Influence of Cytoxan and Poly I·C on Early Antinuclear Antibody Responses in New Zealand Black Mice[1] (36375)

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Employing chicken red blood cell nuclei as substrate for indirect immunofluorescence assay (1), it was possible to detect low levels of antinuclear antibody (ANA) in the plasma of New Zealand Black (NZB) strain mice as early as 2 weeks of age, with strong reactivity demonstrable in 90% of mice by the time they were 5 weeks old (2, 3). These findings suggested that ANA formation might parallel the development of immunologic and hematologic abnormalities of NZB mice believed to provide a milieu favoring autoantibody formation: namely, accelerated immunologic maturation (4, 5), immunologic hyperresponsiveness (6, 7) and elevated numbers of hematopoietic stem cells (8). The present report describes attempts to modify this early ANA development by such agents as cytoxan and polyinosinic·polycytidylic acid, which have been demonstrated to influence ANA levels in older New Zealand mice (9–12).

Materials and Methods. The NZB mice represented the tenth generation in this laboratory from breeding stock (generations 57 and 58) obtained from W. H. Hall, University of Otago Medical School, Dunedin, New Zealand. Animals were injected intraperitoneally at specified ages with varying doses of saline-dissolved cytoxan (Cyclophosphamide, Mead Johnson & Co., Evansville, IN) administered on a milligram per kilogram body weight basis. Polyinosinic·polycytidylic acid (Poly I·C, Lot 001080, Calbiochem, Los Angeles, CA) was dissolved in phosphate buffered saline (pH 7.6) at a concentration of 0.8 mg/ml.

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An indirect immunofluorescence technique using formalinized chicken erythrocyte nuclei as antigen (1, 2) was routinely employed. Samples to be assayed represented 1:20 dilutions of individual mouse plasma in phosphate buffered saline (pH 7.5). Test preparations of plasma-incubated substrate were further incubated with a 1:40 dilution of fluorescein conjugated goat antimouse gamma globulin (Hyland Laboratories, Los Angeles: Lot No. 2226H0026, precipitin titer of 1:16, fluorescein/protein ratio, 3.70 mg/g). Slides were examined with a Leitz fluorescence microscope (primary BG12 and OGI barrier filters) and the intensity of fluorescence rated on a 0 to 4+ scale. Known positive and negative plasma served as controls for each assay.

Results and Discussion. In an early experiment groups of 9 to 10 baby NZB mice were injected once at 6 or at 13 days of age with 75 mg/kg of cytoxan, while control mice received isotonic saline. There was no diminution in the appearance of ANA in mice at 26 days of age as a result of this cytoxan treatment, although these animals were observed to display toxic effects such as hair loss and marked growth retardation.

Multiple administration of cytoxan in adult NZB and (NZB X NZW)F1 hybrid (B/W) mice has been previously reported (9–11) to be highly effective in delaying antinuclear antibody formation and in diminishing the incidence of associated glomerulonephritis. It thus seemed feasible to study the efficacy of cytoxan in the present system with a regimen of serial injections utilizing low dosages of the drug. In this connection, 9-day-old NZB mice were started on a course of 20 mg/kg of cytoxan twice weekly. Oc-

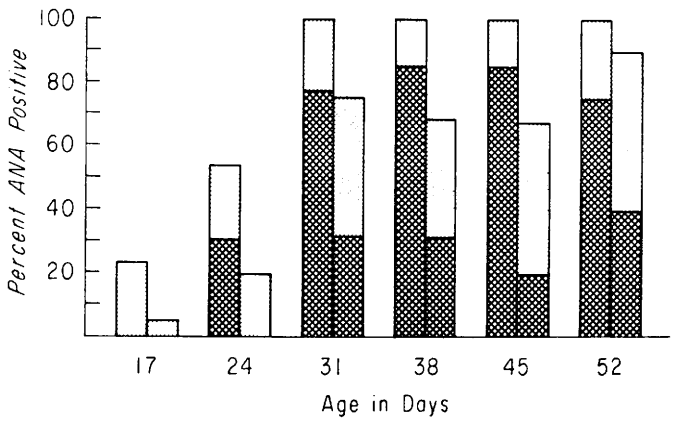

FIG. 1. Percentage of mice demonstrating designated ANA immunofluorescence response with and without cytoxan treatment: (stippled) weakly positive responses (2+); (crosshatched) strongly positive responses (3+ and 4+). Of each pair, left column represents untreated control mice ($N = 13$); right column represents mice injected with 20 mg/kg of cytoxan twice weekly from 9 days of age ($N = 20$).

currence of weak immunofluorescence at 17 days of age was observed to be more prominent in the case of controls than for experimental animals (Fig. 1). From 24 to 52 days of age (Fig. 1) the development of strong (3+ and 4+) ANA reactions in the cytoxan-treated animals was notably diminished compared to the controls. In further results not presented here, similar immunosuppression with continued cytoxan treatment was evident up to 2.5 months of age. After this time ANA reactions increased, and by 4.5 months were similar in strength to control responses. Body weights of cytoxan-treated mice remained about 8% lower than those of controls; and hair loss, evident soon after initiation of treatment, disappeared by the time the animals were 6 weeks of age.

In an examination of the influence of cytoxan on preexisting plasma ANA, 10 ANA positive, 4-month-old NZB mice were injected with 180 mg/kg of cytoxan. Immunofluorescence persisted strongly positive in tests performed 7, 14, and 21 days following treatment. Assays at these times for spleen antibody plaque-forming cells, carried out 4 and 5 days after a primary sheep erythrocyte (SRBC) immunization (13), revealed only slight to moderate immunodepression, responses ranging from 31 to 77% of those obtained for untreated control mice. This indicated the need for a more vigorous immunodepressive regimen if modification of preexisting ANA was to be achieved. In a subsequent experiment, injection of five 4-month-old NZB mice with 300 mg/kg of cytoxan resulted in a 98% depression of plaque responses following SRBC injection 3 weeks later; cytoxan-treated mice yielded an average of 8.6×10^3 plaques/spleen (range = $0.4–11.4 \times 10^3$) compared to 406.6×10^3 plaques/spleen (range = $273–600 \times 10^3$) for 5 control animals. Despite the strongly immunodepressive effect of this cytoxan dose on primary SRBC antibody formation, immunofluorescence assays revealed no reversal or attenuation of plasma ANA activity.

In another experiment it was attempted to induce tolerance to nuclear antigens in 2-month-old ANA positive NZB mice. Table I presents the ANA responses of NZB mice receiving injections of 75 mg/kg of cytoxan alone, or 160 μg of poly I·C followed 24 hr later by 75 mg/kg cytoxan. Plasma ANA was found to be virtually identical for control (cytoxan only) and experimental (poly I·C + cytoxan) groups except for attenuation of strongly positive reactions in the latter on day 14 (Table I). This effect was transient, however, as all subsequent assays demonstrated strong ANA activity for both groups of animals. Steinberg and Talal (12) have

TABLE I. Immunofluorescence with Plasma of 2-Month-Old NZB Mice Receiving Tolerogenic Treatments with Poly I·C and Cytoxan.[a]

Poly I·C, cytoxan treatment (days)	Day of ANA assay	Poly I·C + cytoxan		Cytoxan only	
		Strong ANA[b]	Weak ANA	Strong ANA	Weak ANA
0, 1	0	15/16[c]	1/16	15/15	0/15
7, 8	7	15/16	1/16	15/15	0/15
	14	6/16	9/16	13/15	2/15
	51	13/13	0/13	13/15	2/15
68, 69	92	12/12	0/12	11/13	2/13
	105	10/12	2/12	11/13	2/13
	205	7/8	1/8	8/10	2/10

[a] Poly I·C (160 μg) was injected intraperitoneally 24 hr before ip injection of 75 mg/kg of cytoxan. Control mice received cytoxan (75 mg/kg) only.

[b] Gradation of immunofluorescence used to categorize ANA response: 3–4 = strong, 2^+ = weak, $0–1^+$ = negative.

[c] Number of mice showing given reaction per total mice assayed.

succeeded in markedly diminishing the incidence and intensity of ongoing antibody responses specific to RNA in B/W mice by a series of tolerogenic treatments with poly I·C and cytoxan. The same schedule was observed to reduce anti-DNA antibodies at 20 but not 45 days after the last course of therapy. Since the present assay appears to detect largely anti-DNA activity (2), it is possible that administration of a tolerogen more closely related to DNA would be required to further diminish this response.

The present studies have described the detection of antinuclear antibodies in the plasma of very young NZB mice. This early manifestation of autoimmunity could be depressed for a few months by repeated injection of small doses of cytoxan. This observation, along with previous observations of similar ANA depression following infection of NZB mice with Rauscher leukemia virus (3), would tend to support the view that a conventional humoral immune response is involved in antinuclear antibody formation. In this regard, ANA development appeared to coincide with a period of immunologic hyperresponsiveness (6, 7) and to be coterminous with the existence in NZB mice of elevated numbers of hematopoietic stem cells (8). Thus, the relatively high levels of ANA in this mouse strain could conceivably be a consequence of the presence of unusually large numbers of responsive cells, the latter susceptible to immunodepressive manipulation of the host.

Summary. Early manifestation of antinuclear antibodies (ANA) in the plasma of NZB mice was significantly depressed by twice weekly injection of 20 mg/kg body weight cytoxan from the age of 9 days. Single injection of 75 mg/kg at 6 or at 13 days of age was ineffective in this regard. Similarly, ongoing ANA responses in 4-month-old NZB mice were undiminished by single injection of 180 or 300 mg/kg of cytoxan. A course of administration of 160 μg of poly I·C followed 24 hr later by 75 mg of cytoxan was repeated three times during a period of 56 days in an attempt to induce tolerance in 2-month-old ANA positive mice. Attenuation of ANA response was demonstrable 2 weeks after initiation of treatment, but was not observed at later times during the study, conceivably a consequence of the anti-DNA activity of the antinuclear antibody assayed.

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