SELECTIVE SUPPRESSION OF AUTOANTIBODY RESPONSES IN NZB/NZW MICE TREATED WITH LONG-TERM CYCLOPHOSPHAMIDE

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Autoimmune responses were assayed in 80 cyclophosphamide-treated and control NZB/NZW mice over a period of 1 year. Fluctuation between positive and negative immunofluorescent heterogeneous ANA tests and daily alterations of ANA titers were detected in young mice of both sexes. Although high-dose cyclophosphamide therapy (8 mg/kg/day) failed to prevent the spontaneous appearance of ANA, titered ANA values were partially suppressed in high-dose treated mice. This study permitted sequential comparisons between ANA titers and anti-DNA as useful indices of cyclophosphamide-induced suppression of autoimmune disease. ANA titers were relatively resistant to cyclophosphamide therapy. Antibodies directed specifically against DNA were suppressed in mice receiving high-dose cyclophosphamide. In treated animals, decreased anti-DNA levels were associated with protection from severe glomerulonephritis and renal vasculitis. Treatment with low-dose cyclophosphamide (1 mg/kg/day) appeared paradoxically to stimulate autoantibody production and renal disease/vasculitis.

Evolution of autoimmune disease in hybrid New Zealand Black/New Zealand White (NZB/NZW) mice is characterized by the spontaneous appearance of autoantibodies and immune complex glomerulonephritis (1). Therapeutic studies have demonstrated that selected immunosuppressive drugs effectively modify the course of disease in these animals. In 1966 Russell and coworkers (2) reported suppression of renal disease and prolongation of life in female NZB/NZW mice treated with the alkylating agent cyclophosphamide. Other studies have shown that the therapeutic benefits of cyclophosphamide noted in NZB/NZW mice were associated with decreased numbers of positive immunofluorescent tests for antinuclear antibodies (ANA) (3), and with delay in appearance of antibodies directed specifically against deoxyribonucleic acid (anti-DNA) (4). This evidence has supported the use of cyclophosphamide in treating patients with systemic lupus erythematosus (5).

This report describes the effects of prolonged

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therapy with high (8 mg/kg/day) and low (1 mg/kg/day) doses of cyclophosphamide on autoantibody formation and renal disease in hybrid New Zealand mice. These animals were treated with cyclophosphamide until death. The incidence of neoplasms was 94% in high-dose treated mice, 21% in low-dose treated mice, and 9% in control mice (6). Aberrations in autoantibody responses detected in terminal sera collected from mice dying with lymphoreticular neoplasms will be described in another report (7).

Unexpectedly, variation between positive and negative tests for ANA in individual animals was common in young mice of both sexes. This variability persisted in older control male mice and high-dose treated females and males. Although therapy with cyclophosphamide failed to prevent the spontaneous appearance of positive tests for heterogeneous ANA, titered ANA values were partially suppressed in high-dose treated mice. Assays for anti-DNA showed that this antibody was decreased significantly after prolonged treatment with high-dose cyclophosphamide. In mice treated with low-dose cyclophosphamide the incidence of ANA, elevated ANA titers, and anti-DNA rose steadily over the 52-week period of observation. Paradoxically, low-dose therapy appeared to accelerate autoimmune disease in hybrid New Zealand mice.

MATERIALS AND METHODS

Animals. Breeding pairs of New Zealand Black (NZB) and New Zealand White (NZW) mice were obtained from Mr. W.D. Hall, the University of Otago, Dunedin, New Zealand. Each colony is maintained by brother-sister matings (6).

Treatment Protocol. Eighty littermate female and male NZB/NZW mice aged 4 to 24 weeks were assigned at random to treatment and control groups. Weighed aliquots of cyclophosphamide (Cytoxan, Mead Johnson and Co, Evansville, Indiana) were dissolved in sterile 0.15 *M* NaCl immediately before administration. Each dose was injected in 0.1 ml volume into interscapular subcutaneous tissue of each mouse. Two doses were employed: 10 females and 10 males received daily injections of high-dose cyclophosphamide (8 mg/kg/day), and 10 females and 10 males were given low-dose cyclophosphamide (1 mg/kg/day). Twenty female control mice and 20 male controls received daily injections of 0.1 ml sterile 0.15 *M* NaCl.

All mice were bled from the orbital plexus before injections started, and after 8, 24, and 52 weeks of treatment. Moribund mice were bled, killed, and autopsied. After 93 weeks, 5 surviving mice, aged 100 to 103 weeks, were killed to end the experiment. Blood was collected in plain capillary tubes and allowed to clot at room temperature. After centrifugation, serum was separated and stored in sealed capillary tubes without preservatives at −20°C. This serum was used for ANA and anti-DNA tests.

Table 1. *Fluctuation Between Positive and Negative Tests for ANA in Serial Examinations of Sera from Untreated 5-Week-Old NZB/NZW Mice**

	Mouse Identification Number	Positive Tests
Female mice	946	4 (1–7)†
	947	1 (7)
	948	3 (1–3)
	954	4 (1–3)
	955	1 (3)
	956	3 (1)
		Total 16
Male mice	950	3 (3)
	951	3 (1–3)
	942	2 (3)
	943	4 (1–3)
	944	6 (1–5)
	949	5 (1–3)
		Total 23

*Each mouse was bled on alternate days for a period of 2 weeks. Seven samples of sera from each animal were tested.
†Number of positive tests. Parentheses enclose highest tube number ranges (1–$\log_2$ of serum dilution) giving a positive test for ANA detected by an indirect immunofluorescent technique.

Immunofluorescent Tests for ANA. The indirect immunofluorescent procedure described by Friou (8) and adapted for use in this laboratory (9) was used to test thawed mouse sera for heterogeneous ANA on human leukocyte substrate. Fluorescein-conjugated goat anti-mouse 7S γ-globulin (Hyland Laboratories, Costa Mesa, California) was employed. This antiserum was characterized by a molar F/P ratio of 3.8 and a protein concentration of 18.4 mg/ml (10). Antiserum was diluted 1:20 before use. Each sample of mouse serum was tested undiluted (1:1) and after addition of phosphate-buffered saline (pH 7.0) at dilutions of 1:4, 1:16, 1:64, 1:256, and 1:1024. In fourteen instances, sera diluted 1:1024 gave positive tests. These samples were diluted to 1:2048, and after repeated testing each serum at this dilution was ANA-negative. Each tube of diluted serum was given a number corresponding to 1–$\log_2$ of the dilution. Therefore, undiluted serum (1:1) was designated 1, 1:4 = 3, 1:16 = 5, etc. Results were expressed as the highest tube number giving a positive test for ANA.

Three patterns of nuclear fluorescence were identified in positive tests: rim, homogeneous, and speckled. The typical appearance of each pattern on human white blood cell substrate has been described in another report from this laboratory (9). In many instances more than one pattern was found in individual nuclei on a single slide. This phenomenon was termed a "mixed" pattern.

Day-to-day fluctuation between positive and negative immunofluorescent ANA tests was studied in a separate group of 12 young NZB/NZW mice (Table 1). To test

Table 2. *Characterization of KB Cell-derived ^{14}C-labeled DNA Used in Anti-DNA Determinations*

Nitrocellulose Filter* Studies	Mean (counts/min)
1. Control—0.6 *M* TCA precipitation	2636
2. Test RNA contamination (13)	
RNAase† incubation	2612
NaOH hydrolysis	3005
3. Test DNA purity—DNAase‡ incubation	328
4. Test DNA "double-strandedness"	
2 × SSC§ filtration (14)	733
0.5 *M* NaCl filtration (15)	811
S_1 nuclease|| incubation (16)	2622

Specific activity: 0.06 μCi/μg (11,600 counts/min/μg). Counting efficiency: 88%.

Spectrophotometric characterization (13). 260:280 mμ ratio = 1.9; 260:230 mμ ratio = 2.1.

Protein contamination not detected by Lowry test (17).

*Each test utilized 0.26 μg (0.005 ml) ^{14}C-labeled KB cell-derived DNA, and type B-6 (0.45 μ) nitrocellulose filters, Schleicher and Schuell Inc, Keene, New Hampshire.

†Ribonuclease A, boiled, Worthington Biochemical Corp, Freehold, New Jersey.

‡Deoxyribonuclease I (Bovine pancreas), P-L Biochemicals Inc, Milwaukee, Wisconsin.

§One liter 2× SSC contains NaCl, 17.5 g, and Na citrate, 8.8 g.

||This enzyme degraded specifically 92% of denatured DNA in this test system. Experiments described in this table indicated that > 95% of ^{14}C-labeled DNA used in anti-DNA determinations was double-stranded.

the reproducibility of titered ANA determinations, pooled ANA-positive serum from untreated 12-month-old female NZB/NZW mice was divided into aliquots and frozen. This serum was tested repeatedly on 6 successive days.

Anti-DNA Tests. KB cells of human tumor origin (11) grown in spinner culture (900 ml, 200,000 cells/ml) were exposed to 250 μCi thymidine-2-^{14}C (specific activity 54.1 Ci/mm) (New England Nuclear Corp, Boston, Massachusetts) at 37°C for 72 hours. ^{14}C-labeled DNA was separated from the cells by phenol extraction using the method described by Pincus *et al* (12). The concentration of recovered DNA was calculated by assuming an optical density of 1.0 at 260 mμ for a solution containing 50 μg DNA/ml. Spectrophotometric, chemical, and enzymatic characterizations of the radioactive DNA preparation are listed in Table 2.

DNA-binding tests were performed using a modification of the Farr technique described by Steinberg *et al* (18). Optimal antigen-antibody concentrations used in anti-DNA tests were determined initially by setting up titration curves to study the effects of increasing volumes of mouse sera or ^{14}C-labeled DNA on the binding reaction. Serum was heated at 56°C for 60 minutes to decrease nonspecific binding. A serum aliquot of 0.015 ml and 0.034 μg labeled DNA (400 counts/minute) were employed in each test. After incubation at 37°C and 4°C, addition of saturated $(NH_4)_2SO_4$, and centrifugation, supernate and dissolved precipitate were applied separately to 1-inch squares of Whatman 3-mm filter paper. Each dried paper was placed in 20 ml of toluene scintillation fluid* and counted 10 minutes in a Packard Liquid Scintillation Counter. Percent binding was calculated using the formula:

$$100 \times \frac{\text{precipitate counts/minute}}{\text{precipitate counts/minute} + \text{supernate counts/minute}}$$

Renal Histology. Renal tissue obtained at autopsy was fixed, embedded, and stained as described in a previous report (6). Glomerular lesions in 4-μ sections of renal tissue were counted as described earlier by this laboratory (9) using an adaptation of the method of Pirani *et al* (19). Periarterial collections of lymphocytes were graded on a scale from 0 to 4+.

Statistical Analysis. The MIDAS software package (20) developed by the Statistical Research Laboratory of the University of Michigan was used within the MTS system on the IBM 360/67 computer. Least squares regression analysis was performed as described by Draper and Smith (21). Analysis of variance used to analyze the data derived from groups containing unequal numbers of mice (22) was calculated using the BMDX64 program (23), revised by the Statistical Research Laboratory of the University of Michigan for use on the MTS system. The *P* value was set at 0.05. If the attained significance level was less than 0.05, the hypothesis under consideration was rejected.

A descriptive statistical model† was derived to evaluate titered ANA data. This additive model permitted evaluation of the effects of sex, type of treatment, duration of treatment, and interactions between these parameters on ANA titers. Based upon these calculations, predicted mean ANA titers were derived for each group of mice and compared with the experimental results.

RESULTS

ANA Fluctuation in Young NZB/NZW Mice. Titered ANA tests were performed using sera collected from 12 young untreated mice (Table 1). No animals were consistently ANA-positive or -negative. A large number of positive tests were unexpectedly

*100 ml of toluene containing 4.0 g PPO (2,5-diphenyloxazole) and 0.1 g POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene).

†Initially, least squares regression techniques (21) confirmed that ANA titers did not increase consistently in individual mice as a function of age. Therefore, in subsequent calculations employing analysis of variance, age was not included as a variable that might influence titers. The usual distributional assumptions on the error term were not satisfied. The following additive model was constructed, subject to the usual zero-summability constraints of the 3-way fixed effects analysis of variance (22): $y_{ijkm} = \mu + \alpha_i + \beta_j + \eta_k + \gamma_{ij}^{(1)} + \gamma_{ik}^{(2)} + \gamma_{jk}^{(3)} + \gamma_{ijk} + e_{ijkm}$, where y_{ijkm} = ANA titer of the *m*th mouse at time *k* in the *i*th and *j*th treatment group, and e_{ijkm} is the error term. Least squares estimates (21) of the above parameters were obtained, and parameters were eliminated when the sums of squares for the effect were small compared to the total sum of squares. The model finally considered was: $y_{ijkm} = \mu + \alpha_i + \beta_j + \eta_k^{(2)} + e_{ijkm}$.

found in sera from young male mice compared to those from female mice.

ANA Reproducibility. Aliquots of pooled ANA-positive mouse serum tested on 6 successive days gave consistently reproducible ANA titers at the same end point.

Incidence of Positive ANA. The incidence of positive tests in female control mice increased from 25% to 100% during the 52-week observation period, and fluctuation between positive and negative tests rarely occurred in individual animals in this group. Twenty-five percent of cyclophosphamide-treated female mice were ANA-positive at the beginning of this study. After 24 weeks of treatment the incidence of positive tests was 85%; after 52 weeks ANA were detected in 10 surviving treated female mice. Variability between positive and negative tests occurred in 7 of 10 high-dose treated female animals. In male control mice, 10 of 20 sera collected at the beginning of the study contained ANA. This unexpected finding (24) reflected the early, transient appearance of ANA in young male NZB/NZW mice in our colony (Table 1). The incidence of positive tests for ANA increased from 45% at 8 weeks to 92% at 52 weeks in male control mice. Fluctuation between positive and negative tests occurred in 9 of 20 mice in this group. High-dose and low-dose cyclophosphamide therapy did not prevent the appearance of positive tests for ANA in male mice. In 6 of 10 high-dose male mice, fluctuation between positive and negative tests was noted.

ANA Titers. Median titers of positive ANA tests and ranges within each group of control and treated mice are listed in Table 3. Sequential median titers in ANA-positive female control mice increased from 5 to 9 over a period of 52 weeks. Fluctuating titers were observed commonly in individual untreated female mice. Only 5 of 16 female control mice surviving to the twenty-fourth week of this study had steadily increasing titers. High-dose therapy did not consistently suppress ANA titers in female mice. Median titers increased from 1 to 7 in female mice treated with cyclophosphamide (8 mg/kg/day) for 52 weeks. Sequential titers showed great variation in 9 of 10 high-dose female mice that developed ANA. In 10 female mice treated with low-dose cyclophosphamide (1 mg/kg/day), median titers increased from 5 to 7 during the course of this study. Steadily increasing titers were observed in 7 of 10 low-dose female mice. Median ANA titers in untreated male mice rose from 3 at 0 weeks to 7 at 52 weeks. Fourteen male

Table 3. *Positive Titered Tests for ANA in 80 Cyclophosphamide-treated and Control NZB/NZW Mice*

	Treatment Period (wk)			
	0	8	24	52
Females				
Treated				
High dose	1 (1–3)*	5 (3–7) [3]	3 (1–9) [4]	7 (1–9) [6]
Low dose	5 (5)	5 (3–11) [5]	7 (1–11) [6]	7 (7) [8]
Controls	5 (3–7)	5 (1–9) [5]	5 (1–11) [6]	9 (5–11) [8]
Males				
Treated				
High dose	3 (3)	3 (1–3) [2]	1 (1–5) [2]	5 (1–7) [5]
Low dose	3 (3–5)	3 (1–5) [4]	5 (3–7) [5]	9 (3–11) [7]
Controls	3 (3–7)	5 (1–7) [3]	3 (1–9) [4]	7 (3–11) [7]

*Median (range). Values are expressed as highest tube number (1—$\log_2$ of serum dilution) giving a positive test for heterogeneous ANA determined by an indirect immunofluorescent technique. Brackets enclose predicted cell means derived by least squares analysis (22) and rounded to the nearest integer.

control mice had marked fluctuations in individual ANA titers. As a group, male mice treated with cyclophosphamide (8 mg/kg/day) had the lowest median ANA titers observed in this study. Consistent suppression below comparable control values was evident throughout the first year of high-dose treatment. High-dose treated male mice demonstrated great variability of ANA titers. Median titers increased progressively from 3 to 9 in low-dose treated male mice, a fact suggesting that cyclophosphamide (1 mg/kg/day) may have augmented the autoantibody response in this group of animals.

Based on the descriptive statistical model described in Materials and Methods, predicted mean ANA titers at 8, 24, and 52 weeks were obtained by least squares analysis (21). These values are listed in Table 3. The predicted cell means suggested that mean ANA titers within each group of control and treated mice were expected to increase during the 52-week observation period. It was anticipated that titers in male control and treated mice should be lower than titers in female mice at each bleeding point. Mean ANA titers in high-dose treated mice were expected to be lower than titers in control mice of the same sex. Based upon the use of derived cell means, it was anticipated that these trends would be reproduced if this experiment were to be repeated.

In control and low-dose treated mice, individual

Table 4. *Variable Patterns of Nuclear Fluorescence in Dilutions of ANA-positive Sera from Cyclophosphamide-treated and Control NZB/NZW Mice*

	Treatment Period (wks)			
	0	8	24	52
Females and males				
Treated				
High dose	1/6*	1/7	4/9	4/14
Low dose	3/5	6/11	14/19	5/10
Controls	7/15	12/25	20/27	8/15

*Number of sera with varying ANA patterns in serial dilutions/ total number of positive tests with titers > 1:1.

samples of serum produced patterns of nuclear fluorescence that changed as each subsequent dilution of the same sample was tested for ANA. Undiluted serum from those animals usually yielded a homogeneous or mixed pattern. As each sample of serum was tested after dilution a mixed or rim pattern often emerged. The incidence of changing patterns is shown in Table 4. Variable patterns were found in sera collected from 47 of 82 control mice and in 28 of 45 sera from low-dose treated mice of both sexes. High-dose cyclophosphamide therapy was commonly associated with the production of either homogeneous or rim patterns in titered sera. In these samples, the initial rim or homogeneous pattern produced by undiluted serum usually persisted in serial dilutions of the same sample of serum. Patterns varied in only 10 of 36 ANA-positive serum samples obtained from high-dose treated female and male mice.

Anti-DNA. Mean anti-DNA values, expressed as percent of DNA bound to mouse serum measured at 0, 8, 24, and 52 weeks, are listed in Table 5. Before treatment, the range of mean DNA binding values in each group of mice was 14% to 18%. In the first 24 weeks of the study, mean anti-DNA in female control mice increased from 14% to 28%. Two ANA-positive mice in this group failed to demonstrate an increase in anti-DNA levels during the first year of observation. High-dose cyclophosphamide therapy blunted the expected age-dependent increase of DNA antibodies in female mice. After 24 weeks of treatment, female mice in this treatment group had a mean anti-DNA value of 19%, compared to 28% in female control mice. In low-dose female mice, mean DNA-binding values increased from 15% to 55% during 52 weeks of treatment. One female mouse in this group produced sera with the highest anti-DNA level recorded in this study (82% at 52 weeks).

In untreated male mice, anti-DNA increased steadily from a mean pretreatment value of 15% to 26% after 24 weeks of observation. Nine of 15 male control mice tested after 52 weeks continued to produce DNA antibodies in excess of pretreatment values. An unexpected finding was the similarity between mean DNA binding values in male control NZB/NZW mice compared to values in female control mice after 24 weeks. DNA binding did not increase in male mice receiving high-dose cyclophosphamide. Mean values measured before treatment and after 52 weeks of treatment were the same (16%). At 24 and 52 weeks, mean anti-DNA in high-dose treated male mice was appreciably lower than anti-DNA in comparable control animals. Anti-DNA in low-dose treated male mice resembled values in male control mice.

Table 5. *Mean Anti-DNA in 80 Cyclophosphamide-treated and Control Mice*

	Treatment Period (wks)			
	0	8	24	52
Females				
Treated				
High dose	16 ± 1 (8)*	14 ± 2 (9)	19 ± 1 (10)	15 ± 1 (8)
Low dose	15 ± 1 (9)	23 ± 3 (10)	31 ± 5 (7)	55 (2)
Controls	14 ± 2 (13)	21 ± 2 (20)	28 ± 3 (13)	19 ± 5 (3)
Males				
Treated				
High dose	16 ± 1 (8)	12 ± 1 (10)	17 ± 2 (6)	16 ± 1 (6)
Low dose	18 ± 2 (7)	19 ± 2 (9)	21 ± 2 (9)	21 ± 3 (8)
Controls	15 ± 1 (10)	15 ± 1 (17)	26 ± 3 (14)	25 ± 3 (15)

*Mean ± SE. Values are expressed as percent of DNA bound. DNA binding was determined by a modification of the Farr Technique. Parentheses enclose number of mice tested at each time point.

Data were evaluated by analysis of variance* (22) to determine the effects of sex, therapy, and duration of treatment on anti-DNA levels. These three factors were significant at 0.05. It was concluded that DNA binding increased significantly in all groups of control and low-dose treated mice during the first 24 weeks of this study. Therapy with cyclophosphamide (8 mg/kg/day) was associated with significant depression of anti-DNA in female and male NZB/NZW mice.

Renal Histology. Table 6 lists mean values of glomerular lesions and vascular abnormalities observed

*First, pretreatment values in treated and control mice were compared. Mean anti-DNA values in each group were similar at the beginning of the study (F statistic for each effect < 1.0; $P < 0.05$). Three-way analysis of variance was then employed to analyze anti-DNA data using the fixed effects model: $y_{ijkm} = \mu + \alpha_i + \beta_j + \eta_k + \epsilon_{ijkm}$, where y_{ijkm} = anti-DNA level and η_k = duration of treatment.

Table 6. *Renal Lesions in Cyclophosphamide-treated and Control NZB/NZW Mice*

	Glomerular Lesions	Periarterial Lymphocytes	Arteritis
Females			
Treated			
High dose	18 ± 3*	1 (0–2)†	0/10‡
Low dose	43 ± 4	3 (2–4)	2/10
Controls	44 ± 3	2 (0–4)	1/17
Males			
Treated			
High dose	23 ± 3	0 (0–2)	0/10
Low dose	46 ± 4	3 (1–4)	6/9
Controls	41 ± 3	2 (0–4)	5/15

*Mean number of histologic abnormalities counted in 20 glomeruli ± SE.
†Median (range). Periarterial lymphocyte collections were scored on a scale of 0 to 4+.
‡Number of kidneys with arteritis/number of kidneys examined.

in kidneys from 71 cyclophosphamide-treated and control NZB/NZW mice. Extensive glomerular damage was found in mice injected with saline or with cyclophosphamide, 1 mg/kg/day. Glomerular lesions were reduced significantly in mice treated with cyclophosphamide, 8 mg/kg/day. Two-way analysis of variance (22) showed that the reduced numbers of glomerular lesions in high-dose treated mice, compared to control and low-dose treated mice, were significant at 0.01. This finding was in accord with the work of other investigators (3). The massive collections of round cells that usually surround renal arteries in old hybrid New Zealand mice were markedly reduced in high-dose treated animals. Necrotizing arteritis was present in kidney tissue from 19% of control and 42% of low-dose mice of both sexes. This vascular lesion was not found in renal tissue from 20 high-dose treated mice.

DISCUSSION

This report describes serial assays of autoimmune responsiveness in 80 cyclophosphamide-treated and control NZB/NZW mice followed for a period of 1 year. In contrast to other investigations of autoantibody production in NZB/NZW mice (3,4), this study utilized sera obtained from serial bleedings of individual animals. Its procedure allowed the investigators to detect the varying presence of ANA in certain control and treated mice. A separate study provided new information showing fluctuation between positive and negative ANA tests and varying ANA titers in 5-week-old NZB/NZW mice. A new descriptive statistical model was derived to evaluate the influences of sex, type of therapy, and duration of therapy on titered ANA values. This study permitted comparison of ANA titers with anti-DNA as a useful index of cyclophosphamide-induced suppression of autoimmune disease in hybrid New Zealand mice. Heterogeneous ANA appeared to "resist" cyclophosphamide therapy, whereas anti-DNA levels were suppressed consistently in high-dose treated animals. Finally, this study contributed new evidence suggesting that prolonged administration of low-dose cyclophosphamide paradoxically accelerated autoimmune NZB/NZW disease.

In the course of this study it was discovered that hybrid mice of both sexes had an early period of ANA reactivity characterized by variable presence of autoantibodies. Day-to-day fluctuations between positive and negative tests and variable titers were observed in untreated animals of both sexes tested between 5 and 7 weeks of age (Table 1). The cause of ANA variability in these animals is not known, but inconsistent presence of ANA may reflect the transient presence of an inciting antigen in young NZB/NZW mice. Lambert and Dixon (1) demonstrated a transient nuclear antigen in serum from a single NZB/NZW mouse bled at different times. On the other hand, it may be postulated that young NZB/NZW mice have circulating ANA of low antigen-binding affinity, and these ANA are demonstrated transiently using the indirect immunofluorescent test. Petty and Steward (25) reported that young hybrid New Zealand mice produced low-affinity antibodies to human serum transferrin; the affinity of these antibodies was increased in older mice. The striking early variability of ANA may explain the unexpectedly large number of positive tests in pretreatment sera from young male NZB/NZW mice detected at a single point in time. In these young animals, the incidence of ANA in male mice temporarily exceeded ANA incidence in female mice. In older mice, inconstant positive tests persisted in 45% of control male mice and in 60% of high-dose treated male mice. Older untreated females developed ANA that were persistent. Although every male mouse in this study was ANA-positive on at least one occasion, the pronounced variability of ANA responses in control and high-dose males may have contributed to the decreased severity of their autoimmune disease.

NZB/NZW mouse disease is characterized by spontaneous appearance of autoantibodies followed by deposition of antigen-antibody complexes in renal glomeruli. Elution studies have demonstrated the presence of nuclear antigens and anti-DNA in glomerular

deposits (1). Therefore, anti-DNA has been implicated as a specific agent leading to the development of immune complex glomerulonephritis in these mice. The effectiveness of cyclophosphamide in treating renal disease in NZB/NZW mice may reflect the ability of this drug to suppress humoral antibody formation (4). Russell and coworkers (2) found that parenteral therapy with cyclophosphamide (1.8 mg/week) suppressed antibody levels in female NZB/NZW mice challenged with flagellar antigen. The immunosuppressive dose of cyclophosphamide used in their study was equivalent to the high dose of cyclophosphamide administered to mice on a daily basis in the current series. High-dose cyclophosphamide treatment (8 mg/kg/day) effectively suppressed anti-DNA levels, renal glomerular lesions, and vasculitis. Titered heterogeneous ANA levels were relatively resistant to immunosuppressive therapy. Based on information gained from this study, it was concluded that serial measurements of anti-DNA are more valuable than titered ANA in evaluating active autoimmune disease in NZB/NZW mice.

In low-dose treated mice there was no serologic or histologic evidence that administration of cyclophosphamide (1 mg/kg/day) suppressed the formation of autoantibodies. The high DNA binding levels, abundant collections of periarterial lymphocytes, and increased incidence of arteritis in these animals provided evidence that the low dose of cyclophosphamide used in this study may have paradoxically accelerated autoimmune disease in NZB/NZW mice. Although the immune-stimulating properties of cytoxic drugs are not widely recognized, other investigators have reported that administration of these agents may stimulate humoral antibody formation in certain experimental animals. Chanmougan and Schwartz (26) noted that rabbits treated with the antimetabolite 6-mercaptopurine, 10 mg/kg/day for 7 days, developed hypertrophy of the spleen and lymph nodes. If bovine γ-globulin was injected while hyperplasia persisted, humoral antibody response was enhanced. In another study a single injection of cylophosphamide, 0.5 mg, stimulated antihuman γ-globulin levels in mice (27). The enhancing properties of low-dose cyclophosphamide in this animal model of autoimmune disease are currently under further investigation in this laboratory.

This report has reemphasized the value of high-dose cyclophosphamide in treating the SLE-like disease of NZB/NZW mice. However, the encouraging results were tempered by the high incidence of neoplasia in mice receiving long-term therapy (6). Anti-DNA measurements predicted suppression of renal disease in these animals. The titered ANA, which tended to fluctuate in early or mild disease, was less reliable as a parameter of response to immunosuppressive therapy.

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