Accelerated Mortality in Young NZB/NZW Mice Treated with the Interferon Inducer Tilorone Hydrochloride[1]

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Tilorone, 100 mg/kg, was given once a week to two groups of NZB/NZW mice: young mice aged 3 months without overt disease, and 8-month-old mice with established autoimmune disease. Young treated animals died prematurely with severe glomerulonephritis and vasculitis, but lifespans were not shortened in old treated mice. Thymic atrophy was common in treated animals. Tilorone therapy did not affect autoantibody levels; nevertheless, terminal C_3 values were decreased significantly in young treated mice. Although interferon activity was detected in sera from young treated mice, it provided no protection from spontaneous autoimmune disease. Tilorone-induced suppression of cell-mediated immunity may have caused accelerated disease and premature death in NZB/NZW mice.

INTRODUCTION

First-generation hybrid offspring of New Zealand black (NZB) and New Zealand white (NZW) mice spontaneously develop heterogeneous antinuclear antibodies (ANA), specific antibodies to DNA (anti-DNA), and immune-complex glomerulonephritis (1). These animals carry C-type viral particles (2). It has been postulated that the endogenous viruses stimulate autoantibody production in the host animals, which are immunologically hyperresponsive to nucleic acid antigens (3). The current study was designed to investigate the effects of a potent inducer of interferon on the course of autoimmune disease in NZB/NZW mice. Tilorone (2,7-bis[(diethylamino)ethoxy]fluoren-9-one dihydrochloride) is a low-molecular-weight aromatic amine with activity against RNA- and DNA-containing viruses in mice (4). This drug was fed to young NZB/NZW mice and to older NZB/NZW mice with established autoimmune disease. When treated mice were tested 6 months and 8 months after the study began, interferon activity was detected in their sera. Nevertheless, in the young treated animals autoantibody levels increased, serum complement levels fell, and severe vasculitis and glomerulonephritis caused premature death.

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MATERIALS AND METHODS

Treatment Protocol

The origin and maintenance of NZB and NZW mouse colonies and breeding of hybrid NZB/NZW mice in the Rackham Arthritis Research Unit have been described (5). Tilorone was obtained from Merrell National Laboratories, Cincinnati, Ohio. Tilorone, 100 mg/kg in 0.25 ml of distilled water, was given by gavage once a week to 15 young female NZB/NZW mice aged 4 to 16 weeks (mean 11 weeks). Fifteen female littermate mice serving as controls received distilled water by gavage. To determine the effects of therapy in animals with established disease, 15 old treated female mice aged 29 to 33 weeks (mean age 31 weeks) were treated with tilorone, 100 mg/kg, given once weekly by gavage. All three groups of mice were treated until death. Blood was collected from the orbital plexus before treatment began and after 12 and 24 weeks of treatment. In most instances, moribund animals were bled before they were autopsied. At each bleeding, white blood cells (WBC) were counted in the conventional manner and thin blood films were prepared. Differential counts of 100 leukocytes on each slide were performed. Sera for ANA tests, anti-DNA determinations, and complement assays were stored in sealed capillary tubes at $-20°C$.

Histology

Complete autopsies were done routinely following a protocol described in an earlier publication (5), and tissues were examined by light microscopy for any pathologic process that might have contributed to death. Fibrinoid necrosis of arteries in any organ was recorded. Cross sections through both thymic lobes were examined. An eyepiece micrometer was used to measure widths of thymic medulla and thymic cortex, and cortical:medullary (C:M) width ratios were calculated. Complete cortical atrophy was identified when the cortex was shrunken and depleted of lymphocytes. Fibrosis in each thymus was assessed in Carnoy-fixed tissue stained by van Gieson's method. Histologic abnormalities in 20 glomeruli in each section of renal tissue were counted using a quantitative method adapted for use in this laboratory (6).

Autoantibodies

Autoimmune responses were evaluated by measuring heterogeneous ANA in titered mouse serum using an indirect immunofluorescent technique (7). Specific anti-double-stranded-DNA antibodies were quantitated by an adaptation of the Farr technique, using KB-cell-derived ^{14}C-labeled DNA as substrate (7).

Complement

Serum levels of the third component of complement (C_3) were determined by single radial immunodiffusion of 0.002 ml of serum in 1% agar containing 0.005 M EDTA and 5% antiserum (Cappel Laboratories, Downingtown, Pa.). The antiserum was raised in rabbits inoculated with zymosan−mouse complement complex according to the method of Mardiney and Müller-Eberhard (8). Separate immunoelectrophoretic analyses in 0.005 M EDTA agar of fresh cold EDTA-treated mouse plasma and mouse serum stored 18 hr at room temperature showed that the antiserum detected both β_{1C}- and β_{1D}-globulins. Although the antiserum

reacted with C_3 and at least one of its breakdown products, this determination will be referred to as a test for C_3. A pool of serum from healthy outbred albino mice (Spartan Co., Haslett, Mich.) was assigned a value of 100 C_3 units/0.002 ml, and four dilutions of this reference serum were included on each diffusion plate. After incubation at room temperature for 72 hr, the diameters of precipitin rings formed by the reference serum plotted against individual dilutions of the same serum formed straight lines on a semilog graph. Serum C_3 levels in individual mice were read from these standard curves and expressed in arbitrary C_3 units. Percentage coefficient of variation (C) was calculated to determine the reproducibility of precipitin ring diameters produced by each dilution of reference serum on 38 individual diffusion plates. For serum diluted 1:8, C = 4.8%; for 1:16, C = 4.5%; for 1:32, C = 5.0%; for 1:64, C = 6.2%.

Interferon

Because tilorone-induced interferon activity varies in different strains of mice (9), a pilot study was performed to detect interferon in NZB/NZW mouse serum. Thirty-four 4-month-old female NZB/NZW mice were given tilorone, 100 mg/kg, by gavage. Interferon activity assayed by a standard test-tube method (10) was present in pooled serum samples obtained 16 and 24 hr after treatment. Control mice and young tilorone-treated mice in the current study were tested at the intervals listed in Table 3. The microassay described by Dahl and Degré (11) was adapted to test this serum for interferon activity on mouse L-929 cells in individual wells of Micro Test II tissue culture plates (Falcon Plastics, Oxnard, Calif.). The result of each test was expressed as the reciprocal of the PD_{50}, the dilution of test serum that protected 50% of cells from viral cytopathogenic effect (CPE). The TCD_{50} (tissue culture dose of virus that destroyed 50% of cells) was determined by back titration on each individual plate. The challenge dose of VSV virus used on each plate ranged from 56 to 170 TCD_{50}. In each determination, pooled standard serum with a high level of antiviral activity from a separate group of tilorone-treated mice was included as a positive control. Plain medium was used as a negative control.

Statistical Analysis

χ^2 calculations and student's t tests were performed using the MIDAS software package (12) within the MTS system on the AMDAHL 470V/6 computer.

RESULTS

Mortality

Figure 1 illustrates cumulative deaths in control and treated mice. In accord with earlier studies in this laboratory (5), control mice began to die with renal disease and vasculitis at 27 weeks of age. The mean age at death was 45 weeks; the oldest survivor died at 77 weeks of age. An unexpected finding was the accelerated death rate in young mice treated with tilorone. The first death in this treatment group occurred in a 24-week-old female mouse, 8 weeks after tilorone therapy began. The mean age at death in young treated mice was 35 weeks; the oldest surviving mouse died at the age of 60 weeks. When the shortened mean lifespan in young treated mice was compared with the lifespan in control mice, the

difference was significant (P = 0.047). Postmortem examinations showed that 13 young treated mice died with accelerated renal and vascular disease. Two animals were found autolyzed, and the causes of death could not be determined. Tilorone therapy had no effect on mice in the older treatment group (Fig. 1). In these animals, the mean age at death was the same as that of control mice (45 weeks). Nine old treated mice died with renal disease and arteritis. Two animals died with hemorrhage, and one mouse had a large uterine abscess and widespread amyloidosis. Despite daily surveillance for evidence of illness, two old mice died unexpectedly and were autolyzed. Early autolysis in four autopsied mice in this group prevented detailed evaluation of their thymic and renal histologies. Two control mice and one old treated mouse died when ether anesthesia was given before a routine bleeding. These three iatrogenic deaths were omitted from longevity data and the graph in Fig. 1.

Histologic Abnormalities

The most notable anatomic lesions were found in thymic tissue, kidneys, and arteries. In untreated animals, the mean C:M ratio was 0.92 ($\pm$ 0.14 SE). Complete cortical atrophy was found in one control mouse. In young tilorone-treated mice, cortical thinning was common and the mean C:M ratio was 0.58 ($\pm$ 0.16). In 4 of the 13 thymus glands examined in this group, the cortical areas were completely atrophic. Thymic cortical atrophy was most severe in the older treated female mice. The mean C:M ratio was 0.22 ($\pm$ 0.09). Compared to control animals, this value was significant (P = 0.002). Three of nine thymus glands from old treated mice had acellular cortices. When van Gieson-stained sections of thymus were examined, excessive fibrous tissue was not identified in the cortical areas. Loss of lymphocytes, rather than cortical fibrosis, caused atrophy of the thymic cortex. An increased amount of fibrous tissue surrounded blood vessels in the thymic medulla in 5 of 12 control mice, in 8 of 13 young treated mice, and in 4 of 9 old treated mice.

Every control and treated animal had severe glomerulonephritis that was
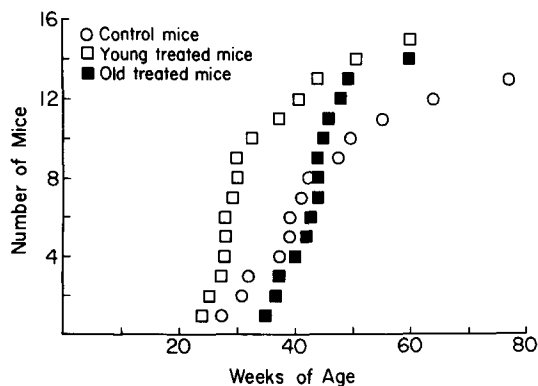

FIG. 1. Cumulative deaths are illustrated in young and old female NZB/NZW mice treated with tilorone, 100 mg/kg/week, until death. Control mice received water. Mean lifespan in young treated mice was shortened significantly compared to control mice (P = 0.047).

characteristic of NZB/NZW renal disease (5). Thickening and hypercellularity of the mesangial stalk, glomerular hypercellularity, thickening of the glomerular basement membrane, and crescent formation were found in kidney tissue from each animal. In many instances, abundant fibrinoid change was found in the glomeruli. Control mice (mean glomerular lesion count, 53 ± 2) and young treated mice (mean lesion count 50 ± 2) had severe renal disease. In old treated mice, the mean lesion count was 38 ± 3. The unexpected decrease in severity of glomerular disease in old mice may have reflected loss of diseased renal tissue in six autolyzed mice. Generalized arteritis was common. Fibrinoid necrosis of small and medium arteries was present in kidneys and lymphoid organs (spleen, lymph nodes, and thymus) in 9 of 13 control mice. Widespread arteritis was present in 11 of 13 young treated female mice. In old treated mice, arteritis was found in five of nine animals with complete autopsies.

Autoantibodies and Complement

In control NZB/NZW mice, median titered ANA values increased from 0 to 7 in the first 24 weeks of this study (Table 1). A similar increase in ANA titers was observed in young treated mice. Mean anti-DNA levels in untreated mice showed an age-dependent increase from 16 to 38% in the first 24 weeks of observation; mean anti-DNA values in young treated mice increased from 16 to 27%. Autoantibody levels in old treated mice were not included in Table 1 because these values

TABLE 1

AUTOANTIBODIES AND C_3 LEVELS IN TILORONE-TREATED AND CONTROL MICE

Mice	Weeks of treatment			
	0	12	24	Terminal
ANA[a]				
Control	0(0–5)	5(0–7)	7(3–9)	5(1–9)
	[15]	[15]	[11]	[12]
Young treated	0(0–5)	3(0–7)	7(3–11)	3(0–11)
	[15]	[13]	[6]	[12]
Anti-DNA[b]				
Control	16 ± 0.6	24 ± 3	38 ± 5	21 ± 1
Young treated	16 ± 0.6	20 ± 2	27 ± 4	19 ± 1
C_3[c]				
Control	120 ± 7	68 ± 7	70 ± 9	104 ± 17
Young treated	116 ± 16	89 ± 15	63 ± 10	55[d] ± 8

[a] Median (range). Values are expressed as the highest tube number (1-$\log_2$ of serum dilution) giving a positive test for heterogeneous ANA using an indirect immunofluorescent technique. Brackets enclose the number of mice tested at each bleeding.

[b] Mean ± SE. A modification of the Farr technique was used to test sera for specific antibodies to double-stranded DNA. Values are expressed as percentage of ^{14}C-labeled DNA bound to 0.015 ml of mouse serum.

[c] Mean ± SE. Serum C_3 levels were determined by radial immunodiffusion. Values were expressed as arbitrary C_3 units, determined by comparing precipitin ring diameters produced by NZB/NZW sera with diameters produced by twofold dilutions of standard mouse serum.

[d] Compared to control mice, $P = 0.005$.

were not directly comparable to control values. At the beginning of the study, ANA titers and anti-DNA levels reflected active autoimmune disease in these older animals. The median ANA titer increased from 5 (range 0–9) before treatment to 9 (range 5–11) after 12 weeks of treatment. The terminal median ANA titer was 7 (range 5–9). Mean anti-DNA levels in old treated mice increased from the mean pretreatment level of 33 ± 4% to 46 ± 2% after 12 weeks of therapy. The mean anti-DNA value in terminal serum was 23 ± 2%. In accord with the report of Miller and associates (13), C_3 decreased with age in unmanipulated female NZB/ NZW mice (Table 1). In control mice, mean C_3 levels fell from 120 to 70 units during the first 24 weeks of the study. C_3 values in young treated mice were similar to values in control animals. In terminal serum, the mean C_3 level in young treated mice (55) was reduced significantly compared to the mean level in untreated control mice (104); $P = 0.005$. C_3 levels assayed in old treated mice were consistently lower than C_3 levels in the reference mouse serum used as a standard on each immunodiffusion plate. Mean C_3 levels in the older mice were 62 (± 12) before treatment and 86 (± 17) after 12 weeks of treatment. The mean terminal C_3 value in this group was 92 (± 22).

Leukocyte Counts

During the course of this study, mean total WBC counts fell in treated and control mice. Peripheral circulating large and medium lymphocytes (LML) decreased throughout the treatment period, and mean absolute counts of these cells did not differ significantly from cell counts in control mice (Table 2). Twelve weeks after the study began, the mean small lymphocyte (SL) count of 656 in young treated mice was significantly smaller than the corresponding mean SL count of 1152 in control mice ($P = 0.03$). When terminal peripheral blood smears were examined, mean numbers of SL were 197 in control mice and 787 in young treated female mice. This difference was significant at the 0.02 level. In old treated female mice, there were no appreciable changes in mean absolute WBC, SL, or LML counts in the first 12 weeks of the study. Terminal cell counts in the older mice were almost identical to terminal cell counts in control mice. Despite a careful

TABLE 2

PERIPHERAL CIRCULATING LEUKOCYTES (WBC), SMALL LYMPHOCYTES (SL), AND LARGE AND MEDIUM LYMPHOCYTES (LML) IN TIORONE-TREATED AND CONTROL MICE

Mice	Weeks of treatment											
	0			12			24			Terminal		
	WBC	SL	LML	WBC	SL	LML	WBC	SL	LML	WBC	SL	LML
Control	6765	701	5201	5091	1152	3288	3840	306	2580	3376	197	1654
	±308[a]	±118	±247	±510	±175	±372	±395	±44	±302	±552	±58	±223
Young treated	5512	631	4327	3852	656[b]	2659	4047	376	2740	5154	787[c]	2546
	±573	±82	±483	±456	±118	±310	±272	±88	±268	±713	±216	±478

[a] Mean absolute cell count (cells/mm^3) ± SE.
[b] Compared to control mice, $P = 0.03$.
[c] Compared to control mice, $P = 0.02$.

search for morphological abnormalities, no granules or vacuoles were noted in cytoplasm of mononuclear cells.

Interferon Assays

After a prolonged course of treatment, NZB/NZW mice were not refractory to the interferon-inducing properties of tilorone. Table 3 describes interferon activity in control and young treated mice tested 24 and 32 weeks after treatment began. In accord with the findings of De Clercq and Merigan (14), the greatest serum interferon activity was found 16 hr after tilorone was administered.

DISCUSSION

Other investigators have attempted to control infection with C-type virus and suppress autoimmune disease in New Zealand mice by administering antiviral drugs to these animals. Gabriel (15) treated NZB/NZW mice with methisazone (1-methylsantin 3-thiosemicarbazone), a compound that directly inhibits multiplication of RNA and DNA viruses (16). The mean lifespan was 10.6 months in the treated mice, compared to a mean lifespan of 9 months in a group of control mice. This evidence that a viral inhibitor might prevent early deaths in autoimmune NZB/NZW mice was supported by data from another study employing therapy with an interferon inducer. Lambert and Dixon (17) treated a group of female NZB/NZW mice with Statolon, a double-stranded natural RNA (18). Although ANA appeared spontaneously, ANA titers were decreased in treated mice compared to control mice after 4 months and 7 months of therapy. Steinberg *et al.* (19) administered a synthetic double-stranded RNA, polyinosinic–polycytidilic acid (poly I–poly C), to hybrid New Zealand mice. Although recipient female mice produced interferon, they developed anti-poly I–poly C antibodies, high levels of anti-DNA, and severe renal disease. This experiment showed that a ribonu-

TABLE 3
Serum Interferon Activity in Tilorone-Treated and Control Mice

Mice	N^a	Total duration of therapy (weeks)	Time after oral dose of tilorone (hrs)	Serum interferon titer[b]	Standard mouse interferon titer[c]
Control	6	24	16	<25	600
Young treated	3	24	16	38	600
Control	5	32	0	<25	800
			16	<25	800
Young treated	2	32	0	<25	800
			6	<25	800
			16	200	800
			24	44	800
			48	<25	800

[a] N = Number of mice tested at each interval.

[b] Pooled serum from mice in each group was tested for ability to protect mouse L-929 cells from bovine VSV-induced CPE using a microassay technique. Test results were expressed as the reciprocal of the PD_{50} (the dilution of test serum that protected 50% of cells from CPE).

[c] Twofold dilutions of pooled standard serum from a separate group of tilorone-treated mice were included on each microtiter plate as a positive control.

cleotide compound used as an inducer of interferon could act as an antigen, stimulating autoantibody formation and accelerating disease in NZB/NZW mice.

In the current study, it was anticipated that using tilorone as an interferon inducer would avoid the stimulation of autoantibody formation observed in New Zealand mice treated with poly I−poly C. Interferon activity was detected in sera from young treated mice, but these animals were not protected from autoimmune disease. Autoantibodies appeared spontaneously and increased with advancing age in control and tilorone-treated mice. C_3 levels decreased with age, reflecting increasing severity of immune-complex disease (13). Postmortem examinations confirmed that treated and control mice died with glomerulonephritis and arteritis. Two important observations suggested that continuous tilorone therapy beginning at an early age had an adverse effect on disease in young female NZB/NZW mice. First, lifespans were decreased significantly in the young treatment group compared to the control group. In addition, C_3 levels in terminal sera from young treated mice were lower than mean C_3 levels in dying control mice. In old treated mice, lifespans were not influenced by tilorone therapy. This finding suggested that the course of established disease in older animals was not influenced by tilorone-induced changes in immune response.

The immune system is defective in NZB/NZW mice; their thymic-regulated, cell-mediated immunity is depressed. Thymic suppressor cells may be destroyed spontaneously in adult New Zealand mice, and it has been postulated that loss of thymic suppression accelerates production of autoantibodies in these animals (20). Recently, tilorone was found to influence immune responses in rodents, and the immunoregulatory effects of this drug may explain the shortened lifespan in young treated NZB/NZW mice. Megel and co-workers (21) reported that tilorone therapy enhanced immune responses to sheep red blood cells in mice. In contrast to the apparent stimulation of humoral immunity, treatment with tilorone suppressed cell-mediated immunity in rats. Depletion of thymic-dependent cells may be the mechanism whereby tilorone therapy influences immune responses. Transient disappearance of circulating thymic-dependent lymphocytes has been described in mice threated with tilorone (22). Long-term administration of tilorone to young NZB/NZW mice may have caused premature loss of suppressor thymic function, thereby accelerating autoimmune disease and causing early death.

In the current study, treated mice commonly lost thymic cortical cells. These changes may have reflected stress-induced cortical atrophy in chronically ill animals (23). Nevertheless, other investigators have described transient loss of cells in thymic-dependent areas of spleen, lymph nodes, and Peyer's patches in mice and rats treated with a single dose of tilorone (24). It may be postulated that repeated doses of tilorone given once a week over a period of months had a more pronounced effect on the thymus than one dose, and prolonged treatment may have caused irreversible atrophy of the thymic cortex in NZB/NZW mice.

Although the experimental findings suggested that tilorone destroyed or mobilized lymphoid cells in thymic tissue, there was no evidence that long-term therapy with the drug consistently influenced numbers of peripheral lymphocytes. Twelve weeks after the study began, the mean SL count in young treated mice was decreased significantly compared to that of control mice. When testing was

repeated 12 weeks later, SL counts in treated mice were similar to those of controls. The mean terminal SL count of 787 in young treated mice was greater than the SL count of 197 in terminal blood smears from control mice ($P = 0.02$). Fluctuation of peripheral lymphocyte counts may be partially explained by our practice of bleeding mice 24 to 72 hr after the weekly dose of tilorone was administered. In "normal" experimental animals without autoimmune disease, oral tilorone was found to produce peripheral lymphocytopenia lasting 48 hr (22).

Based upon the work of Rohovsky et al. (25), it was anticipated that granules and vacuoles would be observed in the cytoplasm of mononuclear cells from treated mice. However, these morphologic changes were not found in WBC from the hybrid New Zealand mice in this study. This observation should stimulate further investigation into the relationship between tilorone-induced cytoplasmic lesions and functional classification of lymphoid cells. If granules and vacuoles were markers of thymic-dependent lymphocytes, the absence of such markers in tilorone-treated NZB/NZW mice might reflect a specific decrease in numbers of circulating thymic-dependent cells.

ACKNOWLEDGMENTS

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