphorylated form of ZDV is responsible for its toxicity because of the inhibition of thymidylate kinase, which lowers intracellular thymidine pools (23). This toxicity has been shown to affect human and murine hematopoietic progenitors in the bone marrow (6, 16) and manifests itself clinically by causing anemia, neutropenia, pancytopenia, and granulocytopenia in up to 45% of patients receiving the drug (14, 29, 54, 66). ZDV has been shown to cause apoptosis by the induction of mitochondrial toxicity through such mechanisms as DNA polymerase gamma inhibition and mitochondrial membrane hyperpolarization (3, 11, 21, 43).
Through in vitro studies, the toxicities of SMX have been attributed to the oxidative metabolites SMX-hydroxylamine (SMX-HA) and nitroso-SMX (SMX-N0), with the parent drug causing little or no toxicity (60). The parent is converted intracellularly to these metabolites by cytochrome P450 iso-enzyme 2C9 and is then detoxified by reducing species, including cysteine and glutathione (12, 13). Treatment with SMX-TMP has been shown to cause a high incidence of adverse reactions in HIV patients, including hypersensitivity reactions and bone marrow suppression, which manifests as neutropenia and thrombocytopenia (31, 55, 71).

We have previously shown that the coadministration of ZDV and SMX-TMP results in the depletion of several cell types from the bone marrow of healthy mice (18). Cells that mature in the bone marrow, including B cells, neutrophils, and monocytes, were all affected. These cells undergo a higher apoptosis rate as a result of drug treatment, and this leads to significantly diminished populations by day 14 of exposure (18). B-lymphocyte and monocyte populations in the spleen were also decreased, consistent with the work of other investigators (18, 22).

The components of immune function that are necessary for the clearance of Pneumocystis murina from mice have been extensively studied. It has been shown in murine models that the absence of CD4+ T cells results in the inability to mount an effective response to Pneumocystis (4, 35). In addition, mice that lack functional B lymphocytes have been shown to be highly susceptible to Pneumocystis infection (36, 46, 50). Because B cells are depleted from the bone marrow of mice that receive ZDV and SMX-TMP, we used this infection model to assess the impact of drug toxicity on the host response to an opportunistic pathogen. The purpose of the current work was to assess the effects of this drug combination on the ability of mice to respond to an infectious challenge. We demonstrate that the host response to pulmonary Pneumocystis infection is blunted in mice that undergo 21 days of exposure to ZDV plus SMX-TMP. Both cellular and humoral responses were shown to be altered as a result of combination drug exposure, although the efficiency with which Pneumocystis was cleared from the lungs was not significantly affected.

MATERIALS AND METHODS

Mice and experimental design. Four- to six-week-old BALB/c mice were obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and quarantined for at least 7 days before manipulation. C.B-17 severe combined immunodeficient (SCID) mice, originally obtained from NCI (Indianapolis, IN) and qua...
determined to be statistically significant when a P value of <0.05 was obtained. Data are expressed as the means ± standard deviations (SDs).

RESULTS

Morbidity with combination dosing. Day-to-day subjective observations of the mice showed an increase in lethargy, failure to groom, hunched appearance, and an overall decrease in health in the group treated with ZDV plus SMX-TMP compared to the conditions of the mice in the other groups; these findings became marked by approximately day 7 of dosing. During all infection experiments conducted, 6 of a total of 24 mice in the combination treatment groups died after being infected with \textit{Pneumocystis}. The median time to death was 4 days postinfection, with a range of 1 to 7 days postinfection. Of all infection experiments completed, 1 of 24 mice in the control group died (1 day after infection), whereas all mice dosed in the single-drug groups survived until the time of killing. Only data for mice that survived to the scheduled time points were included in the analyses.

Bone marrow recovery after drug discontinuation. We have previously reported that ZDV plus SMX-TMP ablates B-lineage cells in the bone marrow. To determine whether discontinuation of the drugs would result in the recovery of bone marrow B cells, we examined B-lineage cell subtypes at 10, 14, and 24 days after dosing discontinuation (which correspond to days 6, 10, and 20 postinfection, respectively). Total bone marrow cellularity had recovered to control levels by day 6 postinfection (data not shown). However, when examining individual cell types, we found that B-lymphocyte populations were still depleted to a significant degree at this time point (Fig. 1). The cell types presented in Fig. 1A through D correspond to their order of maturity, with the number of cells harvested at each time point shown. Pro-B cells (B220⁺/CD43⁺/BP-1⁺/HSA⁺), pro-B-cell (B220⁺/CD43⁺/HSA⁺), early pre-B-cell (B220⁺/CD43⁺/BP-1⁺/HSA⁺), and late pre-B-cell (B220⁺/CD43⁺/IgM⁺) subtypes in panels A through D, respectively. Data represent the means ± SDs for four mice per group and are representative of three separate experiments. *, P < 0.05 compared to the results for the control treated with vehicle only.

![Figure 1](https://example.com/figure1.png)
populations in the BALF (Fig. 2A and B) were observed at any time point as a result of single-drug or combination drug exposure compared to the populations in the BALF of the control animals. Likewise, the numbers of alveolar infiltrating T cells that displayed an activated phenotype (CD44^hi/CD62L^lo) among the treatment groups were not different from those in the control mice (Fig. 2D and E).

At days 10 and 20 postinfection, the numbers of CD19^+ B cells and activated B cells (CD80^+ and/or CD86^+) displayed no differences in the lung digests among the groups (Fig. 2C and F). B cells were not measured in the BALF, as B-cell infiltration into the alveolar space in response to Pneumocystis infection is generally negligible (25, 35, 36, 46). At day 6 postinfection, the total and activated B-cell populations were significantly lower in the single-drug treatment groups than in the control group, but this was not the case for the animals that received the combination treatment. These differences were due to a wide range of total cell counts in the lung digests at day 6 postinfection and not because of differences in the percentages of these cell types.

Total TBLN cellularity was decreased in the animals that received both ZDV and SMX-TMP at days 10 and 20 postinfection (data not shown). The CD4^+, CD8^+, and CD19^+ cell populations in the TBLNs were significantly reduced at days 10 and 20 postinfection in the combination dosing group compared to those in the control mice (Fig. 3A to C). Activated CD4^+ T cells were fewer at day 10 postinfection in the combination group, but the difference at day 20 postinfection did not reach statistical significance (Fig. 3D). Activated CD19^+ B cells did not increase in response to the infectious stimulus on days 10 and 20 postinfection like the other groups did (Fig. 3F).

**Pneumocystis clearance kinetics and antibody titers.** Pneumocystis antigen-specific serum IgG and IgM concentrations were measured by a semiquantitative ELISA to evaluate the humoral response to pulmonary infectious challenge after dosing with ZDV plus SMX-TMP. Figure 4A shows the significantly lower Pneumocystis-specific IgG endpoint titer observed in the mice that received combination exposure compared to the titer in the infected control mice at day 20. Additionally, there was a trend toward a significant decrease at day 10 postinfection. Despite these decreases in the IgG titers, the IgM titers were not significantly affected in the combination treatment group (Fig. 4B). Decreased specific antibody levels corresponded to a trend toward a higher lung Pneumocystis burden on day 20 postinfection in the combination exposure group compared to that in the control group ($P = 0.080$). Although there were no statistically significant differences in the numbers of Pneumocystis nuclei present in the lung digests of the combination treatment animals compared to the numbers in the controls at any time point (Fig. 5), the data for day 20 reflected a mean Pneumocystis burden that actually increased from that on day 10 in the
mice treated with the combination, whereas the *Pneumocystis* counts continued to decline in each of the other groups. The group exposed to only SMX-TMP had a *Pneumocystis* burden significantly lower than that in the combination treatment group (Fig. 5).

**DISCUSSION**

Our previous work demonstrated that ZDV plus SMX-TMP exposure significantly impairs developing B-lymphocyte popu-
lations in the bone marrow of mice (18). We have now shown that this toxicity alters the humoral immune response to an infectious challenge, although the overall clearance of the infection was not significantly affected. We have demonstrated that mice treated with ZDV plus SMX-TMP prior to Pneumocystis infection exhibit diminished B- and T-lymphocyte activation in the draining lymph nodes of the lungs. TBLN populations of CD19⁺ B cells, and CD4⁺ and CD8⁺ T cells were significantly reduced compared to those in the control mice on days 10 and 20 postinfection. B cells are responsible for differentiation into plasma cells that will secrete antibody against antigens associated with invading organisms; and as a result of the decreased cell numbers in the draining lymph nodes, Pneumocystis-specific serum IgG titers were significantly lower in the mice that were exposed to the combination of ZDV and SMX-TMP.

At day 20 postinfection, mice receiving SMX-TMP had a significantly lower Pneumocystis burden than those exposed to both ZDV and SMX-TMP. Although the systemic half-life of SMX and TMP in rodents is not known, based on the half-life in humans (9 h for SMX and 10 to 12 h for TMP), SMX-TMP should have been removed systemically prior to inoculation with Pneumocystis (61, 62, 67). However, the possibility exists that residual drug could remain in the intracellular compartment in the pulmonary tissues. Additionally, the metabolites of SMX have been shown to covalently bind to human proteins and immune cells, potentially altering the local disposition in tissues such as the lungs (58, 60, 65). Despite these possibilities, the toxicity of the combination with concurrent ZDV dosing ablates any benefit observed from previous SMX-TMP exposure.

Pulmonary infection with Pneumocystis is cleared in normal mice, with clearance requiring the use of a combination of cellular and humoral components of adaptive immunity. It has been shown that mice cannot mount an effective host response to Pneumocystis without the presence of CD4⁺ T cells (4, 35, 68). Alveolar macrophages likely be the effector cells responsible for killing Pneumocystis, since depletion of alveolar macrophages in rats resulted in the inability to clear infection (44). Additionally, it has been demonstrated that mice deficient in B cells are also susceptible to Pneumocystis infection and are rendered unable to resolve a primary infection (36, 46, 47, 50). In the present study, the numbers of lymphocytes infiltrating into the site of infection were not altered to a significant degree.

Several investigators have demonstrated that the IgG produced by B cells facilitates the clearance of Pneumocystis in murine models of infection (26–28, 34, 74). Work from our laboratory recently demonstrated, however, that Pneumocystis-specific IgG plays an important, but not critical, role in the defense against Pneumocystis (46). This corresponds with the findings of the present study, in that the mice that received both ZDV and SMX-TMP were still able to clear the Pneumocystis from the lungs, despite reduced serum IgG concentrations. Our data indicate that the clearance of Pneumocystis was slowed, although not to a significant degree. This is consistent with other data from our laboratory, in which we found a delay in the clearance of Pneumocystis in mice unable to produce Pneumocystis-specific class-switched antibody (46). The fact that the titers of IgM (which is constitutively ex-pressed) were not significantly reduced indicates that B cells from mice receiving both ZDV and SMX-TMP may have a decreased ability to switch classes to produce IgG.

We and others have previously reported that this drug combination did not affect the peripheral T-cell numbers in the spleen (18, 22). Here, in the context of infection, total and activated CD4⁺ T-cell frequencies were lower in the TBLNs of the animals receiving the combination treatment. This could be a secondary effect stemming from the B-cell depletion. Investigators have shown that T-cell responses are dependent on interactions with B cells in secondary lymphoid organs in response to many infectious stimuli, including Salmonella, Bordetella pertussis vaccine, and Pneumocystis (45, 46, 70). Linton et al. demonstrated that B cells play a critical role in regulating the clonal expansion of CD4⁺ cells by evaluating antigen-specific cytokine secretion by CD4⁺ cells in healthy and B-cell-deficient mice (45). Ugrinovic et al. suggest that T-cell responses to Salmonella infection are dependent on B-cell antigen presentation (70). Additionally, our group demonstrated that T-cell expansion and activation were reduced in the TBLNs and in the lungs of B-cell-deficient and CD40-knockout mice in response to Pneumocystis infection (46). These data support the findings of our previous work, which show that ZDV plus SMX-TMP affects only the frequencies of the cell types that mature in the bone marrow (18). This suggests that the CD4⁺-cell depletion seen here is directly due to reduced lymphatic B-lymphocyte populations.

Clinical studies illustrate that HIV not only causes damage to T-cell populations but also affects B-lymphocyte function. Patients with advanced AIDS are often hypergammaglobulinemic due to an increased number of plasma cells that spontaneously secrete Ig (42, 51). The B cells in these same patients, however, did not respond adequately to T-cell-independent B-cell mitogenic stimulation (42). Despite being in a hyperactivated state, the ability of B cells to produce specific antibody responses to antigens is reduced, as shown in several clinical investigations (1, 2, 5, 38, 69). In one in vitro study this has been attributed to the fact that B cells from HIV-infected individuals cannot up-regulate CD70 after being stimulated with activated T cells, which impairs CD70-dependent immunoglobulin synthesis (73). Additionally, others have demonstrated that HIV viremia impairs the ability of B cells to deliver appropriate costimulatory signals to T cells (49). The decreased ability to mount a proper humoral response likely contributes to the increase in certain bacterial infections in this patient population. Furthermore, the decrease in the antigen-specific IgG titer in HIV-infected individuals has been positively correlated with the CD4⁺ T-cell count and has been inversely correlated to the viral load (17).

To date, no studies have addressed the contribution of adverse drug reactions to this dysfunction. The patients with lower CD4⁺ T-cell counts are more likely to be taking SMX-TMP, because of clinical guidelines for the treatment and prophylaxis of Pneumocystis pneumonia that require caregivers to place patients on this or an alternative agent when CD4⁺ counts drop below 200 cells/mm³. This supports the possibility that the toxicity of the combination drug treatment demonstrated in our mouse model has a clinically significant effect on B-lymphocyte responses.

There is an increased incidence of treatment-limiting ad-
verse events associated with SMX-TMP therapy in patients infected with HIV. These incidences have been reported to be up to 80% (10, 30, 41). This has been attributed by some investigators to an HIV-driven depletion of glutathione, which has been shown to increase the intracellular concentrations of SMX-HA and SMX-NO in lymphocytes in vitro (8, 9, 59).

However, this has not been confirmed in vivo (64). We are investigating the hypothesis that the presence of ZDV alters the intracellular concentration of SMX-HA and SMX-NO in B lymphocytes in the bone marrow of mice. In this regard, we have found that serum SMX concentrations are elevated in mice also receiving ZDV, which could be due to an interaction that affects the drug disposition at the cellular level (18).

Our previous work has demonstrated that bone marrow depletion occurs in mice treated with ZDV plus SMX-TMP (18). B lymphocytes were depleted primarily at the late pre-B-cell stage of development, due to a cell-cycle specific block as cells proliferated from the early pre-B-cell stage to late pre-B cells (18). The data in the current paper characterize the recovery of the B-lineage subtypes in the bone marrow once the drugs have been discontinued. Overall bone marrow cellularity was restored by day 10 postexposure, with all subpopulations except B-lineage cells being restored. Because pro-B and late pre-B cell populations were not fully recovered until after day 14 postdosing, the altered responses of B cells in the TBLNs could be due to this residual bone marrow depletion in the mice receiving ZDV plus SMX-TMP.

The doses used in the animal experiments corresponded with the doses used in studies by other investigators (22, 52, 53). The doses administered were approximately 8 to 10 times the doses given to humans, on a mg/kg basis, for Pneumocystis pneumonia prophylaxis and HAART; however, the doses were more comparable to clinical doses based on body surface area (32, 40). Interspecies scaling in drug dosing has received much attention as it is a continual problem when animals are used to model and investigate conditions in humans (7, 48). Mice have been shown to require much higher doses of cytotoxic drugs than humans to produce similar levels of cell death (37, 63). The extent to which the concentrations of SMX-HA, SMX-NO, and ZDV in mouse bone marrow correlate with those in the bone marrow of humans treated with these agents is unknown. This issue makes it difficult to predict the clinical significance of such results, making investigations with humans extremely important.

These findings demonstrate that the host response to infectious challenge is altered in mice that received ZDV plus SMX-TMP. This suppression of humoral immunity could affect the morbidity and mortality of HIV-infected patients who receive both of these agents. A clinical trial that assessed the ability of patients receiving these drugs to produce specific antibody in response to an immune stimulus supports our findings reported here (D. J. Feola, unpublished observations). This iatrogenic effect, if it is clinically significant, could have an impact on the outcomes of infections with other opportunistic and true pathogens that affect this patient population. Alternative antiretroviral agents that cause much less bone marrow toxicity than that caused by ZDV are available, and patients at high risk of bone marrow suppression could potentially benefit from the use of alternative agents for the treatment of their chronic HIV infections.

ACKNOWLEDGMENT

This work was supported by Public Health Service Grant HL 64524 from the National Heart, Lung, and Blood Institute.

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