

## ACCELERATED APPEARANCE OF NEOPLASMS IN FEMALE NZB/NZW MICE TREATED WITH HIGH-DOSE CYCLOPHOSPHAMIDE

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Prolonged immunosuppressive therapy with cyclophosphamide increases the prevalence of neoplasms in NZB/NZW mice, an animal model of systemic lupus erythematosus. The current study was designed to compare the oncogenic properties of high dose cyclophosphamide with a low dose therapeutic regimen. Female NZB/NZW mice received life-long therapy with "high dose" cyclophosphamide, 16 mg/kg/day, or "low dose" cyclophosphamide, 5.7 mg/kg/day; control mice received saline. High dose therapy clearly accelerated appearance of neoplasms. Seventeen of 19 mice treated with high-dose cyclophosphamide developed neoplasms at the mean age of 61 weeks. Fifty-seven percent of these tumors were mammary carcinomas. Neoplasms appeared in all mice treated with low dose; mean longevity in this treatment group was 80 weeks (compared to high dose treated mice,  $P < 0.001$ ). Carcinomas, pulmonary adenomas, and lymphomas were the most common tumors in mice receiving low dose therapy. Positive tests for ANA were suppressed in high dose treated mice. AntiDNA antibody levels and glomerulonephritis were decreased significantly in both groups of cyclo-

phosphamide-treated mice compared to controls. It was concluded that the high daily dose of immunosuppressive drug was related to early oncogenesis in autoimmune NZB/NZW mice.

Hybrid New Zealand Black/New Zealand White (NZB/NZW) mice spontaneously develop heterogeneous antinuclear antibodies detected by indirect immunofluorescence (ANA) (1), specific antibodies to DNA (antiDNA) (2), and immune complex glomerulonephritis (3). Disease is accelerated in females, and 50% of female NZB/NZW mice die with renal failure at 10 months of age (1). These animals are accepted as models of systemic lupus erythematosus (SLE). Early experiments in this laboratory showed that long-term treatment with the potent immunosuppressive drug cyclophosphamide effectively suppressed antiDNA, prevented glomerulonephritis, and prolonged lifespans in female NZB/NZW mice. Ninety-four percent of mice receiving cyclophosphamide, 8 mg/kg/day, developed neoplasms. Only 9% of untreated control mice died with malignancies. Neoplasms appeared in mice receiving therapy with cyclophosphamide for periods ranging from 30 to 93 weeks (4). The high incidence of malignancies in these old mice suggested that cyclophosphamide caused neoplastic transformation in treated animals. However, it may be argued that NZB/NZW mice developed neoplasms as a consequence of aging after their lives were prolonged artificially by immunosuppressive treatment.

In the current study, additional groups of NZB/NZW mice received life-long therapy with "low dose" cyclophosphamide, 5.7 mg/kg/day, or "high dose" cyclophosphamide, 16 mg/kg/day; control mice received saline. If neoplasms were associated with aging in New

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**Table 1.** Comparison of neoplasms in NZB/NZW mice treated with cyclophosphamide

Therapy	Mice with neoplasia/total mice in group	Mice with multiple neoplasms	Total neoplasms	Classification of neoplasms					
				Lymphomas	Mammary carcinomas	Other carcinomas	Pulmonary adenomas	Sarcomas	Other neoplasms
Cy, 16 mg/kg/day	17/19*	8	28	2	16	4	0	6	0
Cy, 5.7 mg/kg/day	15/15*	11	27	6	5	3	7	4	2†

\* Mice dying of iatrogenic causes or lost because of autolysis were excluded from this table.

† Cutaneous histiocytoma (1), chondroma (1).

Zealand mice with lives extended by therapy, tumors would appear in both high dose and low dose treatment groups after prolonged exposure to cyclophosphamide. On the other hand, if cyclophosphamide were oncogenic, neoplasms might appear earlier in mice treated with the larger dose of cyclophosphamide. The significantly earlier appearance of tumors in high dose treated mice compared to low dose treated mice supported the concept that cyclophosphamide has oncogenic properties in NZB/NZW mice.

## MATERIALS AND METHODS

**Animals.** Breeding and maintenance of New Zealand Black (NZB), New Zealand White (NZW), and NZB/NZW mice in the Rackham Arthritis Research Unit were described in another publication (4).

**Treatment protocol.** Cyclophosphamide (Mead Johnson and Co., Evansville, Indiana) was dissolved in sterile 0.15M NaCl immediately before use and given by subcutaneous injection to 2 groups of female NZB/NZW mice. Twenty-one mice (mean age 9 weeks  $\pm$  0.1) were injected with cyclophosphamide, 16 mg/kg/day. Seventeen mice with a mean age of 7 weeks ( $\pm$  0.4 SE) received cyclophosphamide, 5.7 mg/kg/day. Fifteen female control mice (mean age 11 weeks  $\pm$  1) received daily injections of 0.1 ml 0.15M NaCl. All 3 groups of mice were treated until death.

Results of therapy in mice treated with the small dose of cyclophosphamide and in control mice were reported elsewhere (5). These animals are included here to permit comparison with the 16 mg/kg/day treatment group. Control mice were entered into the study 8 months after treatment was started in the 5.7 mg/kg/day treatment group. Six months later the 16 mg/kg/day treatment group was established. Because of the increased longevity in mice receiving low-dose cyclophosphamide, 94% of these animals were alive when saline injections were started in the control mice. Therefore, lifespans overlapped in all 3 groups of mice.

**Procedures.** Mice were bled by puncture of the orbital plexus before treatment began and after 24 and 52 weeks of therapy. Terminal blood samples were obtained from 49 animals. Sera were stored in sealed capillary tubes at  $-20^{\circ}\text{C}$ . Mice were examined daily. They were killed when they were moribund or when they developed neoplasms. Complete autopsies were performed and tissue was processed, stained, and examined by light microscopy using methods described in earlier publications from this laboratory (4,6). Renal disease was

scored by counting numbers of specific histologic abnormalities in 20 glomeruli in a  $4\mu$  section from each kidney (7,8). The presence of arteritis was recorded, and periarterial lymphocyte collections were graded on a scale of 0 to 4+: 0 = no lymphocytes, 1+ = few lymphocytes, 2+ = lymphocytes surrounded 50% of arterial wall, 3+ = lymphocytes surrounded 75% of arterial wall, 4+ = lymphocytes surrounded entire artery.

**Autoantibodies.** Heterogeneous ANA were detected in undiluted mouse serum tested on human leukocyte substrate (9). AntiDNA antibodies were measured using a modified Farr assay; values greater than 20% binding indicated the presence of an abnormal amount of antiDNA (9).

**Statistical analysis.** Student's *t* test and  $\chi^2$  analysis were calculated as described by Snedecor and Cochran (10).

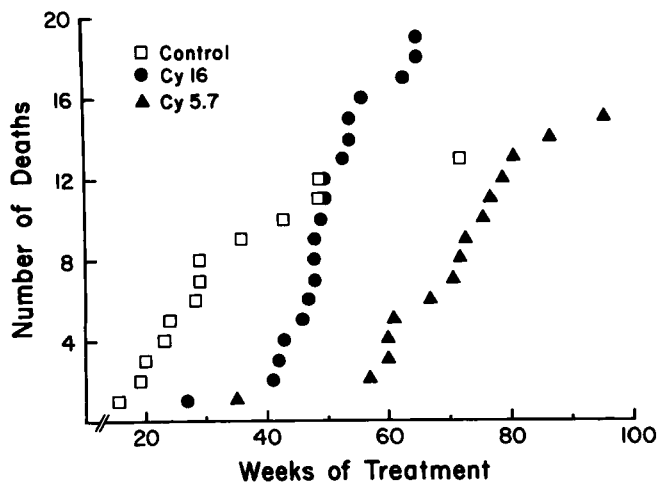
## RESULTS

**Longevity.** Two mice from each treatment group and 2 mice from the control group died of iatrogenic causes or were lost because of autolysis. These 6 animals were not included in descriptions of longevity, neoplasms, and renal pathology.

The mean age at death in control mice was 46 weeks  $\pm$  4; one untreated mouse survived to 77 weeks of age. Lifespans were prolonged significantly in cyclophosphamide-treated mice compared to control mice. In the 16 mg/kg/day treatment group, the mean age at death was 59 weeks  $\pm$  2 ( $P < 0.005$ ). In the group treated with cyclophosphamide, 5.7 mg/kg/day, mean longevity was 80 weeks  $\pm$  3 weeks. Mice in the high dose treatment group had significantly decreased mean lifespan compared to mice treated with the low dose of cyclophosphamide ( $P < 0.001$ ). The early deaths in high dose treated mice reflected premature appearance of neoplasms.

**Causes of death.** All 13 control mice that were followed until spontaneous death had vasculitis and severe proliferative glomerulonephritis. These lesions are characteristically present in untreated NZB/NZW mice (1). Control mice did not have neoplasms.

Table 1 lists the results of postmortem examinations in treated mice. Neoplasms were found in 17 of



**Figure 1.** Cumulative deaths in 47 cyclophosphamide-treated and control mice are illustrated in this graph. Six mice dying of iatrogenic causes or lost by autolysis were excluded. Mean lifespans in each group of treated mice were prolonged significantly compared to control mice. Mice receiving the higher dose of cyclophosphamide (16 mg/kg/day) had accelerated appearance of neoplasms and died earlier compared to mice treated with cyclophosphamide, 5.7 mg/kg/day.

the 19 mice that were not lost by iatrogenic death or autolysis in the 16 mg/kg/day treatment group. Multiple neoplasms appeared in 8 mice. A total of 28 neoplasms were found in mice receiving the larger dose of cyclophosphamide. One mouse died with pneumonia, and one mouse had extensive liver infarcts. All 15 mice that were treated with cyclophosphamide, 5.7 mg/kg/day until death, died with malignant neoplasms or were killed when tumors were found by physical examination. Eleven of these mice had more than one neoplasm, and a total of 27 neoplasms were identified in the 15 mice in this treatment group. High dose cyclophosphamide therapy accelerated oncogenesis in NZB/NZW mice. High dose mice were treated for a mean period of 50 weeks  $\pm$  2 before neoplasms caused death. This period was increased significantly compared to low dose treated mice, whose mean duration of therapy was 73 weeks  $\pm$  3 ( $P < 0.001$ ).

Early mortality, reflecting accelerated appearance of neoplasms in the 16 mg/kg/day treatment group, is illustrated in Figure 1. The first neoplasms in a high dose treated mouse were found after 26 weeks of therapy in an animal with pulmonary adenocarcinoma and squamous cell carcinoma of the bladder. One half of the mice in this group died with neoplasms by the forty-ninth week of treatment; the last survivors developed tumors after 65 weeks of therapy. The first mouse in the low dose treatment group to develop a neoplasm died with a reticulum cell sarcoma after 35 weeks of treatment. Fifty percent of mice receiving cyclophosphamide, 5.7 mg/kg/day, were dead with neoplasms in the seventy-second week of treatment. The oldest mouse in this treatment group died after 96 weeks of therapy.

Another characteristic that differentiated mice in the high dose treatment group from low dose treated mice was the classification of neoplasms (Table 1). Two lymphomas appeared in the high dose treatment group. Sixteen mammary carcinomas were found in this treatment group; 4 additional carcinomas involved bladder or vulva. Pulmonary adenomas were not identified in high dose treated mice. In contrast, 6 mice that were treated with low dose cyclophosphamide developed malignant lymphomas. Eight carcinomas, 7 pulmonary adenomas, 4 sarcomas, one local histiocytoma, and one chondroma were identified.

**Cumulative doses of cyclophosphamide.** Total doses of cyclophosphamide given to mice with lymphomas and other neoplasms are listed in Table 2. High dose therapy with cyclophosphamide, 16 mg/kg/day, was associated with two lymphomas. These malignancies appeared in mice that received total doses of 158 and 161 mg of cyclophosphamide during 47 and 48 weeks of treatment. In mice treated with cyclophosphamide, 5.7 mg/kg/day, 6 lymphomas appeared after a mean treatment period of 62 weeks. The mean cumulative dose of drug in these mice was 74 mg. Other neoplasms in this treatment group appeared after a mean treatment period of 76 weeks, wherein the mice received a mean total cyclophosphamide dose of 90 mg.

**Antinuclear antibodies.** After 24 weeks of treat-

**Table 2.** Cumulative doses of cyclophosphamide in NZB/NZW mice

Therapy	Lymphomas		Other neoplasms	
	Duration (wks)	Total dose (mg)	Duration (wks)	Total dose (mg)
Cy, 16 mg/kg/day	47, 48	158, 161	47 (26-65)*	168 (87-218)
Cy, 5.7 mg/kg/day	62 (35-76)	74 (42-90)	76 (60-96)	90 (71-114)

\* Mean. Parentheses enclose range.

**Table 3.** Autoantibodies in control and cyclophosphamide-treated NZB/NZW mice

Therapy	Weeks of treatment*			
	0	24	52	Terminal
<i>ANA</i>				
Controls	5/15 (33)	11/11 (100)	1/1 (100)	12/12 (100)
Cy, 16 mg/kg/day	15/19 (79)	7/19† (37)	0/3 (0)	16/18 (89)
Cy, 5.7 mg/kg/day	11/20 (55)	18/20 (90)	15/15 (100)	11/14 (79)
<i>AntiDNA</i>				
Controls	16 ± 0.6‡	38 ± 5	45	21 ± 1
Cy, 16 mg/kg/day	24 ± 1	22 ± 1§	16 ± 1	27 ± 2
Cy, 5.7 mg/kg/day	13 ± 0.4	16 ± 1§	22 ± 2	16 ± 2

\* Numbers of mice with positive indirect immunofluorescent tests for heterogeneous ANA/number of mice tested. Parentheses enclose percent of mice with positive tests at each bleeding point.

† Compared to control mice,  $P < 0.005$ .

‡ Mean ± SE. A modified Farr technique was used to test sera for antiDNA. Values are expressed as percent of  $^{14}\text{C}$ -labeled DNA bound to 0.15 ml of mouse serum.

§ Compared to control mice,  $P < 0.005$ .

ment, ANA tests were positive in 100% of control mice and 90% of low dose treated mice (Table 3). In high dose treated animals, 37% of mice were ANA-positive after 24 weeks of therapy; the incidence of positive tests was decreased significantly compared to controls ( $P < 0.005$ ).

**AntiDNA antibodies.** In control mice, mean antiDNA levels increased from 16% to 38% in the first 24 weeks of the study (Table 3). In mice treated with cyclophosphamide, 16 mg/kg/day, mean antiDNA values decreased from 24% to 16% during 52 weeks of therapy. Mean antiDNA values in mice treated with cyclophosphamide, 5.7 mg/kg/day, were 16% after 24 weeks of therapy and 22% after 52 weeks of treatment. Cyclophosphamide-induced suppression of antiDNA was evident in both groups of treated mice after the first 24-week period of treatment. At this point, mean antiDNA levels in high dose ( $P < 0.001$ ) and low dose ( $P < 0.001$ ) treatment groups were decreased significantly compared to control mice.

**Renal lesions.** Glomerular abnormalities in control and treated mice are listed in Table 4. Severe proliferative glomerulonephritis in control animals caused the mean glomerular lesion count of  $53 \pm 2$ . Renal arteritis was found in 9 of 13 control mice. Significantly lower mean glomerular lesion counts and the absence of arteritis in both groups of treated mice reflected the protective effect of cyclophosphamide therapy. In mice that received the high dose of cyclophosphamide, the mean glomerular lesion count was  $14 \pm 1$ . The mean glomerular lesion count in mice treated with low dose cyclophosphamide was  $21 \pm 2$ .

## DISCUSSION

The use of cytotoxic drugs to treat patients with inflammatory diseases of connective tissue has raised the important question of whether these patients are at increased risk to develop neoplasms. To gather additional information pertaining to tumor occurrence in immunosuppressive therapy of autoimmune disease, an ongoing project in this laboratory has examined neoplasia in NZB/NZW mice receiving the alkylating agent cyclophosphamide. Female NZB/NZW mice were chosen for this study because their autoimmune disease is analogous to SLE in humans (3).

Early therapeutic studies reported by Russell and Hicks showed that cyclophosphamide treatment was associated with neoplasms in 29% of female NZB/NZW mice in the long-term treatment group (11). Additional experiments in this laboratory verified the oncogenic potential of cyclophosphamide in hybrid New Zealand mice. Lifelong treatment of NZB/NZW mice with cyclophosphamide, 8 mg/kg/day, suppressed antiDNA and prevented development of severe glomerulo-

**Table 4.** Glomerular lesions and renal arteritis in control and cyclophosphamide-treated NZB/NZW mice

Therapy	Glomerular lesions	Arteritis
Controls (saline only)	$53 \pm 2^*$	9/13†
Cy, 16 mg/kg/day	$14 \pm 1‡$	0/19
Cy, 5.7 mg/kg/day	$21 \pm 2‡$	0/15

\* Mean ± SE. Lesions were counted in 20 glomeruli in a  $4\mu$  section of renal tissue.

† Number of kidneys with arteritis/number of kidneys examined.

‡ Compared to control mice,  $P < 0.001$ .

nephritis (9). Lifespans were prolonged significantly in treated female mice; mean longevity in this group was 77 weeks compared to longevity of 51 weeks in female control mice ( $P = 0.0005$ ). Neoplasms arose in 100% of treated females, compared to tumor incidence of 6% in control female mice (4). The increased incidence of neoplasms in cyclophosphamide-treated mice was confirmed by other investigators (12,13).

Untreated NZB/NZW mice have a low background incidence of malignancy (4,5). However, these animals may succumb to autoimmune disease at an early age before neoplasms appear. The appearance of neoplasms in NZB/NZW mice with lives prolonged by cyclophosphamide therapy may have resulted from age-related changes in immunologic response. NZB/NZW mice lose T-cell function after the age of 18 weeks (14), and old mice lack an important immunologic defense mechanism that might protect them from developing cancer. Furthermore, New Zealand mice carry type C viruses capable of inducing lymphomas or sarcomas. Injections of murine leukemia virus obtained from NZB lymphoblasts resulted in formation of lymphomas in genetically susceptible BALB/c  $\times$  NZB  $F_1$  hybrid mice (15). In another study, budding type C viral particles were identified in a sarcoma that arose spontaneously in an untreated NZB/NZW mouse. Inoculation of this potent virus produced rapid development of tumors in BALB/c mice, rats, and hamsters (16). It is postulated that type C viruses proliferate and induce neoplasms in old mice with age-related suppression of protective immune systems.

In this laboratory therapeutic experiments with hydrocortisone showed that neoplasia was an important consequence of prolonged therapy of New Zealand mouse disease. Female NZB/NZW mice treated with hydrocortisone sodium succinate, 10 mg/kg/day, had extreme prolongation of life which exceeded lifespans in cyclophosphamide-treated mice. AntiDNA antibodies were produced throughout the first year of treatment, and delayed onset of renal insufficiency appeared to result from the potent antiinflammatory action of hydrocortisone. Neoplasms arose in 76% of hydrocortisone-treated mice. The pattern of oncogenesis in mice treated with corticosteroids differed from that in cyclophosphamide-treated mice. Fifty-three percent of the neoplasms in mice treated with hydrocortisone were sarcomas. Although this study did not separate the effects of aging from possible oncogenic effects of prolonged corticosteroid therapy, it supported the theory that tumors are common in aged, treated female NZB/NZW mice (6).

Other studies have shown that cyclophosphamide is carcinogenic in both mice and rats. When A strain mice were treated with cyclophosphamide, 5 mg/kg/day, twice a week for 15 weeks, tumors were found in 38% of treated mice compared to 18% of control mice (17). Furthermore, cyclophosphamide therapy is associated with damaged bladder epithelium in mice (18) and bladder neoplasms in rats. Fischer rats treated with the bladder carcinogen, N-2-fluorenylacetamide, received a single intraperitoneal injection of cyclophosphamide followed by a pyridoxine-deficient diet. These animals had accelerated appearance of bladder carcinomas and a higher incidence of carcinomas compared to rats treated with the carcinogen and deficient diet alone (19).

The theory that cyclophosphamide therapy is directly responsible for tumor genesis in NZB/NZW mice is supported by results of treating male New Zealand mice in this laboratory. In male NZB/NZW mice receiving lifelong treatment with cyclophosphamide, 8 mg/kg/day, autoimmune disease was suppressed but lifespans were not prolonged. Mean longevity was 90 weeks in treated males and 76 weeks in control males ( $P = 0.08$ ). Nevertheless, tumors arose in 89% of treated male mice compared to a significantly lower tumor incidence of 13% in untreated male mice ( $P = 0.003$ ) (4). Hahn and associates have reported that cyclophosphamide is more oncogenic than azathioprine in New Zealand mice. NZB/NZW mice received long-term treatment with cyclophosphamide or azathioprine-plus-prednisolone and lifespans were prolonged in both groups of mice. Neoplasms were found in 61% of cyclophosphamide-treated mice. In contrast, none of the mice that received azathioprine-plus-prednisolone developed neoplasms (12).

Sullins and associates studied neoplasia in NZB/NZW mice treated with 3 doses of cyclophosphamide—1.5, 3.5, and 12 mg/kg/day. Incidences of lymphomas in each treatment group were 17%, 40%, and 89% respectively. The incidence of other neoplasms was not recorded. Although numbers of lymphomas increased with size of the daily dose of cyclophosphamide, there was no correlation between incidence of lymphomas and cumulative dose of drug or number of weeks in the treatment period (20). The current study confirmed the finding that lymphomas were not associated with total dose of cyclophosphamide or duration of therapy. However, we found that mammary carcinomas were the most common neoplasm in mice receiving the higher dose of immunosuppressive drug. The different in-

cidence of lymphomas versus carcinomas in the two studies is not readily explained. Mice bearing localized mammary tumors might have developed lymphomas at a later time if they had continued treatment.

In summary, extended therapeutic studies have shown that oncogenesis in New Zealand mice is accelerated by prolonged treatment with large daily doses of cyclophosphamide. Cyclophosphamide is the most oncogenic immunosuppressive drug in NZB/NZW mice; hydrocortisone therapy is associated with a smaller number of neoplasms. The pattern of oncogenesis in cyclophosphamide-treated mice is influenced by the daily dose of drug. Earlier studies in this laboratory showed that a very low dose of cyclophosphamide, 1.5 mg/kg/day, did not suppress autoimmune disease in NZB/NZW mice. This ineffective dose also failed to produce neoplasms. Mice receiving larger doses (5.7 and 8 mg/kg/day) were protected from antiDNA antibodies and glomerulonephritis. Nevertheless, these immunosuppressed animals had a high incidence of neoplasms (4). In the current study, a very large daily dose of cyclophosphamide (16 mg/kg/day) accelerated the appearance of neoplasms. This finding confirms the oncogenic properties of cyclophosphamide in NZB/NZW mice.

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