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HIGH INCIDENCE OF NEOPLASMS IN FEMALE NZB/NZW MICE TREATED WITH PULSE DOSES OF CYCLOPHOSPHAMIDE

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ABSTRACT

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The immunosuppressive properties of cyclophosphamide prevent formation of anti-DNA antibodies and prolong lifespans in autoimmune NZB/NZW mice, an animal model of systemic lupus erythematosus. In the current study, NZB/NZW mice were treated with weekly doses of cyclophosphamide to determine if intermittent pulses of the drug were effective therapy. Life-long treatment with cyclophosphamide, 56 mg/kg/week, was started at the mean age of 6 weeks; results were compared with saline-injected control mice. Pulse therapy with cyclophosphamide suppressed anti-DNA antibody levels, prevented severe glomerulonephritis and prolonged longevity. Seventeen of 19 treated mice developed neoplasms; 7 of these immunosuppressed animals had 2 to 4 separate neoplasms. Examination of earlier studies in this laboratory in which NZB/NZW mice were treated each day with cyclophosphamide showed that daily and weekly therapeutic regimens had similar immunosuppressive and oncogenic effects.

INTRODUCTION

Female New Zealand Black/New Zealand White (NZB/NZW) mice spontaneously develop antibodies to DNA (anti-DNA) (Steinberg et al., 1969) and immune complex glomerulonephritis (Lambert and Dixon, 1968), and they are accepted as models of systemic lupus erythematosus. Fifty percent of female NZB/NZW mice die with renal failure at the age of 10 months (Howie and Helyer, 1968).

A series of experiments in this laboratory showed that prolonged daily administration of the potent alkylating agent cyclophosphamide, in doses ranging from 5.7 to 16 mg/kg, suppressed autoimmunity and prolonged lifespans in hybrid New Zealand females. An unfortunate complication of therapy was the appearance of neoplasms in 89 to 100% of treated mice (Walker and Bole, 1973; Walker and Anver, 1978).

Because long-term treatment with cyclophosphamide given in daily doses resulted in a high incidence of tumors, it was postulated that intermittent therapy might modify oncogenesis. In the current study, therapeutic efficacy and oncogenic potential of weekly doses of cyclophosphamide were evaluated in female NZB/NZW mice. Pulse therapy on a weekly basis suppressed formation of

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anti-DNA, prevented glomerular disease and prolonged lifespans. Neoplasms appeared in 89% of treated mice. Intermittent cyclophosphamide therapy was therefore similar to daily therapy. Pulse doses did not modify the oncogenic properties of cyclophosphamide.

METHODS

Animals

Breeding and maintenance of New Zealand Black (NZB), New Zealand White (NZW), and NZB/NZW mice were described in another publication (Walker and Bole, 1973). Hybrid mice used in this study were produced by mating NZB females and NZW males. These animals were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

Treatment protocol

Cyclophosphamide (Mead Johnson and Co., Evansville, IN, U.S.A.), 56 mg/kg in 0.1 ml volume, was dissolved in sterile 0.15 M NaCl immediately before use and given weekly by subcutaneous injection to 22 female NZB/NZW mice (mean age 6 weeks ± 1 SEM). Fifteen female control mice with mean age 11 weeks (± 1) were injected daily with 0.1 ml 0.15 M NaCl. Injections continued until spontaneous death. Control mice were entered into the study 3 months after the treated groups began receiving cyclophosphamide. Because therapy prolonged lifespans, all cyclophosphamide-treated mice were alive when saline injections began in control animals.

Blood samples were collected from the orbital plexus before treatement and after 24 and 52 weeks of treatment. Treated and control animals were examined daily; they were bled, killed by cervical separation, and autopsied when they developed neoplasms or appeared moribund.

Histologic studies

Complete necropsies yielded tissue which was fixed, embedded and stained for light microscopic examination using methods described earlier (Walker and Bole, 1973). Severity of glomerulonephritis was scored by counting numbers of specific abnormalities in 20 glomeruli in a 4-micron cross section of each kidney. Infiltrates of lymphocytes around renal arteries were graded on a scale of 0 to 4+: 0 = no cells, 1+ = few cells, 2+ = cells surrounded 25-50% of arterial wall; 3+ = cells surrounded 75% of arterial wall; 4+ = cells surrounded entire arterial wall (Walker et al., 1978).

Anti-DNA

Serum was stored at -20°C in sealed capillary tubes and tested for anti-DNA

antibodies using a modified Farr technique. ^{14}C-labeled E. coli DNA (Amersham Corporation, Arlington Heights, IL, U.S.A.) was reacted with heat-inactivated mouse serum using a method described previously (Walker and Bole, 1975). In this laboratory, values greater than 20% binding indicate the presence of anti-DNA antibodies.

Statistical analysis

Student's t test was calculated as described by Snedecor and Cochran (1967).

RESULTS

Longevity

Three treated mice and 2 control mice died of iatrogenic causes or were lost because of autolysis. These animals were excluded from the study. In the control group mean longevity was 46 weeks $\pm$ 4, and lifespans ranged from 27 to 77 weeks. The first death in cyclophosphamide-treated mice occurred at 32 weeks of age; the last animal died at the age of 94 weeks. Mean survival in treated mice was 70 weeks $\pm$ 4. Lifespans were prolonged significantly in treated mice compared to controls ($p<0.001$).

Causes of death

In the controls, vasculitis and severe glomerulonephritis caused death and no neoplasms were found at necropsy. In contrast, 17 of the 19 treated mice that were not lost by iatrogenic death or autolysis had 1 or more neoplasms (Table 1).

The first malignancies to appear were malignant lymphomas; 4 of these lesions appeared 38 to 49 weeks after the study began. Two widespread sarcomas also appeared relatively early in the treatment period. Localized lesions in subcutaneous areas, pelvis, breast, and lung appeared late in the treatment period after mice received therapy for 58 to 86 weeks. Two treated mice died with ovarian hemorrhage.

To determine if cyclophosphamide administered in weekly doses was comparable to daily therapy, mice treated with both regimes were examined for longevity and neoplasms. Table 2 compares NZB/NZW female controls, female mice treated with cyclophosphamide 8 mg/kg/day in an earlier study in this laboratory (Walker and Bole, 1973), and pulse-treated females in the current study.

Anti-DNA antibodies

In control mice, mean anti-DNA increased from 16% $\pm$ 1 at the beginning of the study to 38% $\pm$ 5 after 24 weeks of observation. At 52 weeks, one surviving untreated animal had 45% DNA binding. Mean anti-DNA in terminal sera from control mice was 20% $\pm$ 1. Intermittent therapy with cyclophosphamide suppressed

TABLE 1

Neoplasms in female NZB/NZW mice treated with weekly doses of cyclophosphamide, 56 mg/kg

Number of affected mice		Treatment period (wks)
	Generalized Neoplasms	
4	Malignant lymphoma	28-49
1	Fibrosarcoma	50
1	Sarcoma	63
1	Malignant lymphoma[a]	70
	Local Neoplasms	
1	Mammary carcinoma, sebaceous gland adenoma, pulmonary adenoma	58
1	Squamous cell carcinoma	70
1	Subcutaneous sarcoma	70
1	Mammary carcinoma, squamous cell carcinoma, pulmonary adenoma	74
1	Mammary carcinoma, sarcoma, hemangiosarcoma, pulmonary adenoma	77
1	Mammary carcinoma, fibrosarcoma, pulmonary adenoma	79
2	Sarcoma (pelvis)	70,88
2	Subcutaneous sarcoma, pulmonary adenoma	82,86

[a]This mouse also had a pulmonary adenoma.

TABLE 2

Comparison of longevity and neoplasms in female NZB/NZW mice treated with daily and weekly doses of cyclophosphamide

Treatment groups[a]	Mean, range longevity (wks)	Mice with Neoplasms (%)	Mice with Multiple Neoplasms (%)	Classification of Neoplasms[b] (% of total neoplasms)			
				Ly	Carc	Sarc	Other
0	46 (24 - 103)	3	0	100	0	0	0
8	77 (58 - 108)	100	22	42	42	8	8
56	70 (32 - 94)	89	37	17	21	34	28

[a]Treatment groups; 0 = control mice injected with saline; 8 = cyclophosphamide, 8 mg/kg/day; 56 = cyclophosphamide, 56 mg/kg/week. Each group contained 9-19 mice.

[b]Abbreviations: Ly = lymphoma; Carc = carcinoma; Sarc = sarcoma.

anti-DNA during the first year of the study. Mean DNA binding values were 21% ± 1 pretreatment, 19 ± 1 after 24 weeks, and 20 ± 1 after 52 weeks of treatment. At the 24-week interval, anti-DNA in treated mice was decreased significantly compared to controls ($p<0.001$). In 15 treated mice with terminal serum available for testing, mean anti-DNA was 30 ± 2.

Renal lesions

Severe glomerulonephritis in control mice accounted for the mean glomerular lesion score of 53 ± 2; 69% of these animals had arteritis involving renal vessels, and heavy 3-4+ periarterial infiltrates were found in 47% of control kidneys. The protective effect of weekly doses of cyclophosphamide was evident in treated mice, whose mean glomerular lesion score was suppressed significantly to 22 ± 2 (compared to control mice, $p<0.001$). Renal vasculitis was not present in treated animals, and scant periarterial lymphocytes graded 1+ were identified in 16% of treated mice.

DISCUSSION

The earliest reports of favorable outcome in autoimmune NZB/NZW mice treated with cyclophosphamide (Russell et al., 1966; Russell and Hicks, 1968) were confirmed by other investigators who used intermittent or daily dose regimes. Weekly injections of 1.8 mg cyclophosphamide suppressed glomerulonephritis and prolonged longevity in female NZB/NZW hybrids (Russell and Hicks, 1968; Casey, 1968; Horowitz et al., 1969). In subsequent experiments, therapeutic effectiveness was achieved with doses ranging from 50 to 240 mg/kg, administered at intervals of 7 days to 1 month (Hahn et al., 1975; Morris et al., 1976; Levy et al., 1978). Autoimmunity was also suppressed with daily doses of cyclophosphamide, 5 to 8 mg/kg; administration of equivalent doses in weekly, 10-day, or monthly schedules had similar protective effects (Hahn et al., 1975; Morris et al., 1976).

The oncogenesis which complicates cyclophosphamide therapy in New Zealand hybrids was noted in the early report of Russell and Hicks (1968), when neoplasms were discovered in 29% of animals receiving prolonged therapy with cyclophosphamide, 1.8 mg/week. Subsequent descriptions of intermittent cyclophosphamide therapy in female NZB/NZW mice mentioned tumors in 2 to 86% of treated animals, compared to tumor occurrence of 0 to 3% in controls (Casey, 1968; Horowitz et al., 1969; Hahn et al., 1975; Morris et al., 1976). In one report malignancies were found in 72% of mice receiving daily therapy, whereas 55% of mice treated on a weekly basis had tumors (Hahn et al., 1975). This information suggested that daily treatment had a greater oncogenic potential compared to weekly therapy. However, careful determination of the true incidences of neoplasms in daily versus intermittent treatment groups was hindered

because some animals were killed at intervals before neoplasms appeared, and other mice died spontaneously and were not necropsied.

In the current study, mice were examined carefully on a daily basis and only 2 treated animals were lost because of autolysis and iatrogenic death. Reliable information concerning causes of death was therefore available in 91% of mice receiving pulse therapy, and the favorable therapeutic outcome and undesirable oncogenic properties of the weekly dose regime were verified.

The etiology of neoplasms in cyclophosphamide-treated NZB/NZW mice is not known. Age-related loss of cell mediated immune defenses (Krakauer et al., 1976) and the presence of oncogenic C-type viruses in New Zealand mice (Gazdar et al., 1972; Croker et al., 1974) are important factors which may contribute to oncogenesis in these immunologically flawed animals. Although NZB/NZW mice may be predisposed to develop neoplasms, malignancies were not common in females in the Ann Arbor colony because early death with renal failure obscured the true occurrence of neoplasms (Walker and Bole, 1973; Walker and Anver, 1978).

Results of therapeutic experiments in this laboratory supported the theory that increased longevity was associated with oncogenesis in female NZB/NZW hybrids. Artificial prolongation of life in NZB/NZW females treated with varied agents such as frentizole (Walker et al., 1982) and high-dose hydrocortisone (Walker et al., 1978) was associated with tumor incidence of 29 to 76%. Despite these findings, other evidence suggests that therapy with cyclophosphamide may contribute directly to oncogenesis in hybrid New Zealand mice. Female NZB/NZW mice treated with a high dose of cyclophosphamide (16 mg/kg/day) have earlier appearance of neoplasms compared to NZB/NZW females treated with 8 mg/kg/day (Walker and Anver, 1979). A therapeutic study utilizing long-lived NZB/NZW males provided additional evidence that cyclophosphamide is a carcinogen. Long-term administration of cyclophosphamide, 8 mg/kg/day, was associated with a tumor incidence of 89%. In contrast, neoplasms were observed in 13% of male controls (Walker and Bole, 1973).

The current study confirms the oncogenic potential of cyclophosphamide in female NZB/NZW mice. Longevity was similar in mice treated with weekly and daily cyclophosphamide, and the same pattern of neoplasia was observed in both groups. Malignant lymphomas and sarcomas were found relatively early, after 28 to 70 weeks of treatment. Later in the course of therapy (50 to 86 weeks) localized neoplasms appeared as solitary or multiple lesions. Lymphomas formed a greater percentage of the total number of neoplasms in mice treated daily, and sarcomas were the most common lesions in mice receiving weekly therapy. Despite these minor differences, the similar results of weekly and daily treatment support the conclusion that treatment intervals are of minor importance in determining outcome in female NZB/NZW mice treated with cyclophosphamide.

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