Efficacy of dinitrochlorobenzene and 3'-azido-3'-deoxythymidine for treating acquired immunodeficiency disease

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Received February 25, 2008

Abstract

Background. 3'-azido-3'-deoxythymidine (AZT) has been the nucleoside of choice in treating infections with human immunodeficiency virus (HIV), which is responsible for acquired immunodeficiency disease syndrome (AIDS). In addition, 1-chloro-2,4-dinitrobenzene (dinitrochlorobenzene or DNCB) has been used in trial experiments for treating HIV infection. In each case therapeutic benefits have been realized due to the inhibition of viral replication by AZT, and increase in T lymphocytes (CD8+ cells) and decreased viral replication with DNCB. Combination therapy of AZT with other nucleoside and therapeutic agents results in added benefits for alleviating infection with HIV. These agents have been used in pregnant females for controlling transmission of the virus into the fetus with some beneficial results. However, long-term use of AZT is minimized due to its toxic effects on the hematopietic and immunologic systems. The toxic nature of DNCB has not been as well characterized. Our purpose was to evaluate the toxic effects of AZT and DNCB on dams and progeny when these agents are applied to mice at mid-pregnancy in order to provide realistic baselines for their use in therapy of HIV infection.

Materials and Methods. Pregnant mice were allowed to drink water spiked with 0.2 mg AZT/ml (oral exposure) or drink spiked water alternating every 3 or 4 days with intravenous (iv) injection of 0.5 ml AZT solution at 0.2 mg/ml (oral-iv exposure). In other pregnant mice DNCB was applied to the shaved area behind the ears with 3 doses of DNCB in ethanol at 90 μg/ml, 22.5 μg/ml and 2.25 μg/ml.

Results. In mice given AZT, there occurred a depression in reproductive parameters including decrease in litter size, depressed peripheral red blood cells and white blood cells, and a suppressed proliferation of progeny spleen cells in an allogeneic mixed lymphocyte response. For the progeny of dams exposed to DNCB there was a dose response reduction in body weight gain, while survival of the progeny and dams was unaffected. Conclusion. These results demonstrate that toxicity, as determined within the limits of this experiment, was more severe in the AZT-exposed mice. Additional works to more precisely delineate toxic events, particularly with DNCB, are necessary. Knowing the disposition toward toxicity, treatment regimens with these compounds in combination together or with other therapeutic agents will be more accurately defined. [Life Science Journal. 2008; 5(3): 41 – 49 (ISSN: 1097 – 8135)].

Keywords: 3'-azido-3'-deoxythymidine; dinitrochlorobenzene; acquired immunodeficiency syndrome; efficacy

1 Introduction

Since the advent of acquired immunodeficiency disease (AID), several therapeutic drugs have been used to combat replication of the retrovirus which causes the AID syndrome (AIDS). Still, the drug currently of choice is 3'-azido-3'-deoxythymidine (AZT) used alone in a suitable treatment regimen or in combination with other therapeutic agents[1]. Although various regimens have merit in the inhibition of viral replication, nevertheless, these kinds of treatment are limited, and, in addition, AZT has toxic side effects, particularly harmful to the hematopietic and
immune systems. The potentially harmful side-effects include anemia, neutropenia, bone marrow myelotoxicity, and depression of cell-mediated immunity\[2\]–[7]. Despite its short-term beneficial effects, the side-effects limit long-term therapy with AZT. Similar toxic effects have been observed in mice injected with a C-retrovirus\[2,4,8–11\].

Vertical transmission (from mother to fetus) of human immunodeficiency virus (HIV) has been a critical problem for several years. Methods have been devised to determine the risk of transmission which has been evaluated, in one study, as 0.097 (95% confidence intervals; 0.03 – 0.163)\[12\]. Treatment of pregnant women with AZT has been accelerated because this agent provides the most effective means in controlling vertical transmission of HIV, and because it has been shown that there is a dramatic > 3-fold reduction of infection in the offspring of these women\[13\]. However, because the side effects induced by this agent, still arguably are a significant deterrent for continued treatment, and thus for long range therapeutic benefits, it is important to evaluate the kinds of alterations in biological systems that can occur in the primiparous individuals and in their progeny.

Within the last 10 years a drug that induces delayed-type hypersensitivity, 1-chloro-2,4-dinitrobenzene (DNCB or dinitrochlorobenzene) has been evaluated for its protective ability against viruses and other pathogens\[14,15\]. In addition, it has been shown that DNCB exposure in humans leads to increase in CD4⁺, CD8⁺, and natural killer cells with a concomitant decrease in HIV replication\[15\].

AZT as a nucleoside analog is effective in reacting against viral DNA, and DNCB is capable of stimulating T cell activity of the host leading to production of T cytotoxic cells (CD4⁺), T killer cells (CD8⁺), and to an increase of T helper cells (CD4⁺), events that could affect the proliferation of the retrovirus which causes AIDS. Thus, it would appear feasible to utilize AZT and DNCB in treating HIV infection in humans.

Establishing treatment regimens with AZT and DNCB is necessary, but of equal importance is to evaluate the side effects of these compounds. For this we have chosen the murine system since many of the changes that occur in mice exposed to AZT are very similar to those that occur in the human system. Other than being a potent immunostimulating agent, little, if any, is known on the toxicity of DNCB. On the other hand, toxicity of AZT has been somewhat characterized, and includes several changes of concern. Some of these are toxic syndromes such as damage to peripheral blood cells and to the bone marrow. In animals it has been reported that exposure to AZT leads to diminished reproductive capacity in females, and adverse effects in the offspring when given to dams at pregnancy. In humans there is some indication that birth defects occur\[16\]. Despite its short-term beneficial effects, its side effects limit long-term therapy with AZT. Thus, it is quite important to reveal changes in biologic systems of primiparous and pregnant individuals and their progeny which could affect directly and/or indirectly biological sequelae that would impact on efficacious treatment regimens in humans. For this purpose we have used the murine system to evaluate the toxic effects of AZT and DNCB.

As indicated, in the murine model side-effects induced by AZT are similar to those that occur in humans. This includes peripheral blood cell and bone marrow toxicity\[2,5,6,9\] and severe pigmentation of the extremities\[17–19\]. Thus, because of striking similarities between humans and mice following exposure to AZT, the mouse model used in this study may provide needed information for base-line studies on biologic and immunologic side-effects that occur in humans. In this context, this is, most likely, no less true for DNCB. Our results on some side effects induced by AZT and DNCB in mice are presented in this report.

2 Materials and Methods

2.1 Animals and treatment

C3H/Anf (Cumberland View Farms, Clinton, TN) or C3H/HeJ (Jackson Labs, Bar Harbor, ME) mice (H-2\(^{k/k}\)) 5 to 6 weeks old were separated by gender and placed 5 animals/cage. At 11 or 12 weeks of age 2 females were housed with one male, and the females observed for vaginal plugs daily from 8 to 9 AM. When the plug was observed (day 0 of gestation) the female was removed and housed alone. Throughout the experiment, animals were housed in the animal facility of the Morehouse School of Medicine (MSM) under the guidelines of the Animal Care Committee MSM, and the Public Health Services. They were tested frequently for pathogens and received food and water ad libitum. The pregnant dams were separated into 5 groups as follows: those receiving AZT orally (through drinking water) (oral); animals receiving AZT orally + intravenous (iv) injection (oral-iv); mice receiving topical application of 90 μg/ml DNCB; dams receiving topical application of 22.5 μg/ml DNCB; those receiving topical application of 2.25 μg/ml DNCB.

In mice receiving oral AZT exposure, the animals drank from a graduated bottle filled to the 100 ml mark with AZT dissolved in water at 0.2 mg/ml. For the oral-iv group, exposure from drinking AZT spiked water was alternated with a 0.5 ml iv injection of AZT at 0.2 mg/ml.
3 to 4 days apart. During the period of iv injection, the animals drank unspiked water. Exposure began 7 days before mating, continued throughout mating, and post-partum. Age-matched virgin females also were exposed to oral and oral-iv AZT. Consumption of AZT was described in detail elsewhere\(^7\). Consumption of AZT was accomplished by a competitive inhibition immunoassay (Sigma, St. Louis, MO) according to the manufacturer’s specifications.

Exposure to DNCB was as follows: the site of application was shaved, and the contact sensitizer applied topically, at the doses listed above in a volume of 0.5 ml (in ethanol), to a 1 inch square of skin behind the ears of the animals every other day until parturition. In one scenario, the drug was applied starting on the 3rd day of pregnancy, and in the other, application began on the 12th day (mid-gestation). Application of the drug was discontinued after parturition, and was not given to the progeny. The doses applied were equivalent to the doses commonly used in human subjects. A control group received ethanol only. The ethanol was allowed to evaporate quickly using a fan focused on the treated area. No licking of the site occurred on observing the animals for 3 hours after application.

### 2.2 Reproductive capacity

The biological activity of reproductive parameters for AZT and DNCB dams and progeny was determined by several end-points. These included mating activity of the females, appearance of a vaginal plug, frequency of pregnancy, maintenance of pregnancy, frequency of births, number of pups in a litter, survival of pups at birth, and sex ratio. Survival of the pups at weaning was also measured. Reproductive activity of virgins, or the mating behavior of primiparous mice and pups was not determined.

### 2.3 Body weight

Changes in body weight over time were measured for the progeny and dams treated with AZT and DNCB.

### 2.4 Quantization of peripheral blood cells

Leukocytes (L) and erythrocytes (E) in the peripheral blood were determined by drawing blood from a tail vein in a white blood cell (WBC) or red blood cell (RBC) diluting pipette. Dilution of L cells was in Turk’s solution and the RBC were diluted in Hayem’s solution. The cells counted in a hemocytometer, and the blood profile of L cells and E cells was expressed in number of cells/mm\(^3\). This procedure was described in detail elsewhere\(^20\).

### 2.5 Assay for the allogeneic mixed lymphocyte response (MLR)

The MLR was done for mice exposed to AZT, but was not done for the DNCB animals. MLR methodology has been described in detail elsewhere\(^7\). Briefly, \(1 \times 10^6\) C3H splenic responder (R) cells were cultured with \(1 \times 10^6\) (BALB/cxDBA/2) F1 stimulator (S) cells that had been inactivated with mitomycin C. Three or four days after cultivation at 37 °C under 5% CO\(_2\) in a humidified atmosphere, the cultures were pulsed with 0.5 μCi \(^3\)H-thymidine (S.A. 6.7 Ci/mM) (DuPont), recovered 16 to 20 hours later, and radioactivity determined in a Beckman liquid scintillator. Proliferation, as indicated by \(^3\)H-thymidine uptake into DNA, is expressed in counts per minute (CPM).

### 2.6 Changes in colorization of AZT treated dams

This phenomenon has been observed by others in animals and humans\(^7,17-19\). Increased dark (black) pigmentation was observed in the dams and virgins one day after AZT treatment. Temporal changes in this color were noted for the tail, the foot-pads, the ears, the lips, and the genital region, which increased in intensity throughout the experimental period (as long as AZT was applied). A cohort of mice exposed only to water did not show changes in black pigmentation. In the DNCB-exposed animals, pups were not examined for the presence of black colorization, nor was colorization noted.

Since black colorization increased with dose-time accumulation of AZT, we examined the profile of the dendritic melanocytes under the skin. For this, after anesthetization of the mice, they were sacrificed and the foot-pads removed. They were fixed in cacodylate buffer containing 10% formalin. The tissue was incubated in 0.1% L-3,4-dihydroxyphenyalanine (DOPA) buffered solution containing 10% formalin. The tissue was incubated in 0.1% L-3,4-dihydroxyphenyalanine (DOPA) buffered solution pH 7.4 overnight, processed and imbedded in paraffin. Serial sections were cut at 5 – 10 microns, placed on glass slides, processed, and counter-stained with neutral red\(^21\). The sections were scanned at 10 × magnification to outline an individual dendritic melanocyte (ocular magnification 2 × ). When and if difficulty was encountered in visualizing a melanocyte, the magnification was increased 40 × or 100 ×.

### 2.7 Statistical evaluation

Test for significance was performed using analysis of variance, and significance was established by Student’s \(t\)-test using Dunnett’s modification for comparison of the groups and Bonferroni’s modification where one group was compared to another.
3 Results

3.1 Consumption of AZT

As we showed earlier [7], there was no difference in daily consumption of water for normal mice and virgins; ingestion was from 5 to 7 ml per day, while the primiparous mice drank 10 to 11 ml per day during pregnancy, and after birth to weaning more than 25 ml per day (Figure 1).

![Figure 1. Water consumption by female mice. Open bars: virgin mice; closed bars: primiparous mice. Each bar represents from 40 to 50 mice.](image)

Thereafter, the primiparous mice after weaning drank an average of 10 ml per day up to 4 months. Thus, from birth to weaning the primiparous mice consumed more than 105 mg AZT, and from 6 to 7.5 weeks the females consumed approximately 12 mg AZT. For these time intervals the virgin females drank about 25 ml.

3.2 Reproductive changes, survival and body weight

Significant disruptions occurred in several different reproductive parameters in AZT-exposed mice, punctuated by reduced frequency of pregnancy, reduced maintenance of pregnancy, and reduced litter size (Table 1). Of the ten end-points measured in AZT-exposed mice, 8 showed a significant difference from the controls, drinking water only. In addition, the sex ratio suggested a more toxic effect of AZT on male fetuses and pups.

![Table 1. Changes in reproductive activity of female mice exposed to AZT](image)

A striking picture was observed for body weights of progeny from DNCB-exposed pregnant dams. When DNCB was applied beginning on the 3rd day of pregnancy, there was a semblance of a dose-response effect of DNCB where 22.5 μg/ml application influenced a decrease in body weight gain in the progeny, while 90 μg/ml did not (Figure 2). This dose-response effect was further emphasized for progeny from dams receiving varying dose of DNCB at 12 days of pregnancy. Surprisingly, the most striking decrease in body weight gain with time was observed in the progeny from dams painted with the lowest dose of DNCB at 2.25 μg/ml (Figure 2).

3.3 Peripheral blood cells

Changes in peripheral RBC and WBC with time in primiparous mice and virgins on AZT revealed significant depression of RBC days 10 through days 60 in primiparous dams, while for the WBC there was an early increase followed by a significant decrease on days 35...
which lasted until days 60 after chronic exposure to AZT (Figure 3A). In virgins RBC remained depressed throughout the experimental period, but after an early depression of WBC (days 10 to 30), these cells were enhanced up to 60 days (Figure 3B). For the mice on water only (virgins or primiparous), blood RBC profile remained above $11 \times 10^6$ cells/mm³. It is interesting to note that the influence of AZT on the peripheral blood cells showed mirror images between the WBC of primiparous mice and virgins. In the former, WBC were initially enhanced (10 to 30 days after exposure), while in the latter they were depressed.

Thereafter, in the former WBC were depressed while in the latter they were enhanced. This suggests that AZT attacks, at the origin of WBC, differently for primiparous mice relative to virgins (different stem cells?).

3.4 The MLR

The MLR of virgin females on oral-exposure with AZT is markedly reduced at the time intervals shown (1, 4, 7 months) but enhanced after oral-iv-exposure (Table 3), and as we have shown earlier[7]. For progeny from primiparous mice exposed to oral AZT, proliferation in the MLR was enhanced at 1 and 4 months, but at 7 months it was suppressed. Oral-iv-exposure resulted in severe suppression. The accumulated amount of AZT during drinking for primiparous mice was 54 mg at 1 month, 275 mg at 4 months, and 350 mg by 7 months. For virgin females it was 34, 134, and > 200 mg, respectively by 7 months.

3.5 Hyperpigmentation and melanocyte profiles in AZT-exposed animals

Black pigmentation in the tissue sites depicted

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Table 2. The effect of DNCB given to dams at mid-pregnancy on progeny survival, and survival and body weight changes in the dams

<table>
<thead>
<tr>
<th>Expt group</th>
<th>No. of pups</th>
<th>% overall survival</th>
<th>% DNCB 3 days</th>
<th>Survival 12 days</th>
<th>No. of dams</th>
<th>% DNCB 3 days</th>
<th>BW increase</th>
<th>% DNCB 3 days</th>
<th>Survival 12 days</th>
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<td>Control</td>
<td>13d</td>
<td>92</td>
<td>83</td>
<td>100</td>
<td>2d</td>
<td>107</td>
<td>109</td>
<td>100</td>
<td>100</td>
</tr>
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<td>19e</td>
<td>95</td>
<td>100</td>
<td>92</td>
<td>3</td>
<td>108</td>
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<td>100</td>
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<td>2f</td>
<td>107</td>
<td>ND</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

*DNCB: dinitrochlorobenzene applied topically in µg/ml for the µg doses shown. #: Frequency of body weight (BW) increase for 1 mother only for each experimental group at each time interval of initial DNCB application. #: Survival values for the number of mothers used in each group. #: Number of pups born in litters of 2 mothers. #: Number of pups or fetuses in litters from 3 mothers. #: After the initial application of DNCB at 12 days of pregnancy (2.25 µg/ml), one mother died after the 2nd application.

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Figure 2. Body weights of progeny. Open circles: control (ethanol only); closed circle: 90 µg DNCB/ml; open squares: 22.5 µg DNCB/ml; closed squares: 2.25 µg DNCB/ml. Vertical lines are standard deviations from the mean.
Figure 3. RBC and WBC. Open circles: virgin RBC water only; closed circles: virgin RBC AZT-exposure; open squares: primiparous WBC water only; closed squares: primiparous WBC AZT exposure. Peripheral blood cell changes in primiparous (A) and virgin (B) mice during AZT exposure. Vertical lines: standard deviation from the mean. Each point represents 15 to 20 determinations.

above increased with time as the amount of AZT increased. There were no difference in the pattern of color change between primiparous mice and virgins. Increase in pigmentation with time and amount of AZT accumulated is clearly shown in serial sections of footpad tissue stained by the DOPA method (Figure 4). In addition, there was a sustained increase in dendritic melanocytes harboring the black pigment with time and accumulation of AZT dose (shown in Figure 4).

4 Discussion

We can conclude from the data with DNCB that topical administration of pregnant mice is not associated with abortions. However, the results clearly demonstrate that mice exposed to AZT and DNCB at pregnancy exhibit, concomitantly, several changes induced by these agents. This observation confirms that of others on generally singular physiologic alterations described following AZT therapy in humans and in mice,[4,5,17,22–24], and extend the work in that multiple systemic changes occur at the same time. It is also clear that exposure to AZT or DNCB in pregnant dams has disruptive effects on biological, immunological, and biochemical parameters in the dams and their progeny. From our study DNCB toxicity does not seem as severe as for AZT. However, additional work needs to be done to evaluate more precisely the effect of DNCB on biologic systems other than survival and body weight.

The changes induced by these agents are manifested very shortly after exposure. With AZT, depression in peripheral blood RBC and WBC of the primiparous dams and virgin mice were observed within 5 days after exposure (amount of AZT accumulated was about 5 mg); abnormal reproductive changes occurred within 7 days (31 mg AZT). For DNCB, depressed progeny body weights with time occurred shortly after application of the agent (7 days, at 11.25 mg) from the dams which were topically treated with 22.25 mg/ml, and 3.15 mg from the dams receiving 2.25 mg/ml; for the depressed MLR of the progeny, assayed at 1 month after the dams drank spiked water, 31 mg AZT accumulated in the dams, and hyperpigmentation occurred almost immediately after exposure (day one at 1.5 mg). It should be noted, however, that dam and pup survival following DNCB treatment was normal, and, for the dams, body weight increased as expected (data not shown). It is noted that the consequences of hyperpigmentation are not evident. It is interesting that intensity of pigmentation increased with time-dose of AZT. This change also occurs in humans treated with AZT. It is possible that the amount of AZT
Figure 4. Hyperpigmentation of foot pads of mice on AZT (40 ×). Top left photomicrograph is of a foot-pad from a mouse that drank about 250 ml of water. One day AZT through 5 months AZT: Note the increase in pigmentation with increase in cumulative amount of AZT. The dendritic macrophages are clearly seen in the 5 months photomicrograph (bottom right). The dense accumulation of pigmented cells (e.g., 35 months AZT) obscures the outlines of melanocytes.

present at any one time after application can be predicted by the intensity of pigmentation as shown in Figure 4, i.e., pigmentation acting as a dosimeter for AZT treatment.

Alterations in reproductive capacity of primiparous females exposed to AZT are similar to observations by others\cite{22,24} with some differences. We observed a high frequency of multiple vaginal plugging which may be indicative of fetal resorptions\cite{7}, since, in our mice, the
Table 3. Cell proliferation in the allogeneic MLR by progeny from primiparous dams and by age-matched virgins exposed to AZT

| Experimental groups          | CPM $(10^5)$ determined at time after treatment begun (months) |
|------------------------------|-----------------------------------------------------------------
|                              | 1                          | 4                          | 7                          |
| Control oral; (oral-iv)      | 28.3 ± 2.4                 | 48 ± 3.3                   | 23.6 ± 3.1                 |
| Primiparous oral only        | 33.2 ± 2.8                 | 65.5 ± 5.2                 | 12.0 ± 1.9                 |
| Virgin oral only             | 13.2 ± 1.5                 | 12.2 ± 1.3                 | 8.2 ± 1.5                  |
| Primiparous oral-iv          | 14.2 ± 2.0                 | 28.2 ± 2.7                 | ND                         |
| Virgin oral-iv               | 31.1 ± 3.2                 | 86.4 ± 4.7                 | ND                         |

*: Water oral (oral-iv) are controls (water only) representing 10 to 20 mice/time interval. CPM were pooled at each time-interval for the primiparous mice, and virgin females water controls. The CPM ranged from $(15 – 41) \times 10^5$ at 1 month; $(30 – 60) \times 10^5$ at 4 months; $(10 – 35) \times 10^5$ at 7 months. ± standard deviation from the mean. #: Females on two different AZT treatment regimens, oral only, AZT in drinking water; oral-iv, AZT in drinking water for 1 week alternating with iv injections the second week, and so on. Mice injected with AZT iv drink unspiked water during the week of iv injection. Each group represents 5 to 10 mice. !: Significant differences between water control and AZT-treated mice was at $P < 0.05$ to $P < 0.0001$ by Student’s $t$-test. #: significant differences from $P < 0.005$ to $P < 0.0001$. ND: Not done.

The frequency of pups born is reduced by 40%. At this point, reasons for these aberrations are not immediately apparent.

In lieu of the changes observed with these prospective agents in the treatment of AIDS, this should not be considered a deterrent in using AZT and DNCB for therapy. The beneficial effects of AZT in which it inhibits replication of HIV may offset its harmful effects and provide a situation where its use may be more amenable to protection against the virus. Obviously, a regimen in which it does not lead to undue harm to the individual, but directed toward the elimination of the virus, is advisable. As for DNCB, additional parameters must be studied, despite its less harmful effects whereby the increase in body weights of the progeny is depressed albeit at the smaller dose used in this study. Interestingly, at the lowest dose used, DNCB appears to influence some physiologic activity leading to the inability to maintain and increase body weight of the progeny. Thus, the efficiency of DNCB as a therapeutic agent in HIV-infected progeny (and perhaps other individuals) may depend on how much of this agent is introduced. Within the limits of this study, it would appear that the higher dose would be less toxic (more effective) as a therapeutic agent.

Perhaps an AZT regimen can be used with DNCB in combination. This approach seems feasible, and has not been considered. It seems logical that such a combination may have highly protective effects against HIV. As indicated, AZT inhibits replication of the virus responsible for AID, and, DNCB augments the T cell response, particularly cytotoxic and killer cells (CD8$^+$), stimulates the production of natural killer cells, and is a potent modulator of dendritic antigen processing cells. Perhaps a treatment regimen consisting of a combination of AZT and DNCB would yield fruitful results in further diminishing infection with HIV, most interesting in alleviating fetal transmission if applied to pregnant dams. Therapy with AZT early in its use led to toxic changes which led to cessation of use of this drug. This toxicity also led to the production of other DNA analogs, prompted therapy with combinations of other nucleosides with AZT, and, perhaps here, we have introduced the possibility of combination therapy with AZT and DNCB in alleviating infection with HIV, particularly as it may concern the vertical transmission of the virus.

**Acknowledgment**

We are grateful to Ms. Nicole Downing for editorial assistance and computer expertise.

**References**