STUDIES ON THE ETIOLOGY OF AUTOIMMUNE DISEASE IN NEW ZEALAND BLACK (NZB) MICE

by

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INTRODUCTION

The spontaneous development of lupus nephritis and Coombs' positive hemolytic anemia in mice of the New Zealand Black (NZB) strain has established these animals as a useful experimental model for the autoimmune diseases of the human (1, 2). Antinuclear antibodies (ANA) complexed with DNA represent a major component of the immune depositions in the capillary basement membranes of the glomeruli; this development of ANA and renal lesions mimics the syndrome of human systemic lupus erythematosus. Antierythrocyte autoantibodies, first detected in the young adult NZB, have been implicated in a hemolytic disease characterized by anemia and hepatosplenomegaly.

Autoantibody formation. In considering the factor(s) etiologic to any disease process it is critical to distinguish causal from disease-induced abnormalities. Thus, in the case of NZB autoreactivity, the primordial defect must be sought in the preautoimmune animal. The age-associated appearance of several specific autoantibodies has been clearly delineated in the NZB and indicates that the events triggering autoreactivity must occur very early in life. Antinuclear antibodies have been detected in the plasma of infant NZB mice at 2 weeks of age (3). This appearance is followed by progressive increases in incidence and titer throughout their lifespan. Consistent with this early appearance of ANA is electron microscopic evidence of immune complex deposition in the kidneys of 1-month-old (NZBXNZW)F1 hybrid (B/W) mice (4).

Antibodies reactive with autologous thymocytes (NTA) were also seen in NZB sera within the first month after birth (5). It has been

proposed that NTA may cause thymic dysfunction involving loss of regulatory T-cell or of T-cell surveillance activities, leading to further autoreactivity. Since NTA and anti-RNA autoantibodies have been shown to arise independently in B/W mice (6), no pathogenic role can be attributed at this time to NTA activity.

Plaque-forming cells secreting antibodies against mouse red blood cells have been demonstrated in NZB spleens by 16-18 weeks of age (7), although Coombs' positive hemolytic anemia is seldom detected before 8 months.

NZB Immunologic Hyperresponsiveness. The very early detection of these autoantibodies coincides with the equally early detection of immunologic hyperactivity in terms of responsiveness to a variety of foreign antigenic stimuli. Evans, Williamson and Irvine (8), and Playfair (9), reported an almost mature level of responsiveness in NZB mice immunized with sheep red blood cells (SRBC) at 5 days of age. Young adult NZBs, 1-3 months old, have been shown to be hyper-responsive to a variety of antigens including SRBC (10), proteins (11, 12) and polysaccharides (13). Since autoimmune manifestations and immunologic hyperresponsiveness both appear soon after birth, their coexistence suggested a possible common origin. It has thus seemed reasonable to visualize autoreactivity as a natural extension, or simply one manifestation, of immunologic hyperresponsiveness (14). Hence, low levels of autoantibody naturally present in nonautoimmune strains may reach pathologic proportions in the NZB.

Early immunologic hyperresponsiveness does not appear to be confined to the B-lymphocyte system of the NZB and several lines of evidence point to concomitant increases in activity of certain T-cell

functions. Gazdar et al (15), for example, found that between the ages of 9 days and 6 weeks NZB mice demonstrated superior surveillance activity as measured by their capacity to induce spontaneous regression of Moloney murine sarcoma virus-induced tumors. We (14) have observed that splenocyte stimulation in vitro by the T-cell mitogen, phytohemagglutinin, was greater for 2-3 month old NZB than for BALB/c and DBA/2 mice. In addition, a series of experiments employing concurrent assays of NZB mice of varying ages suggested that this measure of cellular immunity tended to increase from young to middle adult ages with no pattern of deficiency evident until 12 months of age or more (14).

Origin of NZB Immunologic Abnormalities. The immunologic hyperactivity of NZB mice could derive from an abnormality in the immunologic apparatus; specifically, a defect in regulation. Alternatively, an increase in the antigen load or exposure to altered, new, or unique antigens might be causal. Both could act in concert to promote abnormal immunologic reactivity.

a. role of virus. C-type RNA virus has been proposed as an etiologic factor in NZB autoimmunity. This is based on the detection of C-type particles and/or antigens of murine leukemia virus (MuLV) in the lymphoid organs, kidneys and milk of NZB mice (2). Mellors (16) concluded that there was evidence for a relationship between endogenous MuLV and the development of autoimmunity. He observed that NZB mice developed high titers of antibody against Gross soluble antigen (GSA) in later life, while other strains of mice showed significantly lower titers of these antibodies. The appearance of anti-GSA

and the concomitant disappearance of circulating free GSA correlated with the development of autoimmune hemolytic anemia. He speculated that antibody to viral antigen might in some way be implicated in autoimmunity, possibly through viral alterations of erythrocyte antigens making them immunogenic. Vaccination of NZB mice with formalinized cell-free filtrates of old NZB spleens was reported by Mellors (16) to delay positive Coombs' conversion, an observation which we were unable to confirm (14).

Mellors (16) also claimed successful transfer of indirect, but not direct, Coombs' positivity employing enzyme-treated mouse erythrocytes as target cells. Russell, et al (17) were unable to confirm these observations. More recently, Schaap et al (18) reported that none of the immunologic abnormalities of NZB mice could be transferred to BALB/c and NZB mice by highly purified C-type virus from the milk or thymic tissue of NZB mice. As long as one year after inoculation, none of these mice developed a positive direct Coombs' test, ANA, or signs of tumor growth. Moreover, injection of this virus did not influence the time of onset of immunologic abnormalities in the NZB. Histologic examination of thymus, spleen, kidney and liver of virus-inoculated mice did not differ from matched control animals and electron microscopic examination failed to show evidence of increased numbers of C-type particles.

The virus prepared by Schaap et al (18) proved to be identical to the xenotropic agent reported by Levy and coworkers (19). These latter investigators thought the virus might be implicated in the autoimmune disorders of the NZB, either by altering host antigens or by

serving as a source of increased antigen due to an unusual level of activation in NZB tissues. The predominant MuLV of NZB mice has been shown to be xenotropic, and ecotropic virus has not been directly isolated from NZB mice.

Crocker et al (20) demonstrated that injection of Scripps leukemia virus (SLV60A), originally purified from a continuous lymphoid cell line of NZB thymus origin, caused the development of circulating ANA and immune complex glomerulonephritis in (BALB/cXNZB)F1 mice. SLV60A is an ecotropic MuLV which grows equally well in NIH, Swiss and BALB/c fibroblasts. It possesses the antigenic characteristics of Moloney leukemia virus and its etiologic role in NZB autoimmunity is doubtful. Thus, the induction of autoimmune phenomena by either xenotropic or ecotropic agents has not been verified, and Koch's postulates as applied to virologic disease remain unsatisfied.

Linder and Edgington (21) have recently suggested that Mellor's demonstration of indirect Coombs' positivity in Swiss mouse recipients of NZB tissue extracts may be due to the passive transfer of NZB humoral antibody against the murine HB erythrocyte antigen. Enzyme augmentation, as used by Mellors, has been found to be a very sensitive indicator for antibodies directed against these hidden red cell autoantigens. These antibodies do not bind independently to erythrocytes in vivo and are of questionable pathogenetic significance with respect to autoimmune hemolytic anemia.

There have been reports of immunologic imbalance in NZB mice, specifically a depressed cell-mediated immune capacity in parallel with humoral hyperreactivity (22). It has been suggested that the large

amounts of MuLV antigens detected in NZB mice may be the result of diminished T-cell surveillance allowing excessive viral proliferation. B-cell hyperresponsiveness, a possible result of suppressor T-cell deficiency, would result in the formation of large amounts of anti-viral antibody. Virus can, and apparently does, act as antigen in an immunologically hyperresponsive environment, and has been implicated in immune complex renal disease (23). Such an antigenic role for non-specific virus must be conceptually distinguished from a unique agent having an etiologic role in relevant NZB disorders such as immunologic hyperresponsiveness. Here, for example, virus, as observed with the lactic dehydrogenase elevating agent (24), could act as an immunologic adjuvant and create a state of chronic hyperresponsiveness. Alternatively, infection with a unique agent might be thought to target on and damage the thymus, causing loss of the B-cell regulatory functions attributed to that organ.

Speculatively, RNA virus might initially have been incorporated into the genome via reverse transcriptase, vertically passaged, and activated and expressed uniformly in each subsequent generation of NZB mice. Since there is no experimental evidence to support this proposal, it seems more reasonable to visualize the information permitting or promoting autoimmunity to be host DNA which lacks infectious potential following transcription.

b. regulatory disorders. B cell hyperactivity is now generally accepted to play a critical role in the genesis of autoantibodies. Investigations directed to evaluating antibody mediated feedback indicated that, in terms of suppressing SRBC formation, this activity was normal in the NZB (25). Recent studies have supported the proposal of suppressor T-cell regulation of B-cell proliferation (26). Gershwin and Steinberg (27) described a striking example of in vivo intervention in the NZB autoimmune process. Starting at 4 weeks of age NZB mice were given intraperitoneal injections every two weeks of syngeneic thymocytes ($5X10^7$) from 2-week-old mice. These animals remained Coombs' negative, whereas control animals were uniformly Coombs' positive at 8 months of age.

Young adult NZB mice showed high resistance to the lethal effects of ionizing radiation (28). This was associated with an elevated capacity for endogenous splenic recolonization after sublethal x-ray exposure (14, 29). Since such radiation resistance is conferred by the pluripotent hemopoietic stem cell population, we have proposed (28) that the defects in the NZB leading to autoimmune disorders may originate in and/or be expressed by hyperreactive behavior of the stem cell. This might imply that if suppressor cell failure were the regulatory defect leading to NZB disease, such failure might allow excessive stem cell as well as B-cell proliferation.

Hemopoietic Stem Cells. Hemopoietic stem cells are a primitive self-renewing population which gives rise to the various differentiated lineages of the hemopoietic system. It is necessary to characterize these stem cells by function rather than morphological characteristics,

as morphologic criteria for their recognition are not known. In addition, these cells are present in very small numbers and are diffusely disseminated throughout the tissues of the hemopoietic system. It has been estimated that there are about $1.4X10^6$ stem cells in the adult mouse (30).

A particularly valuable technique for functional and quantitative analysis of these cells has been the in vivo spleen colony (CFU) assay of Till and McCulloch (31). The details of this assay are described in the Materials and Methods section. In brief, it involves clonal repopulation of the spleen of a hemopoietically ablated animal by regeneration from individual stem cell precursors. The monoclonal origin of each resultant macroscopic colony has been convincingly demonstrated by karyotypic analysis following transfer of small numbers of stem cells bearing chromosomal markers (30).

The multipotentiality of these stem cells has been demonstrated by the appearance of three main lines of hemopoietic differentiation; erythroid, granulocytic and megakaryocytic. Undifferentiated colonies and mixed colonies are also detected. There is a constancy of the relative proportion of colonies of different morphology, despite different sources of stem cells, suggesting that the pathway of differentiation is not a predetermined property of the donor cells and is, rather, determined by host need for hemopoiesis. Moreover, retransplantation of pure colonies of any one type produces CFUs of all cellular types with a numerical distribution similar to primary colonies. Such primary colonies can also proceed to completely repopulate the lymphoid system of a recipient, with complete reconstitution of

immunologic capacity (30).

Transfer of Autoimmune Disease by Hemopoietic Cells. A number of investigations have dealt with the transfer of autoimmune disease from the NZB to other mouse strains. Their intent was to define the cell populations and other factors responsible for the autoimmune and immunopathologic effects. Holmes et al. (32) observed that transplantation of spleen cells from Coombs' positive NZB mice to young Coombs' negative NZBs has resulted in the early appearance of a transient Coombs' positivity. Cells from lymph nodes, bone marrow and thymus were not effective. Polaskova and Strejcek (33) were unable to alter the subsequent course of development of Coombs' positivity by injecting newborn NZB mice with 1-2 X 10^7 syngeneic spleen cells from Coombs' positive old NZB mice. Denman et al. (34) reported the successful transfer of Coombs' positive hemolytic anemia by spleen plus marrow cell transplants from old diseased NZB or (NZB X C57BL/6)F_1 hybrid mice into BALB/c or C57BL/6 recipients which had received multiple injections of antilymphocyte globulin (ALG). Such adoptive transfer of autoantibody-producing spleen cells from diseased NZBs to healthy syngeneic or allogeneic recipients, with subsequent appearance of disease, provides only limited information as to the factors originally influencing these cells to abnormal immunogenesis.

Other studies have involved transplantation of bone marrow cells from NZB mice to nonautoimmune recipients, these recipients consisting of neonates, ALG-treated, or x-irradiated animals. Allman et al. (35) injected newborn CBA (H-2k) and BALB/c (H-2d) mice iv with 6-10 X 10^6 bone marrow cells from 1-month-old NZB mice (H-2d). Two-thirds of the

CBAs became Coombs' positive, mostly at 2-months to 3-months, but sometimes as early as 1 or as late as 8 months. Coombs' tests remained positive throughout their lifespan. No positive tests were obtained in BALB/c mice. The converted animals retained NZB skin grafts for over 30 days, suggesting a chimeric state, and it was assumed that the survival of viable bone marrow cells or their progeny was the basis for the direct Coombs' test conversion. Successful transfer occurred in the CBA, but not the BALB/c strain, raising the question whether incompatibility at the H-2 locus might have favored anti-erythrocyte autoantibody production.

Denman et al. (34), by injection of marrow from 1-month-old NZBs into ALG-treated BALB/c recipients, achieved Coombs' conversion in 2/21 recipients 60 days after transplant. Strong ANA responses were also present in 18/21 mice. Cell-free material from old NZB spleens injected into BALB/c mice following a course of ALG injection failed to produce positive Coombs' or ANA tests. In these experiments, ALG presumably allowed the establishment of lymphoid chimerism in the histocompatible NZB to BALB/c transfer. The use of ALG to prepare the host for transplantation raises several problems. First, the attainment of lymphoid and stem cell aplasia by ALG treatment may be difficult to achieve, along with marked individual differences in response. Treatment, which requires multiple repeated injections of ALG, must be discontinued several days prior to the grafting of allogeneic cells so that the latter are not affected. This time gap may result in the regeneration of host cells. Furthermore, lymphoid cells which have homed to lymphoid tissues are protected from the peripheral action

of ALG (36). ALG, unless neonatal tolerance to rabbit globulin has been successfully induced in each test animal, is a highly immunogenic foreign protein, and in spite of its immunosuppressive properties, renal deposition of immune complexes has been observed. In addition, chronic and acute stimulation of experimental animals with non-crossreacting foreign antigen has been shown to bring about the appearance of humoral autoantibodies, such as ANA and rheumatoid factor (37).

Reversal of ongoing Coombs' positivity was observed in lethally irradiated old NZB mice after grafting of CBA marrow, but not when young NZB bone marrow was grafted (33). The survival level of irradiated old mice was poor, so that only data for the period up to 8 weeks after grafting were available. Lindsey and Woodruff (38) have induced persisting Coombs' negativity by injection of bone marrow cells from young NZB or (NZB X CBA-T6)F_1 hybrids into lethally irradiated Coombs' positive NZB mice. Since Coombs' positivity is slow to develop even in the intact NZB, this study failed to deal with differences between the potential for NZB marrow and nonautoimmune bone marrow cells to bring about eventual autoimmune conversion.

While some of the above-cited studies suggested that autoimmunity might be innate to the NZB hemopoietic system, the results were oftimes equivocal. The reported discrepancies may have been due to inadequate preparation of the host for transplantation and/or to the use of Coombs' tests as the marker for autoimmunity, a trait which develops relatively late in the NZB lifespan. In some studies, histocompatibility differences at H-2 may have introduced graft-versus-host disease

as a variable. The experimental transfer system employed in the present studies obviated many of the pitfalls of earlier studies by the use of lethal whole-body x-irradiation for host preparation, a sensitive assay for early developing plasma antinuclear autoantibodies, and transplantation between strains which are compatible at the H-2 locus.

MATERIALS AND METHODS

Mice. The NZB colony represented the 20-26th generations in this laboratory of mice received in 1966 as generations 57 and 58 from Otago University Medical School, Dunedin, New Zealand. Age-matched 2- to 3-month-old NZB mice were randomly mated, 2 females with 1 male, and housed in disposable plastic cages on Sanicel bedding. Feed consisted of Purina mouse chow, and the drinking water was chlorinated and acidified to pH 2.4 to minimize pseudomonas infection. Several breeding pairs of BALB/c and DBA/2 mice, originally obtained from the Jackson Laboratories, Bar Harbor, ME ., were also maintained. All strains were housed in the same quarters in order to standardize their environment, microbiological and other, insofar as possible. Breeding was carried out in a separate room. Weanlings were transferred to another animal room where they were held until use.

Radiation Chimeras. Chimeras were prepared following exposure of potential graft recipients to lethal whole body x-irradiation; approximately 850 rads for the DBA/2 strain, 750-800 r for the BALB/c and 800-1000 r for the NZB. Following exposure, mice were injected subcutaneously daily with 0.40 mg of the antibiotic Garamycin (gentamicin sulfate, Schering Pharmaceuticals, Kenilworth. NJ) for 14 days.

Irradiation. Irradiation was performed with an X-ray unit (Picker-Vanguard) operated at 277 kV peak and 15 MA with added filtration yielding a half-value layer equivalent to 1.9 mm Cu. The exposure rate averaged 69 r per minute. Animals were exposed in plastic containers mounted on a turntable which was rotated during exposure. Irradiations

and calibrations were done by the UOHSC Department of Radiation Therapy under the supervision of their physicist, Mr. Ray Fry.

Cells for Transplantation. Bone marrow cell suspensions were prepared by flushing the excised femurs with phosphate-buffered physiological saline containing 100 units of penicillin and 100 µg of streptomycin per ml. Liver and spleen cell suspensions were prepared by teasing cells through a sterile fine-gauge screen. The desired numbers of nucleated cells were injected via the tail vein in 0.25 to 0.60 ml volumes. This transfer was done immediately following irradiation or on the following day (16-18 hr later).

Colony-Forming-Unit (CFU) Assays. Following sublethal exposure to whole body x-irradiation (400-600 rads) the spleen undergoes repopulation by surviving hemopoietic stem cells which are manifest as distinct colonies (erythroid and myeloid) on the surface of the spleen, 7-10 days later. These endogenous colonies are thought to be indicative of the stem cell pool size or of stem cell numbers in cycle at the time of irradiation. The exogenous or transplantation assay involves similar splenic repopulation in lethally irradiated recipients injected intravenously with known numbers of viable donor cells (31). Here, CFUs are generally related to the resting stem cell content of the donor tissue, although repopulation may also be influenced by homing and lodging factors and possibly host inductive, immunologic or histocompatibility factors.

Cytotoxic Drug Treatments. Drug treatment for chimera preparation involved intraperitoneal (ip) injection, 24 hours before grafting, of 50 mg/kg body wt. of busulfan (Myleran, provided by Dr. G.H. Hitchings,

Burroughs Wellcome Co.) suspended in 2.5% carboxymethylcellulose. This was followed by ip injection of 200 mg/kg of cyclophosphamide (Cytoxan, Mead Johnson), 4-6 hr before grafting.

Cytosine arabinoside (Cytosar, Upjohn) was diluted to an appropriate concentration and 18 mg injected ip into each mouse 40 minutes before exposure to 600 rads for endogenous colony assay.

Histology. Tissue samples of thymus, lymph nodes, spleen, small intestine, bone marrow, liver, and kidneys were removed 10 months after x-irradiation and marrow transplantation (12-month-old mice). These were fixed in 7% formalin, embedded in paraffin, and stained with hematoxylin and eosin. In addition, closely adjacent sections of the kidneys were stained with the periodic acid-Schiff reaction and the periodic acid-silver methenamine technique.

Tissue Immunofluorescence. Samples of unfixed kidneys were prepared by placement in a small tissue capsule with embedding medium (Ames Co., Division of Miles Laboratories, Inc., Elkhart, IN) and freezing in liquid nitrogen. The capsule was then mounted in a cryostat and sections were cut at 5 μ. The tissue sections were examined for the presence of globulin within the glomeruli by the direct method, using a 1:40 dilution of fluorescein-conjugated goat antiserum to mouse 7S gamma globulin (Hyland Laboratories, Costa Mesa, CA). Unlabeled goat antimouse globulin was used as a blocking agent.

Antinuclear Antibody Assay. Blood was collected in heparinized capillary tubes by orbital sinus puncture. Samples were spun for 3.5 minutes in a microhematocrit centrifuge and plasma immediately collected and assayed. The indirect immunofluorescence technique was used

routinely for detection of antinuclear antibodies. Chicken red blood cell nuclei were prepared and used as antigen according to the method of Ten Veen and Feltkamp (39). The nuclei were diluted in pH 7.5 phosphate buffered saline (PBS), and single drops of the nuclear suspensions placed on a glass slide and air dried. Following rehydration in PBS, they were overlaid with a 1:20 dilution in PBS of the mouse plasma to be tested. After 30 minutes incubation at room temperature, the slides were washed in three changes of PBS for a total of 15 minutes. Excess buffer was carefully removed, and the test site incubated with a 1:40 dilution of fluorescein-conjugated goat antimouse gamma globulin (Hyland Laboratories, Los Angeles, CA) for 30 minutes at room temperature. The slides were again washed in three changes of PBS and mounted in glycerol adjusted to pH 10 with 1 N NaOH.

Slides were examined with a Leitz fluorescent microscope. The light source was an HB 200 (Osram, Germany) high pressure mercury lamp and a primary BG 12 and an OG1 barrier filter were used. The intensity of fluorescence was rated on a 0 to 4+ scale. Known positive and negative plasma served as controls to each assay. Only reactions of 2+ or greater were considered to be positive.

Typical patterns of nuclear fluorescence obtained with formalinized chicken erythrocyte nuclei as substrate are depicted in Fig. 1. Illustrated are reactions evaluated as 1+ and 4+ which developed with different plasma samples obtained from NZB mice. These patterns of immunofluorescence closely resembled those described (39) for nuclei incubated with sera from human SLE patients. Susceptibility of the response to substrate treatment with deoxyribonuclease, but not with ribonuclease or trypsin, suggested that the response was directed against DNA (3).

Employing this assay it has been possible to detect antinuclear antibodies (ANA) in the plasma of very young NZB mice (3). While 7-10 day old animals gave uniformly negative responses, weak ANA was detected in about 12 % of NZB mice at 14 days of age, with strong reactivity demonstrable in 90 % of mice by the time they were 5 weeks old. Quantification of immunofluorescence indicated that ANA reactivity of NZB plasma increased with age as measured up to 2 years. During the first year of life, mice of C57Bl, DBA, C3H, BALB/c, AKR, A, SJL, DBA/2 and 129/J strains manifested largely negative and weak reactions.

Direct Coombs' Antiglobulin Assay. NZB mice spontaneously produce antibodies directed against their own erythrocytes. Mouse erythrocytes, when coated with these antibodies, can be agglutinated by cross-linking with a heterologous antimouse globulin antibody. Individual blood samples for assay were collected by orbital sinus puncture in a heparinized microhematocrit capillary tube, and the contents immediately washed into a 6 ml test tube with isotonic saline. Samples were suspended to 5 ml in saline and washed by centrifugation three times at room temperature. They were then suspended to equivalent final concentrations on the basis of packed red cell volumes. For example, a sample having an hematocrit of 50% was suspended to 2 ml in saline. One drop from a 12 inch Pasteur pipet was added to the concavity of a Kline agglutination slide. One drop of a 1:20 to 1:40 dilution of rabbit antimouse globulin (Nutritional Biochemicals Co., Cleveland, OH), heat-inactivated at 55^{o} C for 30 minutes, was then added to each red cell sample, mixed with a wooden applicator stick, and incubated for ten minutes at room temperature. After this time, a semiquantitative estimation of agglutination (0-4+) could be made by visual observation of aggregate formation.

Assay for Cells Forming Antibody Against SRBC. The Jerne plaque assay (40) was used to enumerate individual antibody-forming cells from the spleens of immunized mice. Intact mice and chimeras were injected intraperitoneally with 0.20 ml of a 1:10 suspension in 0.85% saline of saline-washed sheep red blood cells (SRBC) (Sterile sheep blood in Alsevers solution, Sheep Blood Supply, Tualatin, OR). This represented a dose of approximately $5X10^8$ SRBC. After five days mice were killed by cervical fracture, spleens weighed, and single cell suspensions prepared by teasing through a fine-mesh screen. The total spleen suspension was brought to a 4 ml volume in medium NCTC-109 (North American Biologicals, Los Angeles, CA) and kept in a 12 ml conical centrifuge tube in an ice bucket until ready for plating. Fifty-milliliter Erlenmeyer flasks containing 10 ml of Difco agar, 0.7% in NCTC-109, were maintained fluid in a 47° water bath. Prior to plating, 0.20 mg of DEAE-dextran (Sigma Chemical Co., St. Louis, MO) was added to each agar flask in order to neutralize the anticomplementary effects of agar (40). Then 0.50 ml of a spleen cell suspension and 0.50 ml of a 1:10 SRBC suspension in saline were added and mixed by swirling. Aliquots of 2.0 ml were immediately plated in triplicate on 100 X 15 mm plastic Petri dishes having a baselayer of solidified 1.4% agar in saline. After 40-60 minutes' incubation in a 37° incubator, 1.5 ml of a 1:10 dilution of guinea pig serum (Lyophilized guinea pig complement, Grand Island Biological Co., Berkeley, CA) was added as a source of complement and plates were incubated at 37° for an additional 40 minutes to allow plaques to develop. Plates were held up to a light source and plaques counted by hand lens magnification. Spleen samples were assayed at two different dilutions, each in triplicate.

Averaged values were calculated and expressed as plaques per spleen.

In Vitro Stimulation of Spleen Cells with T-cell Mitogens. Studies were carried out on the responses of spleen lymphocytes of intact mice and chimeras to the blastogenic effects of phytohemagglutinin (PHA) and concanavalin A (Con A). Spleen cell suspensions were passed through a fine-mesh nylon column and the lymphocytes (over 95% pure) cultured at a final density of 1-2 X 10^6 leucocytes per ml in Hepes buffered medium 199 with added antibiotics and 5% fetal calf serum. A concentration of 10 µg per ml PPHA (Rigas) and 2 µg per ml Con A (Sigma Chemical Co., St. Louis, MO) were used routinely in the different experimental cultures. The cell suspension was distributed in 1.0 ml volumes in 12 X 15 mm capped plastic tubes and incubated at 37°. To determine the extent of DNA synthesis, 0.05 ml of medium containing 1.0 µCi of tritiated thymidine with a specific activity of 6.7 Ci/mM (New England Nuclear Corp., Boston, MA) was added to the culture 20-24 hr prior to harvesting at 72 hr of culture. Experimental and control cultures were carried out in quintuplicate. Following ^{3}HTdR exposure, cells were prepared for liquid scintillation counting by filtration through a glass fiber filter. The entrapped cells were then washed with cold saline, 5% trichloroacetic acid and methanol. Aquasol solution (New England Nuclear) was added to the dried filters in counting vials and their contained radioactivity determined in a Packard Tri-Carb liquid scintillation spectrometer. The mitogenic response for each individual experiment was expressed as a stimulation index, i.e., as the ratio of radioactivity in stimulated to that in unstimulated cultures. Since the cells responding to PHA and Con A are thymus dependent, the response is generally considered to be a measure of cell-mediated immunity (41).

RESULTS

Transplantation Of Autoreactivity

Transfer of NZB Marrow to H-2 Compatible DBA/2 and BALB/c Recipients.

The intravenous injection of 2 X 10^6 bone marrow cells from 3-week-old NZB mice into lethally irradiated DBA/2 mice resulted in the subsequent appearance of plasma antinuclear autoantibodies (Fig 2). Here, although no ANA was evident one week after transfer, 40% of the mice were positive at 3 weeks and 80% at 5 weeks. Thereafter through the seventh month of the experiment, recipient mice remained 90-100% ANA positive. Syngeneic transfer of young DBA/2 marrow did not result in ANA formation, whereas allogeneic transfer of BALB/c marrow resulted in positivity in 2/10 recipients during the second month after transplantation. This latter subsided and then reappeared at 4 months, subsequently increasing in incidence until half were ANA positive 7 months post-transplantation. The reason for this increase is not known. As will be demonstrated later (Table 4), at 10 months these mice were ANA negative and lacked renal disease. Mice in all groups tested negative in direct Coombs' assay for antierythrocyte autoantibodies performed at 5 and 7 months after transfer.

A similar course of ANA conversion was observed in 850 rads irradiated BALB/c mice transplanted with 4 X 10^6 NZB marrow cells. Ten days after transfer all recipients were ANA negative, at 18 days 50% were positive and by 25 days mice showed 100% positivity. Over the same 25-day period, recipients transplanted with syngeneic BALB/c marrow or with allogeneic, H-2 incompatible SJL/J marrow cells remained completely negative.

The capacity of recipients to support the transfer of autoimmune

potential was found to be x-ray dose-dependent (Table 1). DBA/2 mice exposed to 800 rads and transplanted with 4 X 10^6 NZB marrow cells showed a pattern of delayed but 100% transformation to ANA positivity by 6 weeks which persisted to the end of the experiment at 14 weeks. These results were similar to those delineated in Fig. 2. In contrast, mice exposed to 200 rads before marrow transfer showed a low incidence of autoreactive antibody over a 98-day period (Table 1).

This relative inability to transplant NZB autoimmune potential into DBA/2 mice receiving low dose irradiation suggests possible competitive or regulatory activities of residual host hemopoietic tissues. For example, growth of the NZB graft might be restricted by host immune function, or by a lack of available microenvironmental niches in the recipient spleen for donor cell lodging as a consequence of concurrent repopulation by host stem cells. There might also be host-directed homeostatic controls over NZB cells which would act to prevent autoimmune behavior.

Transfer of Spleen Cells from 8-Day-Old NZB Donors. Spleen cells from eight-day-old NZB mice were also capable of transferring autoimmune potential to lethally irradiated recipients (Fig. 3). This contrasted sharply with the results of transfer of DBA/2 and BALB/c spleen cells; these latter induced early transient positive responses in BALB/c hosts which were far less pronounced than those elicited by NZB splenocytes.

These results (Fig. 3) differed from those in which marrow was transferred in that ANA developed in spleen cell recipients within 9 days after grafting in contrast to a 3-week latency period following marrow transplantation. This rate difference in ANA appearance could not be attributed to differences in stem cell content. The NZB spleen and

marrow preparations used in these studies showed similar numbers of colony-forming units by transplantation assay into lethally irradiated NZB and DBA/2 recipients (16 CFU/10^5 spleen cells and 15 CFU/10^5 bone marrow cells). Acceleration with splenocytes may have been due to the presence of T and B lymphocytes available for or committed to autoimmune reactivity, although it should be noted that 8-day-old NZB mice do not manifest circulating ANA (3). The increased lag following marrow transfer may represent the time required for hemopoietic regeneration and for stem cell differentiation into immunocompetent forms.

Transfer of Marrow Across the H-2 Barrier and from F1 Hybrid to Nonautoimmune Parent. The low and irregular ANA positivity (Figs 2 & 3) following marrow and spleen cell grafts between the H-2 compatible BALB/c and DBA/2 strains may share some common basis with the ANA pattern reported for F1 mice grafted with parental spleen cells (42,43). Although the mechanism for such autoimmune conversion has not been established, recognition of and response to minor histocompatibility differences could conceivably be responsible for a significant part of the autoimmunity observed with the cell transfers between the BALB/c and DBA/2 strains. In this regard, the strong autoimmune responses noted after grafting of NZB tissues could possibly reflect the immunologic hyperresponsiveness previously described for this strain (10). If this were the case, a graft-versus-host reaction, distinct in character from the autoimmune abnormality intrinsic to mice of the NZB strain, might explain the apparent transplantability of autoimmune potential.

In order to evaluate the possible participation of a graft-versus-host reaction, two further types of transplantation combinations were employed. In one, NZB marrow (H-2d) was grafted into irradiated mice

of the incompatible SJL/J (H-2s) and C57Bl/6 (H-2b) strains. In the other, 3-week-old (SJL X NZB)F1 and (C57Bl X NZB)F1 mice were employed as marrow donors to the corresponding irradiated nonautoimmune SJL/J or C57Bl/6 parental strain. These are presumably compatible combinations and would not elicit a conventional graft-versus-host reaction. Since F1 hybrids of nonautoimmune strains with the NZB develop ANA to an extent comparable to the NZB, such hybrids provide a definitive source of autoimmune marrow for transfer studies.

As seen in Fig. 4, F1 to parental strain transfers of marrow cells brought about substantial ANA conversion. The responses of SJL/J recipients (Fig. 4A) was comparable to that previously observed in DBA/2 and BALB/c recipients of NZB bone marrow, while C57Bl/6 recipients of F1 marrow (Fig 4B) showed a somewhat lower ANA incidence, possibly associated with their lower x-ray exposure. In both recipient strains, however, NZB bone marrow elicited less ANA formation than did hybrid marrow. Recipients of NZB bone marrow also showed wasting and early deaths, possibly due to graft-versus-host reactions or to inadequate reconstitution by the donor graft (44).

Transfer of Fetal Liver Cells to Lethally-Irradiated DBA/2 Mice. This capacity of preautoimmune NZB marrow and spleen cells to transfer autoimmunity, the unusual proliferative and regenerative behavior previously described for the NZB stem cell (14,28,29), and the spontaneous formation of autoantibodies in close coincidence with hemopoietic reconstitution all suggested that the potential for autoimmunity might be transmitted by information contained in the grafted NZB hemopoietic stem cell. In this regard, fetal liver also constitutes a rich source of stem cells in the mouse (30), and, as noted in Table 2, an inoculum

of 4 X 10^6 nucleated cells was able to convert lethally irradiated DBA/2 recipients to ANA positivity, whereas DBA/2 and BALB/c liver cells were relatively ineffective. Parenthetically, 4 of 12 DBA/2 recipients of NZB fetal liver cells showed positive direct Coombs' tests after 100 days, whereas Coombs' conversion was not evident in any of the bone marrow grafted recipients (Fig 2), a difference for which I at present have no explanation.

Cytotoxic Drug Treatment in the Preparation of Graft Recipients. To determine whether cytotoxic drug treatment could be substituted for lethal irradiation in the preparation of marrow recipients, BALB/c mice were treated with 50 mg/kg of busulfan followed by 200 mg/kg cytoxan. Such treatment was lethal and no spleen colonies or spleen weight recovery were demonstrable 9-10 days later. Transfer of 4 X 10^6 NZB bone marrow cells into these animals resulted in a rapid conversion to ANA positivity, which was evident as early as 1 week after transplantation (Table 3). No Coombs' conversion was evident during the course of this study, which was limited to a 49-day period because of high subsequent mortality of drug-treated recipients. This poor viability precluded the further use of this mode of host preparation and lethal x-irradiation became the method of choice for subsequent studies.

BALB/c mice receiving 200 mg/kg of cyclophosphamide alone did not serve as suitable hosts for ANA development after NZB marrow grafting, reminiscent of findings with mice receiving sublethal as compared to lethal irradiation. The onset of ANA in drug-treated mice that received transplants preceded that in lethally-irradiated recipients by some 2 weeks, and resembled the earlier onset of ANA previously noted following splenocyte transfer. It is possible that the drug-treated

animals retained residual thymus or T-cell functions (45) which served to enhance hemopoietic regeneration (46), or acted to provide helper T-cells to the autoimmune response.

Transplantation of Renal Disease

The early appearance of ANA in the recipients of NZB hemopoietic cells raised the possibility that related abnormalities, such as renal disease, might subsequently appear in long-term transplant recipients.

Autoantibody Responses. Individual plasma ANA responses of surviving transplant recipients from the study depicted in Fig. 2, 10 months after lethal irradiation and bone marrow grafting, are presented in Table 4. Strong ANA reactivity was evident in all animals receiving NZB bone marrow. In the NZB-grafted DBA/2 mice this ANA positivity reflected reactions that had developed in the second month after transplantation and persisted. Irradiated NZB mice grafted with NZB bone marrow had high ANA reactivity throughout the study, similar to that of untreated NZB mice (3).

Transfer of BALB/c marrow to irradiated DBA/2 mice had been previously noted (Fig. 2) to result in sporadic, weakly positive ANA responses. However, at 10 months (Table 4) only two of eight mice showed weak ANA reactivity. Plasma ANA determinations, both early after transfer and on long-term evaluation, were always negative with grafting of DBA/2 marrow to lethally irradiated DBA/2 recipients.

Coombs' tests were negative for all mice except for one of two NZB recipients of NZB bone marrow 10 months after grafting (mouse 191, Table 4). This positive test was not present at a 7-month assay.

Histological Changes. Lethally irradiated DBA/2 and NZB mice receiving marrow transplants from NZB donors manifested histological changes characteristic of NZB glomerulonephritis (Figs. 5, 6 & 7). These consisted of various combinations and degrees of increased nuclear size, focal hypercellularity, irregular thickening of the basement membranes of the glomerular capillaries, thrombi, and intercapillary glomerulosclerosis. There were, in addition, focal atrophy of tubules, thickening of the walls of some small arteries and glomerular arterioles, and scattered aggregates of lymphocytes and plasma cells within the interstitial connective tissue.

The glomeruli of DBA/2 mice receiving marrow transplants from BALB/c and DBA/2 strains did not have thickening of the basement membranes or thrombi. All of the other changes (increased nuclear size, focal proliferation of cells, and intercapillary glomerulosclerosis) were present in various combinations and degrees (Figs. 8 & 9). With the exceptions of the basement membrane thickening and thrombi, these changes are commonly observed at the dosages of total body irradiation employed here (47).

Thickening of the capillary basement membrane was the only constant glomerular alteration by which NZB-like glomerulonephritis could be established in the presence of changes induced by irradiation. With this as the criterion, the data in Table 4 are presented to show the relationship between donor and recipient strain, ANA response, and thickening of glomerular basement membranes. Capillary basement membrane thickening was found only in those mice having a persistent strongly positive ANA response, and this combination only in animals that received a transplant of NZB bone marrow.

There were no significant histological changes in liver, small intestine, bone marrow, and thymus in any of the animals that received grafts. There was preservation of the histological structure of lymph nodes and spleen without excessive proliferation or decrease in the cellular population. Germinal centers were small to moderate size with few mitoses. Plasma cells varied from small to moderate numbers in the medulla of the lymph nodes and white pulp of the spleen. These latter cells were more numerous in the DBA/2 and NZB mice receiving NZB bone marrow.

Direct staining with fluorescein-labeled goat antimouse globulin showed numerous deposits of globulin associated with the glomerular basement membranes of DBA/2 mice receiving NZB marrow transplants (Fig. 10). This staining could be blocked or markedly attenuated by prior treatment of the sections with unlabeled antimouse globulin. The distribution of globulin in these mice that received transplants was similar to the distribution of globulin in untreated NZB mice of comparable age (Fig 11). Glomeruli of normal 11-month-old DBA/2 mice were either negative or had minimal fluorescent staining. Fluorescent staining of glomeruli from DBA/2 mice with DBA/2 marrow transplants was similar to the glomerular staining of normal DBA/2 mice, except for a few small aggregates of globulin in foci of hypercellularity caused by irradiation (Fig. 12).

During the long-term study reported here, the animals did not show retardation of growth or emaciation. At termination of the experiment the thymus, lymph nodes, spleen, and gut-associated lymphoid tissues had persistence of histological structure with neither excessive proliferation nor marked depletion of cells. These histological features

were similar for mice that received transplants in both the syngeneic (NZB→NZB and DBA/2→DBA/2) and allogeneic (NZB→DBA/2 and BALB/c→DBA/2) groups as presented in Table 4. These observations argue against chronic graft-versus-host reactions participating in the generation of the autoimmune phenomena described here; they are more suggestive of a state of tolerant chimerism.

In summary, ten months after transplantation, membranous glomerulonephritis and hyaline thrombi were evident in the kidneys of lethally irradiated DBA/2 recipients of NZB, but not of BALB/c or DBA/2, bone marrow cells. Glomeruli of these mice also showed fluorescent staining for mouse immunoglobulin. These renal changes were associated with the presence of plasma antinuclear antibodies. The results indicated that early ANA development, first observed within 6 weeks after grafting, was clearly predictive of a subsequent NZB-like renal disease.

Transplantation of Additional Immunologic and Hemopoietic Abnormalities in NZB Radiation Chimeras

The successful transplantation of autoimmune potential suggested that the fundamental abnormality leading to autoimmune disease development might be inherent to the NZB hemopoietic stem cell and its differentiated immunocyte progeny, rather than a property of the inductive microenvironment or of other factors in the NZB internal milieu. The following studies were directed to seeking a possible association in the NZB marrow-grafted chimera between ANA formation, immunologic hyperresponsiveness, and elevated endogenous spleen colony formation, phenomena which coexist in the intact NZB mouse.

Immunologic Hyperresponsiveness. It was observed (Table 5) that numbers of direct plaque forming cells (PFC) per spleen for each chimera, as assayed at 41 and 90 days after grafting, closely approximated the PFC capability of intact mice of the donor bone marrow strain. The PFC responses of NZB→DBA/2 radiation chimeras simulated those of intact NZB mice rather than those of intact DBA/2 mice. Thus, the previously reported immunologic hyperresponsiveness of NZB strain mice to SRBC immunization (10) was, as in the case of ANA, a property found transmissible to lethally-irradiated recipients through bone marrow grafting. The results presented in Table 5 further suggested that complete restitution of humoral immune capacity had been achieved in these various radiation chimeras, since the requisite helper T-cell as well as B-cell and accessory cell activities were evidently functioning normally.

While NZB, BALB/c and DBA/2 strains are histocompatible at the H-2 locus, and their chimeras showed normal responsiveness to SRBC immunization with no evidence of graft-versus-host reactions, it is possible that incompatibility at minor loci could have influenced the immunologic behavior noted in Table 5. Immune capacity, for example, may have been stimulated by a mild NZB-induced response against minor histocompatibility antigens of the DBA/2, an activity which would be diminished or lacking in the BALB/c→DBA/2 or DBA/2→DBA/2 chimeras. To test this, the relative PFC responses of (NZB X BALB/c)F1→BALB/c and BALB/c→BALB/c bone marrow chimeras were studied since in such combinations graft-versus-host reactions should not occur. The data presented in Table 6 demonstrated that PFC formation by intact F1 hybrids was intermediate to that of each parental strain with the F1 more responsive than the BALB/c. Similarly, BALB/c recipients of F1 marrow showed increased PFC

numbers compared to BALB/c→BALB/c grafted mice. Hence, minor histocompatibility differences with possible allogeneic effects did not appear to influence the innate capacity for immunologic hyperresponsiveness of the NZB hemopoietic system in terms of humoral immune capacity. Parenthetically, the rate and incidence of spontaneous ANA development in the intact (NZB X BALB/c)F1 mouse was observed to be similar to that of the intact NZB. Comparable ANA development was noted in BALB/c recipients of this F1 bone marrow.

The reconstitution of native PFC-forming capacity in chimeras between H-2 histocompatible strains which I have described here was similar to the results obtained by Agarossi and Doria (48) with syngeneic radiation chimeras. These investigators injected 10^7 bone marrow cells from 3-month-old donors intravenously into 900 rads-treated syngeneic or allogeneic recipients. Using the C3H (H-2k) and C57Bl (H-2b) strains, they observed that PFC responses for syngeneic chimeras regained normal values within 2-3 months whereas the allogeneic chimeras remained far below normal even 5 months later.

Playfair (49) injected lethally irradiated NZB and C57Bl mice with syngeneic fetal liver plus thymocytes and at the same time immunized with SRBC. The PFC response of the NZB 8 days later was significantly higher than that of the C57Bl. Similarly, NZB liver cell grafts were superior to those from the BALB/c strain in terms of subsequent PFC responsiveness following transfer to lethally-irradiated (NZB X BALB/c)F1 hosts. This study indicated that NZB fetal liver possessed increased PFC precursors for SRBC. These PFC precursors are presumably generated from the hemopoietic stem cells present in that organ. I have shown that similar stem cells, bone marrow derived, continue to generate

excessive numbers of precursor cells in the fully reconstituted host (Tables 5 & 6). The results of these different reconstitution studies offer analogies to the observations of immunologic hyperresponsiveness of 5-day-old baby (8,9) and young adult (10) NZB mice to SRBC immunization, and would point to the stem cell as the site at which elevated immunologic reactivity is generated.

Cell-mediated immunity, as measured by in vitro responsiveness of spleen lymphocytes to PHA stimulation, was previously observed (14) to be elevated for young NZB compared to BALB/c and DBA/2 strain mice, and it was of interest to determine whether these differences would be transmissible through bone marrow grafting. Table 7 presents the results of simultaneous assays performed on individual spleens from intact mice and from radiation chimeras of 6 weeks' duration. It can be seen that the relative responsiveness to PHA and Con A for the intact strains, NZB>BALB/c>DBA/2, was reproduced in the corresponding chimeric recipients of NZB, BALB/c and DBA/2 bone marrow cells.

Elevated Endogenous Spleen Colony Formation. Features such as high resistance to the lethal effects of ionizing radiation as indicated by $LD_{50(30)}$ values (28) and elevated endogenous spleen colony formation (14,29) have suggested the existence of an expanded stem cell compartment in NZB strain mice. In this connection, it was speculated (28) that NZB autoimmunity might be one manifestation of a hyperactive immune system which had its origins in abnormalities at the stem cell level. Results consistent with this speculation were presented in the previous section, and suggested that these immunologic abnormalities were derived from the grafted hemopoietic stem cell population. Such observations did not, however, afford information as to whether

the aberrant behavior of the NZB stem cell as seen in the intact animal was an autonomously regulated property of the NZB hemopoietic system or whether it depended on environmental determinants unique to the NZB host.

To resolve this question, radiation chimeras of 35 days' duration and intact donor strain mice were exposed to 600 rads and spleen colonies enumerated 8 days later. The extent of colony formation for each chimera was observed (Table 8) to resemble that of the intact marrow donor strain and not that of the recipient. Both intact NZB mice and NZB→DBA/2 chimeras manifested markedly elevated endogenous colony formation compared to the BALB/c and DBA/2 groups. The somewhat increased CFU responses for each chimeric group relative to the intact donor strain could have been a consequence of re-exposure to x-rays at a time just prior to the completion of host hemopoietic reconstitution, or during an overshoot phase. A still ongoing process of regeneration would presumably recruit additional stem cells into active cycle to provide for self-renewal replacement of stem cells lost through differentiation. In this context, Boggs, et al (50) have reported that stem cells in S phase constitute the subpopulation of the stem cell compartment responsible for endogenous colony formation following irradiation.

These experiments demonstrated that the NZB traits of elevated endogenous spleen colony formation and of immunologic hyperresponsiveness as measured by anti-SRBC plaque formation and splenocyte responses to the T-cell mitogens Con A and PHA, can be transferred to lethally irradiated H-2 compatible nonautoimmune strains through bone marrow grafting. Such transplantability indicates that these characteristics, along with the capacity for autoantibody formation, are innate properties of the NZB hemopoietic stem cell and are not expressed as a result of environmental determinants.

Reversal of the NZB Syndrome in Lethally Irradiated NZB Mice Grafted With Nonautoimmune Marrow Cells

If autoimmune potential is indeed inherent to the NZB hemopoietic system, the disease process should be susceptible to modification or abrogation by substitution of the NZB hemopoietic system with one from a nonautoimmune source. That is, lethally irradiated NZB mice grafted with DBA/2 or BALB/c bone marrow cells should no longer manifest immunologic and hemopoietic abnormalities.

Reversal of ANA Formation in Lethally Irradiated NZB Mice Grafted With Nonautoimmune Bone Marrow Cells. DBA/2 marrow-grafted NZB mice were established, following exposure to x-ray doses ranging from 800 to 1000 rads, and tested periodically for ANA development. As seen in Table 9, marked diminution in the incidence of ANA positivity occured in NZB mice grafted with 3×10^6 DBA/2 bone marrow cells, complete negativity being evident in NZB mice exposed to 1000 rads. Intact DBA/2 mice and DBA/2→DBA/2 chimeras also showed persistent ANA negativity, while intact NZB mice and NZB→NZB chimeras showed high incidences of ANA formation (Table 9). The seeming dependence of ANA reversal on x-ray exposure of the NZB host is consistent with the high radiation $LD_{50(30)}$ values previously reported (28) for young adult NZB mice. In that study, 70% of 3-month-old NZB mice were shown to undergo autologous hemopoietic recovery following whole body exposures to 800 rads, 10% following 950 rads, and 0% following 1000 rads. It is thus reasonable to interpret the renewed ANA formation in a number of NZB mice exposed to 950 rads or less to have resulted from the survival of NZB stem cells in adequate numbers to repopulate and overgrow the DBA/2

graft, with a consequent reversion of the chimeric hemopoietic system to host (NZB) type.

(NZB X DBA/2)F1 hybrids manifest codominance in the expression of ANA, about 45% showing positivity (Fig.13, time 0). Five-month-old F1 hybrids were apportioned into three groups having equivalent ANA responses, exposed to 800 rads and grafted with 4 X 10^6 bone marrow cells from NZB, DBA/2 and F1 donors. As seen in Fig. 13, the ANA responses of these recipients over the ensuing 3 months evidenced remarkable fidelity to donor-type ANA behavior. Suppression of F1-type ANA reactivity by DBA/2 marrow was evident by day 30 post-transplantation, while almost total ANA conversion appeared in recipients of NZB marrow. The results as presented in Table 9 and Fig. 13 strongly support the view that factors or agents inductive of autoantibody formation are lacking in the NZB environment.

Reversal of Immunologic Hyperresponsiveness in DBA/2→NZB Radiation Chimeras. If ANA formation in the NZB is one manifestation of a generalized phenomenon of immunologic hyperresponsiveness, this latter should also be reversed in the DBA/2→NZB radiation chimera. As evidenced by the data of Table 10, average numbers of PFC per spleen for DBA/2→NZB and DBA/2→DBA/2 chimeras were similar to one another and to the previously described (Table 5) values for intact DBA/2 mice and DBA/2→DBA/2 chimeras. PFC per spleen for NZB→DBA/2 mice also showed the relative elevation earlier demonstrated (Table 5) for intact NZB mice and for NZB→DBA/2 chimeras. These results indicated that the characteristic hyperresponsiveness of the NZB strain to SRBC immunization could be reversed by hemopoietic replacement. Studies are currently underway to test whether T-cell mitogen reactivity can also be reversed in the DBA/2→NZB combination.

Reversal of Elevated Endogenous Colony Formation in DBA/2→NZB Radiation Chimeras. Endogenous colony formation for DBA→NZB radiation chimeras following a subsequent sublethal x-ray exposure was observed (Table 11) to be DBA/2-like. This was in agreement with earlier studies (Table 8) suggesting an inherent behavior pattern for the splenic stem cell population without regard to its strain of residence. A confirmatory study is presented in Table 12. Intact (NZB X BALB/c)F1 and (NZB X DBA/2)F1 mice show, respectively, strong and intermediate ANA reactivity. If the NZB environment afforded by these hybrids were inductive of abnormal stem cell behavior, one might anticipate altered endogenous colony formation by engrafted NZB, DBA/2 or BALB/c hemopoietic cells. As seen in Table 12, the endogenous CFU responses characteristic of each donor strain were maintained in these hybrid hosts, further supporting the contention that this capacity is innate to the hemopoietic stem cell compartment.

Characterization of the NZB Stem Cell Compartment

The high radioresistance of the NZB, both in terms of hemopoietic survivals (28) and spleen endogenous colony formation (14,29),suggested that there might be significant quantitative or qualitative abnormalities in the stem cell population associable with the appearance of immunologic disorders.

The richest source of stem cells in the post-natal mouse is the bone marrow, a shift from the liver and spleen occuring soon after birth (30). In order to test for possible variations in this population, the concentration of stem cells in the bone marrow of NZB as well as H-2 compatible strains and hybrids was determined by transplantation assay for mice ranging in age from 8 days to 5 months, the period

during which autoantibody formation is initiated in the NZB. CFU per 10^5 grafted marrow cells were observed not to vary notably with age or strain (Table 13), average values ranging from 12-18 surface colonies per recipient spleen.

Since the microenvironment provided by different lymphoid organs within the same individual may differ significantly in terms of the inductive and supportive milieus they present, concentrations of stem cells within the spleens of NZB, BALB/c and DBA/2 mice were similarly evaluated. As noted in Fig 14B, transplantable splenic CFUs showed marked variation with both age and strain. There was an aging pattern for all strains characterized by high CFU counts in baby mice, rapidly declining with age increase to 1-2 months. This decrease clearly represents the ontogenic shift in murine hemopoiesis referred to previously (30). It should be noted, however, that NZB mice showed significantly greater numbers of transplantable splenic CFUs than BALB/c or DBA/2 animals at all ages studied.

There were comparable differences over this same age span in endogenous CFU development following sublethal (650 rads) x-ray exposure (Fig. 14A), essentially paralleling the age and strain associated variations observed for transplantable CFUs. As described earlier (Tables 8,11 & 12), endogenous colony forming capacity has proven to be an inherent trait of the stem cell population and is not host dependent.

Endogenous and transplantable spleen colony assays seemingly discern between two different physiologic states of the stem cell population. Endogenous CFUs are the result of proliferation and differentiation of a relatively radioresistant compartment which is, however, highly sensitive to the cycle-active drug cytosine arabinoside (Ara-C)(50). This

observation suggested that stem cells engaged in DNA synthesis at the time of x-ray exposure are those responsible for endogenous colony formation. In this regard, I observed that intraperitoneal injection of 9- to 11-week-old NZB mice with 18 mg Ara-C, 40 minutes before exposure to 600 rads, resulted in an 80% reduction in 9-day endogenous CFU formation, from an average of 16.8 to 3.4 CFU per spleen. These Ara-C reduced counts were comparable to the normal counts of 5.3 CFU per spleen obtained with 600 rads-exposed DBA/2 animals. This reduction of CFUs by Ara-C treatment of NZB mice was similar to that reported by Boggs, et al (50) for (C57Bl X DBA/2)Fl mice. The present result, along with the endogenous CFU data presented in Fig. 14A, would indicate that an excessive number of stem cells are in proliferative cycle in the NZB spleen.

On the other hand, injection of donor mice with Ara-C just prior to transplantation assay, or of recipients at the time of transplantation, has been reported (51) not to alter CFU counts, suggesting that this assay detects only G_o or resting stage stem cells. Should this also prove to be the case for NZB, BALB/c and DBA/2 mice, the CFU data of Fig. 14B would then represent a relative quantitation of the resting stem cell population in the spleens of these 3 mouse strains. Hence, both cycling and resting splenic stem cell compartments would appear to be notably elevated for the autoimmune strain NZB compared to the nonautoimmune strain BALB/c and DBA/2 animals. An hypothesis on the etiologic role played by this elevated stem cell population in the induction of autoimmunity will be set forth in the following section.

DISCUSSION

The results reported in this thesis have demonstrated that certain hemopoietic and immunologic abnormalities characteristic of NZB strain mice can be transplanted via NZB hemopoietic stem cell grafts to lethally irradiated, H-2 histocompatible recipients. These disorders can be prevented by transplantation of H-2 compatible normal marrow cells to lethally irradiated NZB mice. The NZB traits that have been found associated with the hemopoietic system are (a) the potential for antinuclear antibody formation and subsequent immune complex renal disease, (b) hyperresponsiveness to SRBC immunization in vivo, (c) splenocyte hyperresponsiveness to T-cell mitogen stimulation in vitro, and (d) elevated endogenous spleen colony formation following sublethal x-irradiation. It is therefore concluded that these characters are innate to the hemopoietic system and do not depend upon environmental determinants. Furthermore, this linkage through the hemopoietic cell system strengthens the postulation of a cause and effect relationship between an expanded stem cell population and the subsequent development of immunologic hyperresponsiveness and autoantibody formation. In this regard, endogenous CFU formation which is an index of the stem cell population in cycle, and transplantable CFU numbers representing stem cells in G_o, were both found to be elevated in the NZB spleen at the time of initiation of autoantibody formation.

Nature of the Hemopoietic Abnormality. These observations would point to a genetic abnormality in hemopoiesis and immunogenesis in mice of the NZB strain. Genetic abnormalities in hemopoiesis have been generally observed as defects in erythropoiesis. One experimental model

of anemia has been mice bearing the Steel allele (30,52). Here, a defective microenvironment exists which is unable to support normal erythropoietic responses, even when stem cells from normal mice are grafted. Stem cells from Steel animals are able to differentiate normally when grafted to a normal host. In contrast, the W series anemics (30) have stem cells which are unable to differentiate normally in any host. These animals, as hosts, are able to support the normal differentiation of engrafted cells from other compatible sources. Metcalf and Moore (30) have proposed that in W mice there exist defects in receptor sites on the surface of the stem cell complementary to the stroma of localization. Conversely, the impairment in Steel mice may be related to a defect in microenvironmental receptors. The defects in both these mouse models are more apparent in the spleen than the marrow, suggesting inter-organ variation in the importance of the interaction between the stem cell and the microenvironment. This latter had a parallel in the apparent localization of stem cell excesses to the spleen, but not marrow, of the NZB strain.

The NZB splenic microenvironment clearly does not offer more avid or supportive sites for stem cell lodgment and proliferation. This is evidenced by the normal (DBA/2-like) behavior in the NZB host of an engrafted DBA/2 hemopoietic system and by the hyperproliferation of NZB marrow in a normal DBA/2 environment.

By analogy with the postulated stem cell receptor abnormality of the W strain, the disorder of the NZB stem cell might be one of increased receptor concentration or avidity, leading to saturation of splenic stromal sites. In the case of H-2 histocompatible stem cells from other strains, only partial occupancy of the stroma due to normal

affinities might constitute the equilibrium state. This possibility can be readily tested by determining the seeding efficiency of stem cells from these various strains (52). In this determination, hemopoietic cells are grafted for transplantation assay. A parallel transfer is carried out which involves retransplantation of recipient spleen cells to a second irradiated recipient within 3 to 24 hours of the first graft. The ratio of CFUs developing in the secondary recipient to those in the primary provides an index of the seeding efficiency of the stem cell population. The data presented in Table 13 indicated similar CFU/10^5 bone marrow cells for NZB and other H-2 histocompatible strains. Although it is possible that retransplantation analysis might reveal a higher seeding efficiency for NZB stem cells, this seems unlikely as it would necessarily denote a lowered relative concentration of stem cells in NZB bone marrow.

The hyperreactive character of both the NZB stem cell population and its T- and B-immunocyte progeny might, on the other hand, suggest the existence of a shared defect in regulation. NZB mice produce not only increased numbers of plaque-forming cells from days 1 to 7 after SRBC immunization, but also achieve a peak response approximately one day later than mice of other strains (10). This would point to an increased burst size, i.e., additional divisions, for each initially stimulated antigen-reactive cell in the NZB. This capacity for additional immunocyte divisions may be directly analogous to the abnormal expansion noted for the NZB stem cell population. Because these hyperproliferative properties are transplantable with hemopoietic stem cells, the regulatory defect appears to be innate to the hemopoietic system. In this regard, NZB cells may possess diminished sensitivity to

feed-back signals, perhaps through lowered surface receptor densities or as a result of structural abnormalities. Detection of defects in receptor sensitivity awaits further definition of specific physiologic feed-back signals which turn off immunocyte and stem cell proliferation in normal animals. The passive administration of hyperimmune antiserum does constitute one such signal to the immune system which we have found to be equally effective in suppressing the initiation of the primary immune response to SRBC in both NZB and BALB/c strains (25).

Alternatively, there may be a lower threshold for antigen-triggering of immunoglobulin (Ig) bearing lymphocytes in the NZB, a phenomenon which may relate to their relative resistance to tolerance induction (53,54). We have observed (10), however, that although NZB mice showed notable hyperresponsiveness to SRBC at antigen doses of 5×10^7 to 1.5×10^9 cells, NZB and BALB/c animals showed similar PFC responses when immunized with a low dose (1×10^7) of SRBC. These latter observations were not definitive, and further studies should be carried out using limited doses of antigen.

Rather than postulate an abnormality in NZB cell receptor function, one might propose an insufficiency in the signal that interacts with this receptor. The studies presented here suggest that the signals emanating from the NZB environment and stromal microenvironment are normal and that any abnormal signal must be associated with the hemopoietic cells themselves. In this regard, a possible decrease in T-cell mediated suppression has been considered and studied for the NZB immunocyte system (27,55,56). In work from Steinberg's laboratory (27), 4-week-old NZB mice were given intraperitoneal injections of 2-week-old syngeneic thymocytes at 2-week intervals for 8 months.

These animals showed a significant reduction in direct Coombs' tests, lymphoid proliferation and histologic evidence of renal disease. Since the NZB stem cell shares this hyperproliferative behavior, defective regulation of the stem cell population might be due to similar failures in some suppressor cell activity. On the other hand, a stem cell chalone (57,58) may be functioning inadequately in the NZB.

If the regulatory defect were suppressor cell or chalone insufficiency, replacement therapy should also be expected to prevent development of the hemopoietic and immunologic abnormalities of the NZB→DBA/2 radiation chimera. In such studies (59), lethally irradiated DBA/2 mice were transfused with 4×10^6 NZB bone marrow cells along with 2×10^7 thymus cells from 3-week-old NZB or DBA/2 donors. At 3 weeks of age NZB thymocytes still manifest normal suppressor function (27). Both groups of recipients showed accelerated and augmented ANA formation as compared to mice receiving NZB marrow alone, suggesting that in this system an enhancement phenomenon predominated over any postulated regulator T-cell influence. This enhancement could have been due to addition of a T-cell population helper to the B-cell response to nuclear antigens; showing a thymus dependence similar to that reported for SRBC-immunized, bone-marrow grafted, irradiated mice (60). Thymocytes might also constitute a source of large amounts of nuclear antigen, or have a trophic effect on B-cell regeneration, and thus accelerate the spontaneous development of ANA in these chimeras. Fractionation and concentration of the suppressor T-cell population (61) may thus be required to demonstrate regulatory activity in this autoimmune system, should this be a controlling mechanism for disease development.

In contrast, the administration of DBA/2 bone marrow cells in conjunction with or 18 hours prior to NZB bone marrow transfer to lethally irradiated DBA/2 recipients resulted in marked suppression of ANA development (59). This result is compatible with a DBA/2-exerted homeostatic regulation over NZB cell responsiveness. It could, on the other hand, be due to competition of DBA/2 and NZB stem cells for microenvironmental niches; the diminished ANA formation resulting from a diminished NZB hemopoietic component. Detailed analysis of the chimeric status and composition of each of such mixed chimeras, along with assays for autoreactivity, should clarify the threshold population of NZB cells required for autoreactivity. It would also indicate the coexistent population of DBA/2 cells needed to exert a suppressive effect, through competition or active feedback control.

The Generation of Autoantibodies. Hemopoietic stem cells are a self-renewing population of primitive pluripotential cells which give rise to the several differentiated cell lines of the hemopoietic system; erythrocytic, granulocytic, megakaryocytic, monocytic and lymphocytic. Immunocytes seemingly differ from other end cell populations in their high degree of subspecialization. There now appear to be many different functional populations of T- and B-cells, perhaps best exemplified by the helper, killer and suppressor activities of thymus-derived lymphocytes. There is, in addition, the acquisition by both T and B populations of a high degree of clonal specificity in terms of antigen recognition and reactivity. The acquisition of this specificity, or the generation of antibody diversity, is now thought by some to result from random somatic mutation of hypervariable regions of the

genome coding for the V genes of light and heavy immunoglobulin chains (62). Germ-line encodation of all specificities represents an alternate view (63). The sequence of events and the inductive factors which trigger stem cells to differentiate along lymphocytic pathways remain unclear, as is the point in this development at which somatic mutation of germ-line encoded V genes may occur. This latter may occur before, at the same time as, or after the stem cell has been committed to differentiation along a lymphocytic line of development. In the following discussion, I will consider the possible involvement of the stem cell per se in the generation of antibody diversity.

Immunologic hyperresponsiveness and the concomitant formation of pathogenic levels of autoantibodies might be speculated to derive from the increased population of cycling stem cells demonstrated to exist in the NZB spleen. The stem cell in active DNA synthesis may constitute a physiologic state at high risk for somatic mutation. This would include mutation of the genome coding for the hypervariable regions of immunoglobulin molecules which determine antibody specificity. An increased pool of mutable cells would be expected to generate increased Ig-bearing antigen-reactive progeny of diverse specificities. Exposure of these cells to antigenic signals would result in their proliferation and differentiation along immunocyte pathways, immunologic hyperreactivity being a direct outcome of the somatic generation of increased populations of potentially responsive cells. Detectable autoantibody formation would be one manifestation of this general phenomenon of immunologic hyperresponsiveness.

It is also possible that somatic mutations generating antibody diversity are not imposed preferentially on the cycling stem cell.

However, entrance into proliferative cycle might be a requirement for the expression of mutations occuring in the expanded G_0 population or for the expression of germ-line encoded antibody diversity. Heightened stem cell activation in the NZB could thus result in the increased availability of antigen-reactive precursors.

Implicit in this role of the stem cell as target of immunologic mutations would be the generation in the NZB of extensive clonal diversity, i.e., of antibody heterogeneity. Somatic mutation, imposed on a susceptible population which is increased over the normal by some 3- to 10-fold, should be expected to generate an enlarged population of antigen-reactive cells. The resultant immunocyte population in the NZB should express the same or increased antibody heterogeneity when compared to mice of other strains. The information for V genes may either be germ-line encoded or generated by somatic mutation. Should the latter be the case, it may be assumed that there is a similar complement of germ-line V genes susceptible to somatic alteration and a constant mutation rate for all strains.

One conclusion might be that this rate of mutation, coupled with a short life-span of the generated antigen-reactive cell, would result in only partial expression at any one time of the total potential of an animal with a small target cell population. The NZB, with its expanded target population, would express a greater fraction of this potential and show increased antibody heterogeneity. That is, the number of different antibody-forming cell clonotypes would be greater.

Alternatively, if the mutation rate were such that antigen-reactive cells of all 10^6 potentialities are present in each animal at all times, then the NZB would be expected to possess replicates of each clonal precursor at a higher incidence than mice of other strains. Here, clonal heterogeneity would be similar to that of other strains, but concentrations of antigen-binding cells should be increased.

In this regard, De Heer and Edgington (64), using competitive plaque inhibition assays for anti-SRBC antibodies and for anti-X and anti-HB erythrocyte autoantibodies, demonstrated a high degree of heterogeneity of antibody binding affinity in the NZB. Compatible results were earlier reported by Petty and Steward (65), who demonstrated that the relative affinities of antibodies produced by New Zealand mice following immunization with human serum transferrin were lower than for several other inbred mouse strains. Further studies, using preautoimmune NZB mice, with competitive plaque inhibition or isoelectric focusing for anti-SRBC and anti-DNA heterogeneity, would provide useful information in this regard. It might be possible to relate, for example, extensive antibody heterogeneity at an early age with the appearance of immunologic hyperresponsiveness and autoantibody formation.

The Role of Exogenous Factors in Autoimmunity. Although I have characterized the abnormality etiologic to autoimmunity in the NZB as a genetically determined insufficiency in stem cell regulation, stem cell population size can also be modified by exogenous factors. Boggs, et al (66) have demonstrated that injection of mice with foreign antigens just prior to irradiation for endogenous colony assay resulted in increased CFU counts. Complete Freund's adjuvant, which stimulates

B- and T-cells, and phytohemagglutinin, a T-cell mitogen, have similar effects.

Rabbits, exposed to chronic and acute stimulation with non-cross-reacting foreign antigens have been shown to develop humoral autoantibodies such as ANA and rheumatoid factor (37). We have observed (unpublished) ANA conversion in DBA/2 strain mice injected with SRBC. The incidence and duration of this autoimmune response proved to be a function of red cell dose and could not be removed by exhaustive absorption of the antiserum with SRBC.

With this background of information, it seems reasonable to assume that a chronic or acute viral infection might, acting as antigen or through some adjuvant behavior, increase the cycling activity of hemopoietic stem cells and thereby precipitate autoantibody formation. Similar infections may be responsible for the onset of autoimmune disorders in the human (67,68). Participation of a virus in the autoimmune disorders of several other species has been described. Aleutian mink disease is characterized by generalized vasculitis, nephritis, mononuclear infiltration of the liver, hypergammaglobulinemia and the production of autoantibodies. It has been transferred by ultrafiltrates of spleen suspensions of infected mink and is accepted as a virus-induced disease (69). Lewis et al (70) have carried out studies which indicated that cell-free filtrates prepared from the spleens of dogs with positive LE cell tests could induce ANA in CAF1 mice. Some lymphoid tumors were also induced. Cultured cells from one of these neoplasms produced C-type RNA viruses (SP104) which induced ANA when inoculated into normal mice. The antigenic determinants of the SP104 virus were found to be present on the lymphocytes of patients with

systemic lupus erythematosus. Reproduction of these results by other laboratories would seem highly desirable.

Identification of an etiologic role for a virus in the disorders of the NZB is difficult, and as mentioned in the introduction, has not been verified. The transplantation studies described here do not support participation of a virus. For example, lethally irradiated NZB mice might have been expected to transmit an infectious agent to the engrafted DBA/2 hemopoietic system causing NZB-like abnormalities. This did not occur. Intact DBA/2 mice and DBA/2 mice irradiated with a sublethal dose of 200 rads and given NZB bone marrow failed to develop persistent ANA titers and NZB-like glomerulonephritis. In addition, when an inoculum of NZB marrow cells capable of causing ANA conversion was coinjected with DBA/2 marrow into lethally irradiated DBA/2 mice, ANA formation was markedly diminished. These observations do not exclude some role for an infectious agent, but place severe limitations on the ability of such an agent to cause NZB disease.

SUMMARY AND CONCLUSIONS

These investigations have explored the factors which predispose mice of the New Zealand Black (NZB) strain to early autoantibody formation. Previous results from this laboratory demonstrated that young adult NZB mice are hyperresponsive to *in vivo* immunization with sheep red blood cells and to splenocyte stimulation *in vitro* by T cell mitogens. These observations led to the speculation that the appearance of autoantibodies in the NZB represented one manifestation of their overall heightened capacity for immunologic reactivity.

Since increases in antibody formation and PHA- and Con A-induced blastogenesis are responses of differentiated B- and T-lymphocytes, it was of interest to determine whether the primitive hemopoietic cells giving rise to these immunocyte lineages might evidence related abnormalities. High resistance of young adult NZB mice to the lethal effects of whole body x-irradiation and elevated endogenous spleen colony formation following sublethal x-ray exposures suggested the existence of a quantitative or qualitative stem cell abnormality. Such an abnormality might conceivably be etiologic to or predictive of aberrant immunocyte behavior.

The thesis studies described here are a continuation of the above. They were oriented to establishing a more definitive relationship between abnormal stem cell activity, immunologic hyperresponsiveness, and autoantibody formation, and to clarifying the nature of the fundamental disorder.

These goals were addressed largely through the use of radiation chimeras. Mice whose hemopoietic systems had been ablated by lethal irradiation, or cytotoxic drug treatment, were reconstituted with a hemopoietic system from a donor of a different strain. This was accomplished by the grafting of stem cell-rich tissues such as adult bone marrow, baby spleen, or fetal liver cells. Various chimeric combinations were established between the autoimmune NZB strain and the nonautoimmune H-2 histocompatible DBA/2 and BALB/c strains. Recipients of NZB hemopoietic cells demonstrated the development of antinuclear autoantibodies and subsequent immune complex glomerulonephritis. They also showed immunologic hyperresponsiveness to B- and T-cell stimuli, and elevated endogenous spleen colony formation. Prevention or reversal of these properties were observed in NZB mice grafted with nonautoimmune donor cells.

These data strongly support the postulated linkage between the hemopoietic and immunologic abnormalities of the NZB. These abnormalities appear to be dependent on a genetic characteristic inherent to the NZB hemopoietic stem cell and its differentiated progeny rather than on host environmental determinants. It was further established that increased numbers of stem cells were in proliferative cycle in the NZB spleen and the resting (G_o) compartment was also enlarged. It is proposed that these stem cells, particularly the cycling compartment, may be the target for somatic mutations engendering immunologic diversity. The amplified immunocyte populations which result could thus account for the heightened immune responsiveness and autoantibody formation of the NZB.

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ADDENDUM TO REFERENCES

The results presented under the headings "Transplantation of Autoreactivity", and "Transplantation of Renal Disease" have appeared in published form as follows:

Morton, J.I., & Siegel, B.V. Early autoantibody formation in lethally irradiated or drug-treated H-2 compatible recipients of pre-autoimmune NZB bone marrow or fetal liver cells. Transplantation, 1974. 17, 624-626.

Morton, J.I., & Siegel, B.V. Transplantation of autoimmune potential. I. Development of antinuclear antibodies in H-2 compatible recipients of NZB bone marrow. Proc. Nat. Acad. Sci. USA, 1974. 71, 2162-2165.

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Table 1

ANA Responses of DBA/2 Mice Receiving Different X-ray Doses and Grafted With 4 X 10^6 NZB Bone Marrow Cells

Days after grafting	X-ray exposure	
	200 rads	800 rads
7	14[a]	0
17	14	0
24	0	86
31	29	71
38	43	100
56	14	100
98	14	100

[a] Percent of mice (7 per group) showing ANA positivity as indicated by reciprocal plasma titers of 20 and greater in the indirect immunofluorescence assay.

Table 2

Autoantibody Formation in Lethally Irradiated DBA/2 Recipients of Donor Fetal Liver Cells

Days after transfer[a]	Recipient mice (%) showing titer greater than 1:20 with source of donor fetal liver[b]		
	NZB	BALB/c	DBA/2
14	13	14	0
25	50	0	17
32	50	--[c]	--[c]
39	83	40	0
46	100	17	0
100	83	20	0

[a] Irradiated (850 rads), 10-week-old DBA/2 mice were injected iv with 4 X 10^6 liver cells from 18-day-old fetuses.

[b] Results are percent of recipient mice showing ANA titers of 1:20 or greater. There were 7-12 mice in each experimental group.

[c] Not done.

Table 3

Autoantibody Formation in BALB/c Recipients of Donor Bone Marrow Cells After Treatment With Busulfan Plus Cytoxan

Days after transfer[a]	Recipient mice (%) showing titer greater than 1:20 with source of donor bone marrow[b]	
	NZB	BALB/c
8	64	0
16	70	0
23	45	9
38	88	43
49	100	17

[a] Two-month-old BALB/c female mice were injected ip with 50 mg/kg of busulfan 24 hr before transplantation and with 200 mg/kg Cytoxan (cyclophosphamide) 4 hr before transplantation of 4 X 10^6 bone marrow cells from 20-day-old donors.

[b] Results are percent of recipient mice showing ANA titers of 1:20 or greater. There were 9-11 mice per group.

Table 4

Autoimmune Disease in Lethally Irradiated Recipients of New Zealand Black Mouse Bone Marrow Cells 10 Months After Transplantation

Mouse number	Chimeric combination[a]	ANA response[b]	Renal involvement[c]
191	NZB⟶NZB	4	22
192		4	24
153	NZB→DBA/2	4	14
156		4	18
160		4	32
162		4	26
165		4	18
167		3	22
152	BALB/c→DBA/2	2	0
155		2	0
157		1	0
159		0	0
161		0	0
163		0	0
166		0	0
168		0	0
154	DBA/2→DBA/2	0	0
158		0	0
164		0	0

[a] Marrow cells were harvested from 3-week-old donors and 2 X 10^6 nucleated cells were injected iv into lethally irradiated 2.5-month-old recipients. Animals represent survivors of 10 mice per original group.

[b] Antinuclear antibody (ANA) responses were graded on a 0-4 scale by indirect immunofluorescence assay.

[c] Renal involvement was scored as percent of glomeruli involved in membranous nephritis. One hundred glomeruli were examined in each recipient.

Table 5

Responses of Intact Mice and Radiation Chimeras to Sheep Erythrocyte Immunization[a]

Strain	Avg. 5-day PFC/spleen X 10^3 (range)	
	41 days	90 days
NZB	217 (116-318)	301 (141-444)
NZB→DBA/2	251 (223-305)	305 (234-341)
BALB/c	67 (62-73)	not done
BALB/c→DBA/2	57 (47-66)	88 (71-115)
DBA/2	59 (37-80)	56 (46-62)
DBA/2→DBA/2	31 (20-46)	68 (37-90)

[a] Chimeras were prepared by grafting 5 X 10^6 bone marrow cells from 3- to 4-week-old NZB, BALB/c or DBA/2 donors into 3-month-old irradiated (800 rads) DBA/2 recipients. Mice were injected ip with 0.20 ml of a 1:10 dilution of washed SRBC on day 41 or 90 following transplantation and plaques counted 5 days later. Each group consisted of 3 mice.

Table 6

Responses of Intact Mice and of Histocompatible Radiation Chimeras to Sheep Erythrocyte Immunization[a]

Strain	Avg. 5-day PFC/spleen X 10^3 (range)	Avg. spleen wt. in mg. (range)
Intact mice:		
NZB	282 (241-350)	144 (134-160)
(NZBXBALB/c)F1	167 (161-174)	117 (114-127)
BALB/c	99 (70-117)	138 (132-142)
Chimeras:		
(NZBXBALB/c)F1 ⟶BALB/c	130 (113-153)	110 (92-136)
BALB/c⟶BALB/c	54 (44-59)	126 (113-142)

[a] Chimeras were prepared by grafting 5 X 10^6 bone marrow cells from 2-month-old BALB/c or (NZBXBALB/c)F1 male donors into 3- to 4-month-old lethally irradiated (720 rads) male BALB/c recipients. Intact, 2- to 3-month-old male mice and chimeras of 5 months' duration were immunized ip with 0.20 ml of a 1:10 dilution of washed SRBC and plaques counted 5 days later. Each group consisted of 4 mice.

Table 7

Stimulation Ratios for PHA- and Con A-Stimulated Spleen Lymphocytes From Intact Mice and Radiation Chimeras

Strain[a]	Stimulation ratios (SR)[b]	
	PHA	Con A
NZB	12.4	21.2
BALB/c	9.5	10.4
DBA/2	1.8	4.8
NZB→DBA/2	4.2	14.1
BALB/c→DBA/2	2.8	8.8
DBA/2→DBA/2	1.5	3.4

[a] Intact mice were 8- to 10-week-old females. Chimeras were assayed 6 weeks after irradiation (720 rads) and grafting of 5 X 10^6 bone marrow cells from 6-week-old donors of the various strains.

[b] $SR = \frac{\text{cpm PHA-stimulated cultures}}{\text{cpm unstimulated cultures}}$

Each value represents the average for 2 mice of assays carried out in quadruplicate.

Table 8

Endogenous Spleen Colony Formation in Intact Mice and Radiation Chimeras[a]

Strain	Avg. CFU/spleen (range)	Avg. spleen wt. in mg. (range)
NZB	13.5 (8-18)	38.5 (36.0-41.2)
NZB→DBA/2	25.3 (19-30)	27.8 (25.4-29.6)
BALB/c	0.5 (0-1)	28.0 (26.2-29.7)
BALB/c→DBA/2	1.0 (0-2)	20.4 (18.4-25.4)
DBA/2	0.5 (0-1)	26.0 (21.3-30.5)
DBA/2→DBA/2	4.3 (1-10)	22.9 (21.5-25.3)

[a] Chimeras were prepared by grafting 5 X 10^6 bone marrow cells from 3- to 4-week-old NZB, BALB/c or DBA/2 donors into 3-month-old irradiated (800 rads) DBA/2 recipients. Intact animals were 2-3 months old at time of assay. Thirty-five days after grafting, chimeras and intact animals were exposed to 600 rads. Eight days later, spleens were collected, weighed, and placed in Bouin's solution for colony counting.

Table 9

Prevention of Antinuclear Antibody Responses in NZB Mice By DBA/2 Bone Marrow Grafts

Strain [a]	Age of mouse or duration of graft	
	2-3 months	5-6 months
Intact DBA/2	0/20 (0%)[b]	0/20 (0%)
DBA/2→DBA/2^{900r}	0/14 (0%)	0/13 (0%)
DBA/2→NZB^{800r}	5/18 (28%)	2/10 (20%)
DBA/2→NZB^{950r}	4/14 (29%)	0/6 (0%)
DBA/2→NZB^{1000r}	0/13 (0%)	0/3 (0%)
NZB→NZB^{900r}	9/13 (68%)	7/9 (77%)
Intact NZB	59/61 (97%)	62/63 (98%)

[a] Mice were exposed to the designated x-ray dose at 2-4 months of age and grafted with 3 X 10^6 bone marrow cells from 3- to 6-month-old donors.

[b] Number of mice per total (percent) showing ANA positivity.

Table 10

Response of DBA/2→NZB Radiation Chimeras to Sheep Erythrocyte Immunization

Chimera[a]	Avg. 5-day PFC/spleen X10^3 (range)	Avg. spleen wt. in mg. (range)
DBA/2→NZB	23.3 (16.4-30.6)[b]	105.8 (104.3-107.5)
DBA/2→DBA/2	35.8 (27.7-44.1)	104.9 (96.4-111.9)
NZB→DBA/2	127.7 (126.0-129.4)	117.8 (116.5-119.0)

[a] DBA/2→NZB chimeras were prepared by grafting 2.5 X 10^6 DBA/2 bone marrow cells into 3-month-old NZB mice exposed to 1000 rads x-rays. Chimeras were of 4 months' duration at the time of plaque assay.

DBA/2→DBA/2 and NZB→DBA/2 chimeras were prepared by grafting 3.5 X 10^6 DBA/2 or NZB bone marrow cells into 5-month-old DBA/2 mice exposed to 967 rads. Chimeras were of 6 months' duration at the time of plaque assay.

[b] Each value represents the average for 4 mice.

Table 11

Endogenous Spleen Colony Formation in DBA/2→NZB Radiation Chimeras and Controls

Strain	Number of mice	Avg. CFU/spleen (range) after 600 r	Avg. spleen wt. in mg. (range)
Intact NZB	12	15.5 (5-38)	39.6 (23.9-59.0)
Intact DBA/2	9	0.3 (0-1)	21.0 (17.0-28.3)
DBA/2→NZB	9	0.6 (0-2)	19.9 (12.3-21.8)
DBA/2→DBA/2	4	2.3 (0-8)	24.6 (20.0-30.2)

[a] Intact NZB and DBA/2 mice were 3 and 4 months old, respectively, at the time of x-ray exposure. For chimera preparation see Table 10, footnote [a].

Table 12

Endogenous Spleen Colony Formation in Radiation Chimeras Providing An NZB-F1 Environment

Chimera[a]	Avg. CFU/spleen (range)	Avg. spleen wt. (range)
NZB⟶ (NZBXDBA/2)F1	24.8 (13-43)	46 (26-80)[b]
DBA/2⟶ (NZBXDBA/2)F1	1.5 (0-3)	21 (19-23)
NZB⟶ (NZBXBALB/c)F1	24.7 (13-35)	40 (30-48)
BALB/c⟶ (NZBXBALB/c)F1	0	26 (20-30)

[a] (NZBXDBA/2)F1 mice were exposed to 950 rads at 4 months of age, grafted with $3X10^6$ bone marrow cells from 4-month-old NZB or DBA/2 donors and exposed to 650 rads for 9-day endogenous colony assay, 51 days after transplantation.

(NZBXBALB/c)F1 mice were exposed to 900 rads at 4 months of age, grafted with 2.2- to 2.5 X 10^6 bone marrow cells from 2- to 4-month-old NZB or BALB/c donors and exposed to 650 rads for 9-day endogenous colony assay 42 days after transplantation.

[b] Spleen weights are in mg. Each value represents the average for 4 mice.

Table 13

Transplantable Bone Marrow CFUs For NZB, BALB/c and DBA/2 Strain Mice and F1 Hybrids

Experiment	Marrow donor[a]		Marrow recipient[b]		CFU/10^5 bone marrow[c] cells (range)
	Age	Strain	X-ray dose	Strain	
A.	8	NZB	900 r	NZB	17.0 (13-26)
B.	16	NZB	900	(NZBXDBA/2)F1	12.2 (10-14)
	16	DBA/2	"	"	15.0 (10-19)
C.	28	NZB	720	BALB/c	18.0 (9-29)
	28	NZB	950	NZB	16.7 (10-24)
D.	35	NZB	850	DBA/2	17.0 (11-22)
	35	(NZBXDBA/2)F1	"	"	14.3 (12-19)
	35	BALB/c	"	"	15.7 (14-17)
	35	(BALB/cXDBA/2)F1	"	"	17.0 (16-18)
E.	63	NZB	900	DBA/2	16.1 (13-21)
	150	DBA/2	"	"	15.3 (10-20)

[a] Each donor marrow pool (femurs from 2-7 mice) was assayed in 4-7 recipients for 8-9 day spleen colony (CFU) formation. 10^5 nucleated cells were injected within 3 hours of recipient irradiation. Donor age is expressed in days.

[b] X-ray dose is expressed in rads (r). Mice were exposed at a rate of 71 r per minute. Recipients were 3-6 months old.

[c] Values represent the arithmetic mean of surface colonies counted on the spleens of 4-7 recipient mice.

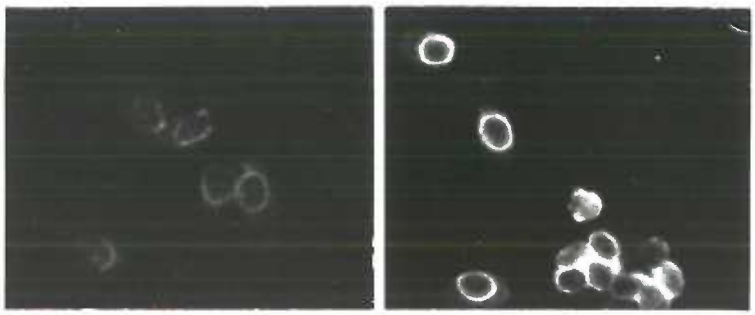

Figure 1. Formalinized chicken erythrocyte nuclei, incubated with different NZB mouse plasma samples and fluorescein-labelled goat antimouse gamma globulin. Reactions are designated as 1+ (left) and 4+ (right).

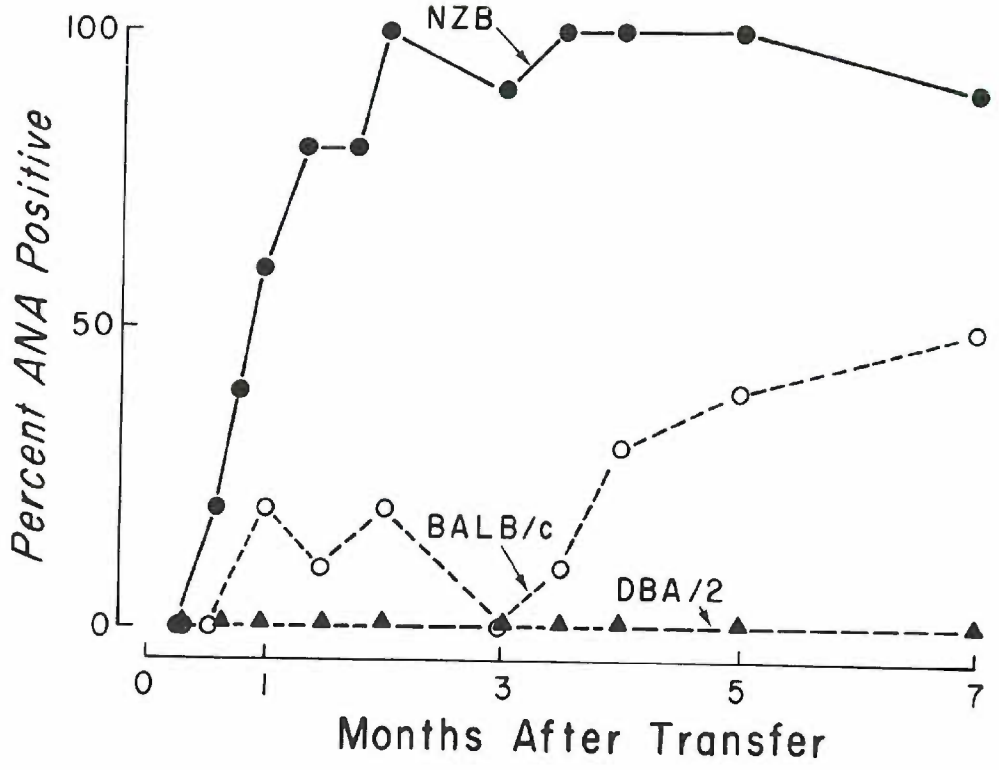

Figure 2. Percent of lethally irradiated (850 rads) DBA/2 mice showing positive ANA responses with time following intravenous injection of 2 X 10^6 bone marrow cells from 3-week-old donor mice. NZB, BALB/c and DBA/2 mice served as donor strains. Each experimental group consisted of 10 mice.

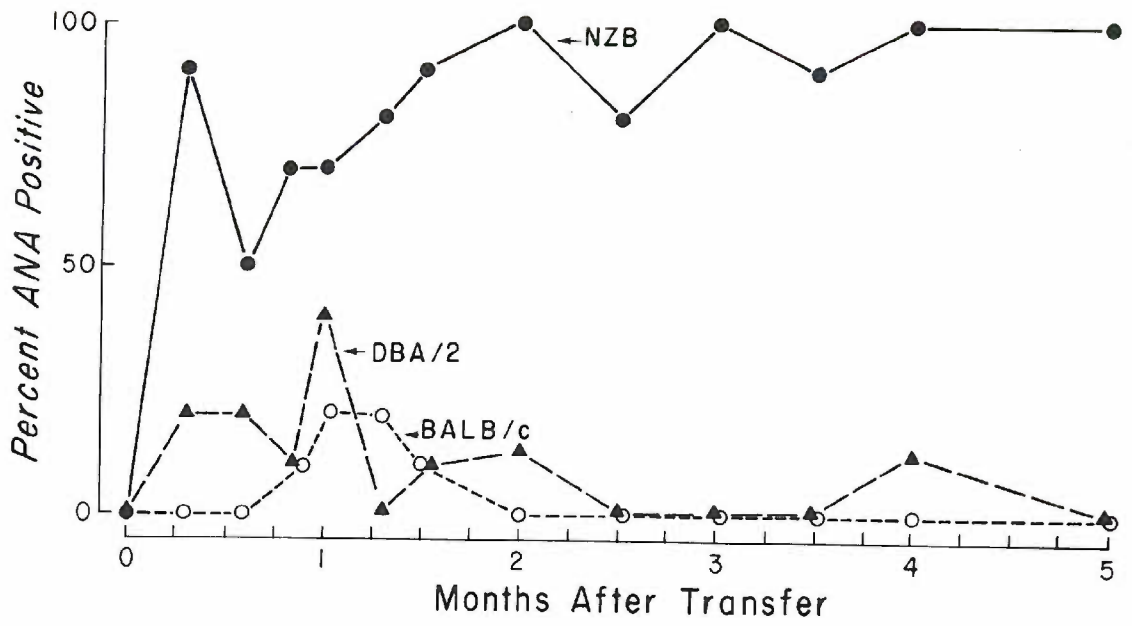

Figure 3. Percent of lethally irradiated (850 rads) BALB/c mice showing positive ANA responses with time following intravenous injection of 5 X 10^6 spleen cells from 8-day-old donor mice. NZB, BALB/c and DBA/2 mice served as donor strains. Each experimental group consisted of 10 mice.

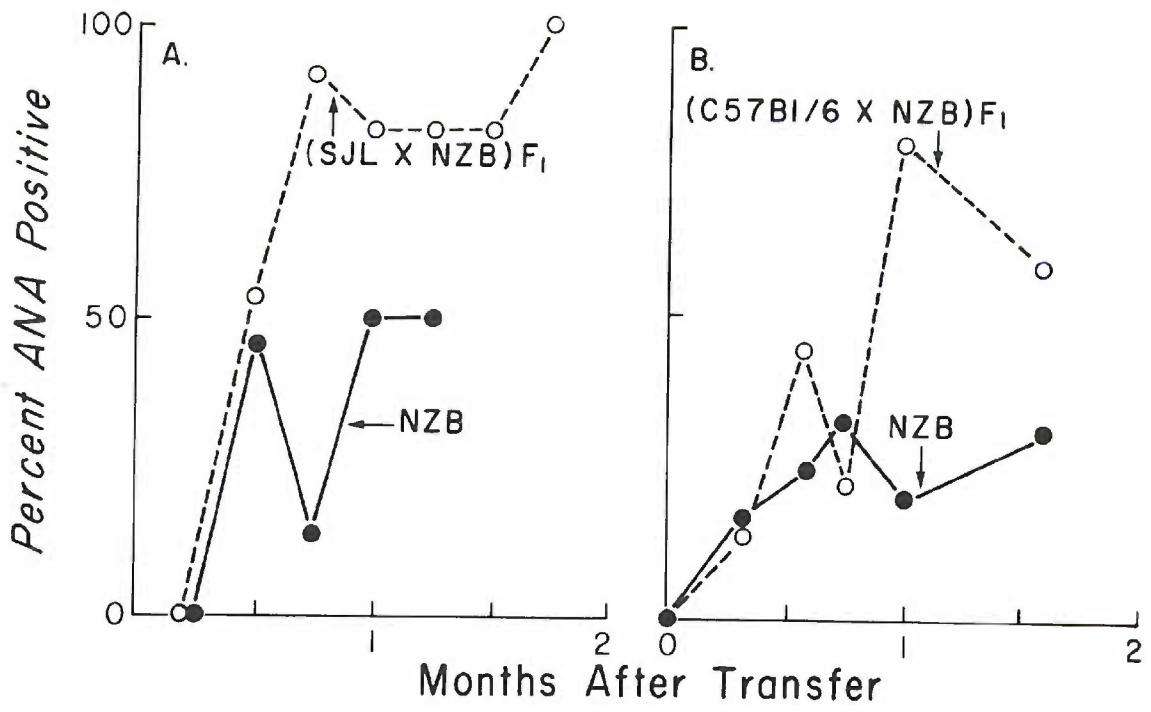

Figure 4. Percent ANA positivity among irradiated, bone marrow grafted recipient mice of two strains not histocompatible with the NZB strain at the H-2 locus. (A) SJL (H-2s) strain recipient mice were exposed to 850 rads and injected with 4 X 10^6 marrow cells from NZB (H-2d), or histocompatible (SJLXNZB)F1 hybrid donors. (B) C57Bl/6 (H-2b) strain recipient mice were exposed to 600 rads and injected with 4 X 10^6 marrow cells from NZB or histocompatible (C57Bl/6XNZB)F1 hybrid donors. Each experimental group consisted of 10 mice.

Figures 5, 6 & 7. Representative glomeruli from DBA/2 mice (Figs. 5 & 6) 10 months after lethal irradiation and transplantation with NZB bone marrow. There are prominent, irregular thickened basement membranes and a thrombus in a capillary lumen. These two features were unique to the DBA/2 and NZB mice that received NZB marrow and resemble the changes observed in untreated NZB mice of comparable age (Fig. 7). Periodic acid-Schiff reaction; X 680.

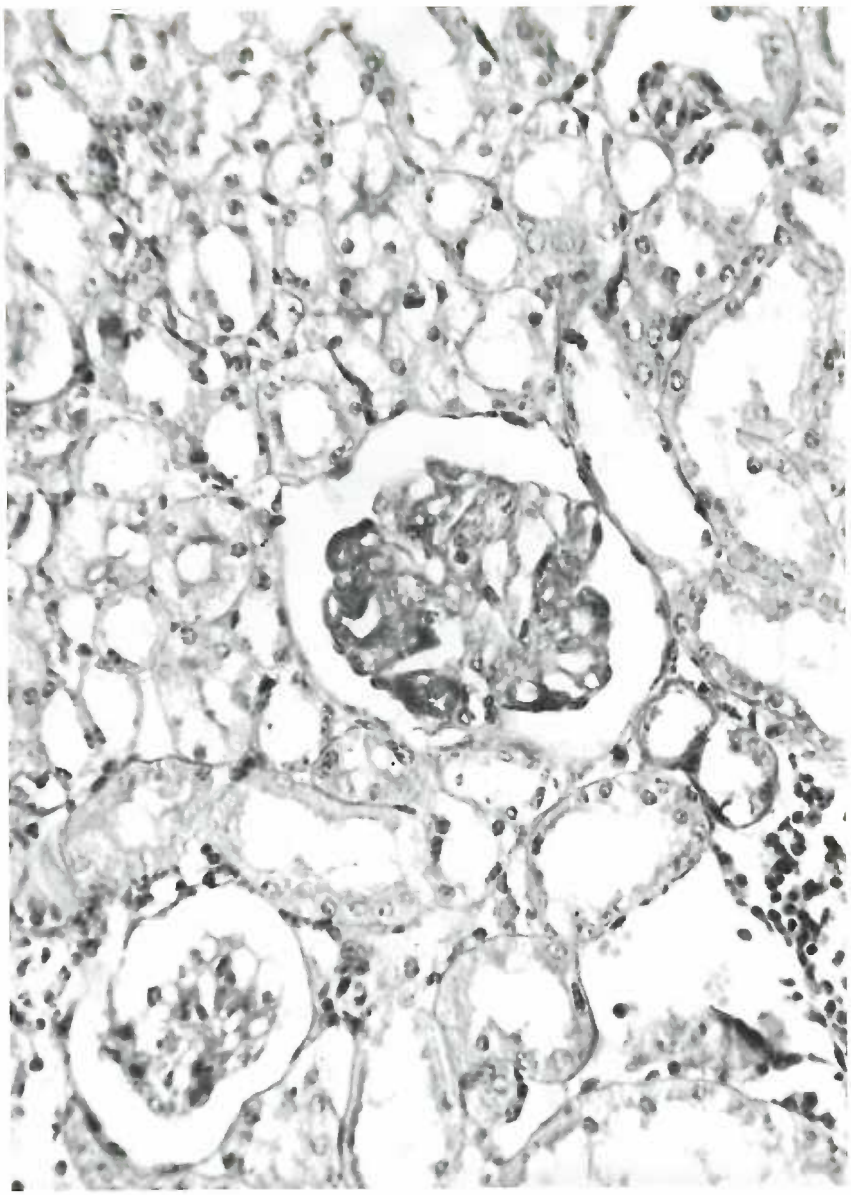

Fig. 5

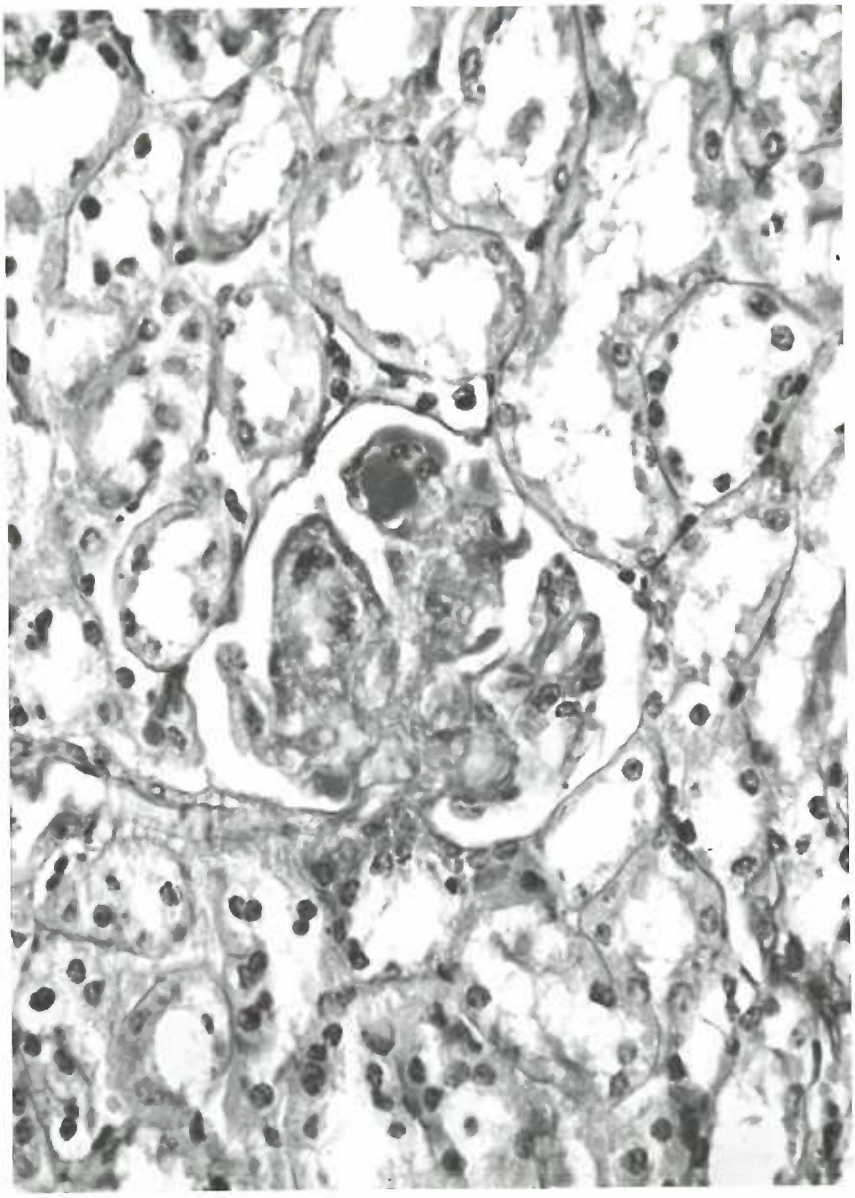

Fig. 6

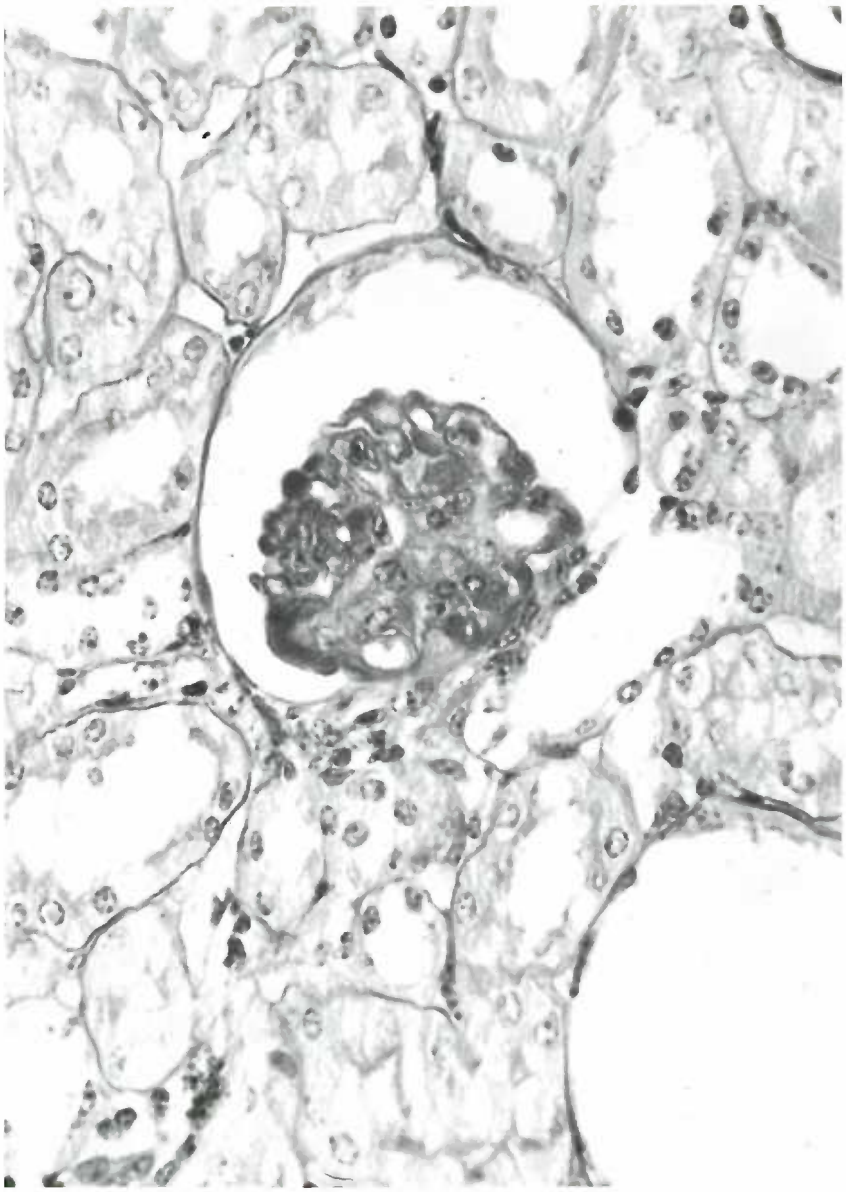

Fig. 7

Figures 8 & 9. Representative glomeruli from DBA/2 mice 10 months after lethal irradiation and transplantation with BALB/c marrow. These illustrate the spectrum of changes present in all lethally irradiated mice that received marrow transplants. Note the focal increase in number of nuclei, variability of nuclear size, and intercapillary sclerosis. There is absence of basement membrane thickening and capillary thrombi, features present only in mice receiving NZB marrow transplants. Periodic acid-Schiff reaction; X 680.

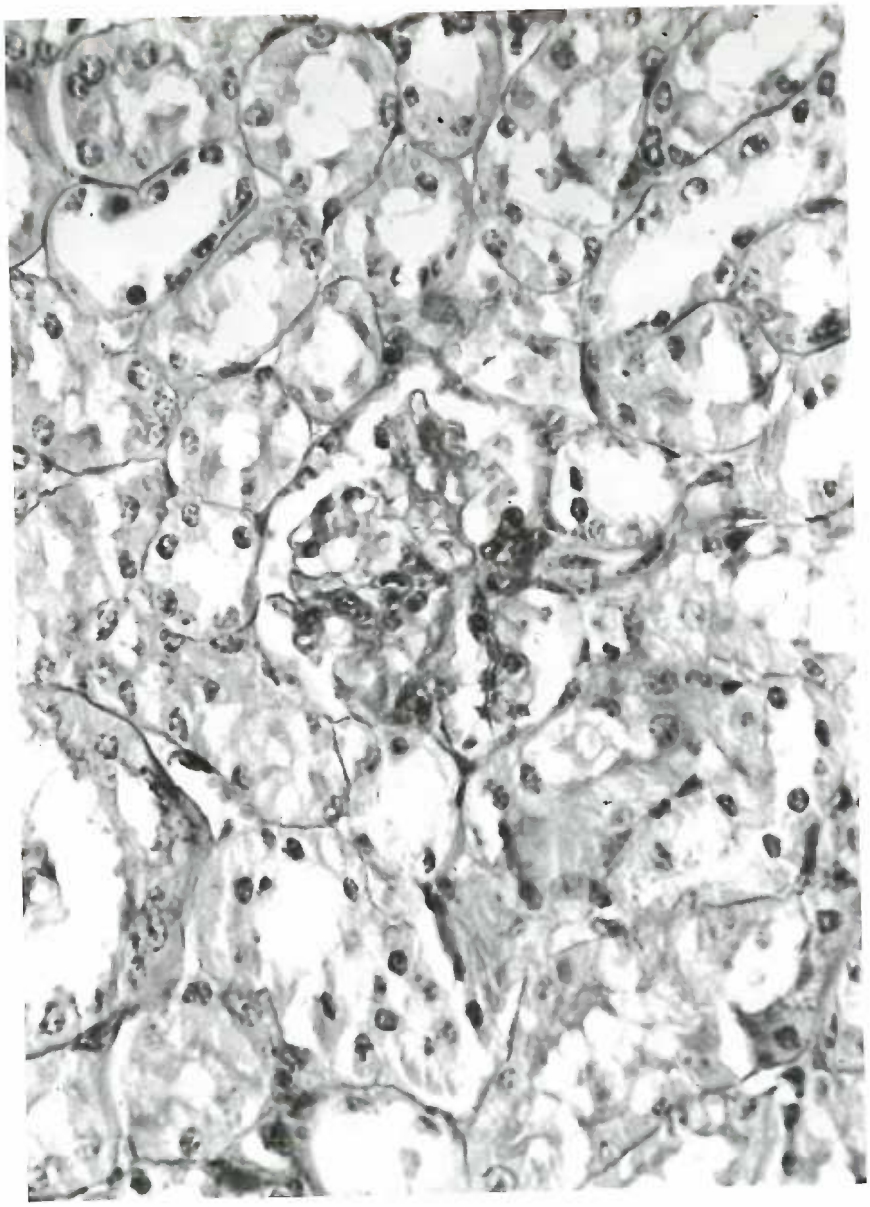

Fig. 8

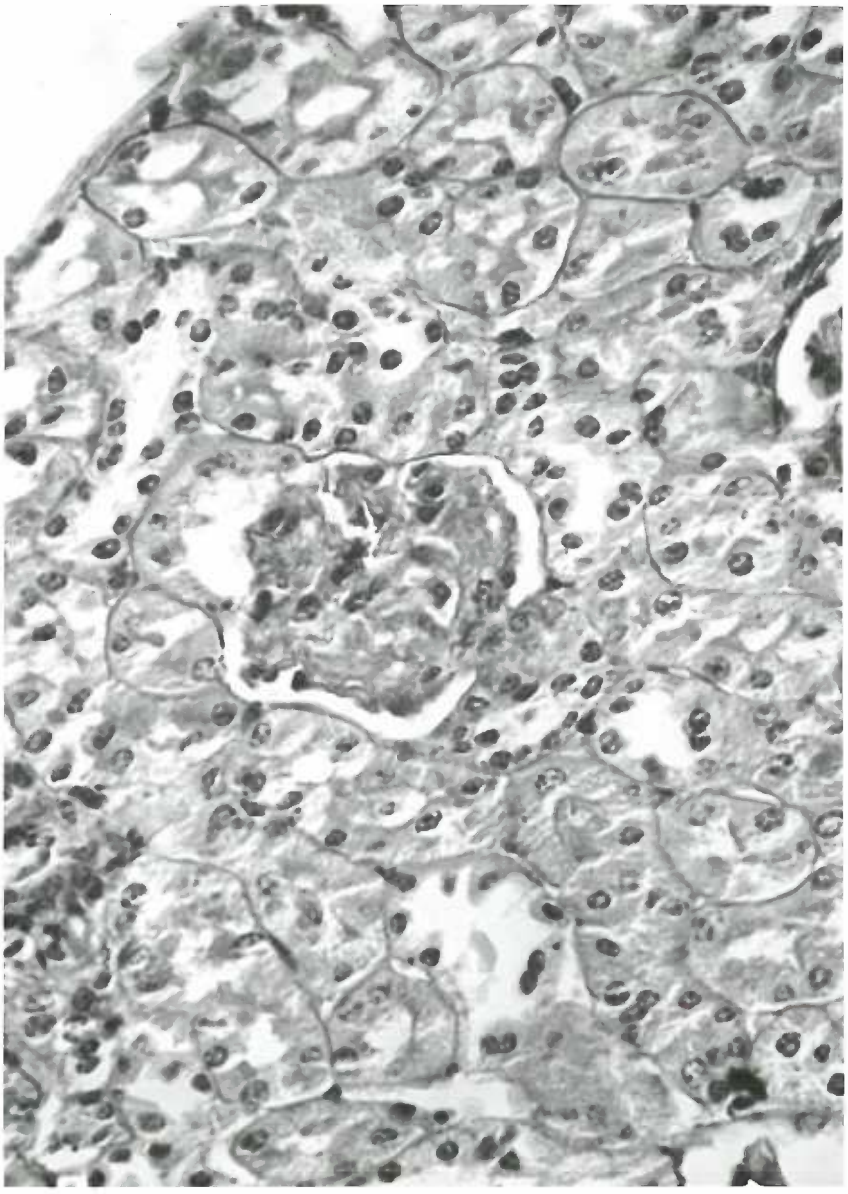

Fig. 9

Figure 10. Glomerulus from a lethally irradiated DBA/2 mouse that received an NZB marrow transplant. There are irregular deposits associated with the basement membranes that stain with fluorescein-labeled goat antimouse globulin. X 320.

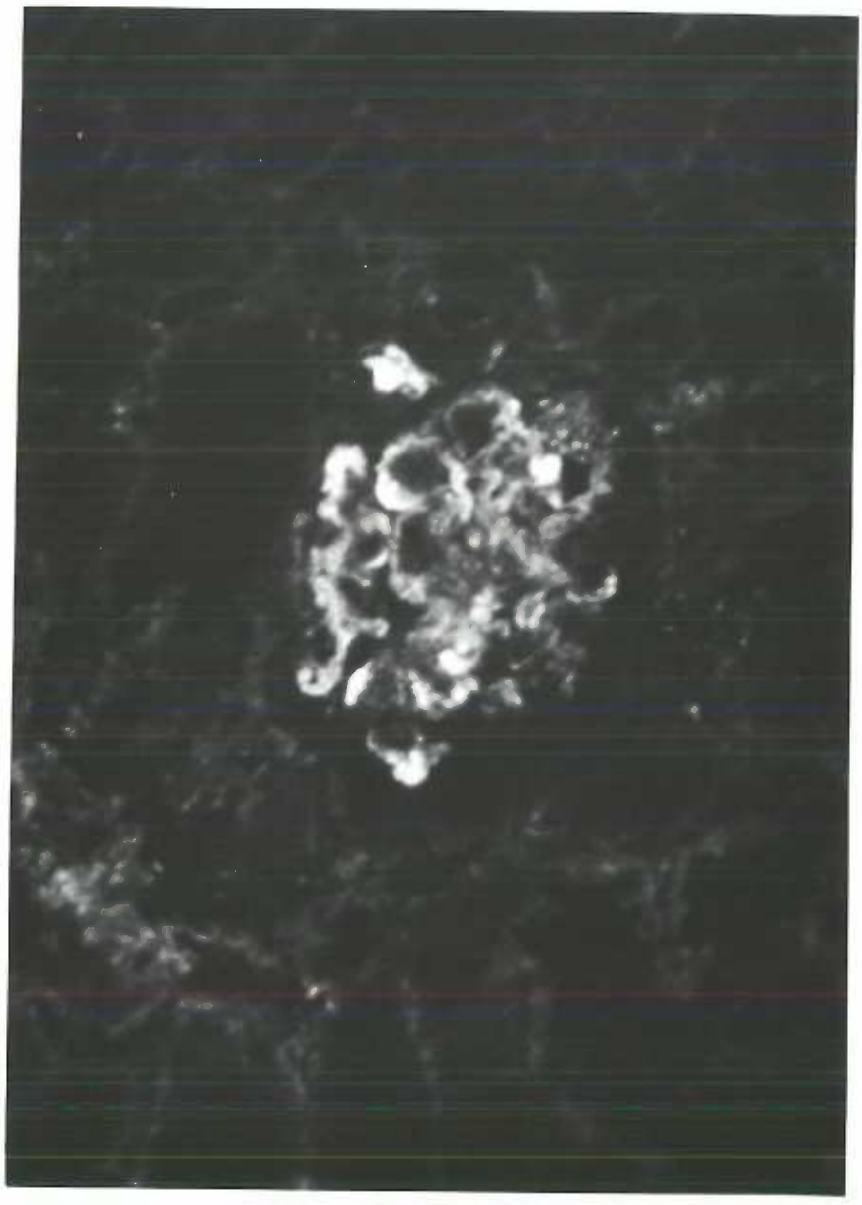

Fig. 10

Figure 11. Glomeruli from an untreated 10-month-old NZB mouse after staining with labeled goat antimouse globulin. The pattern of fluorescent staining is comparable to that seen in Fig. 10. X 320.

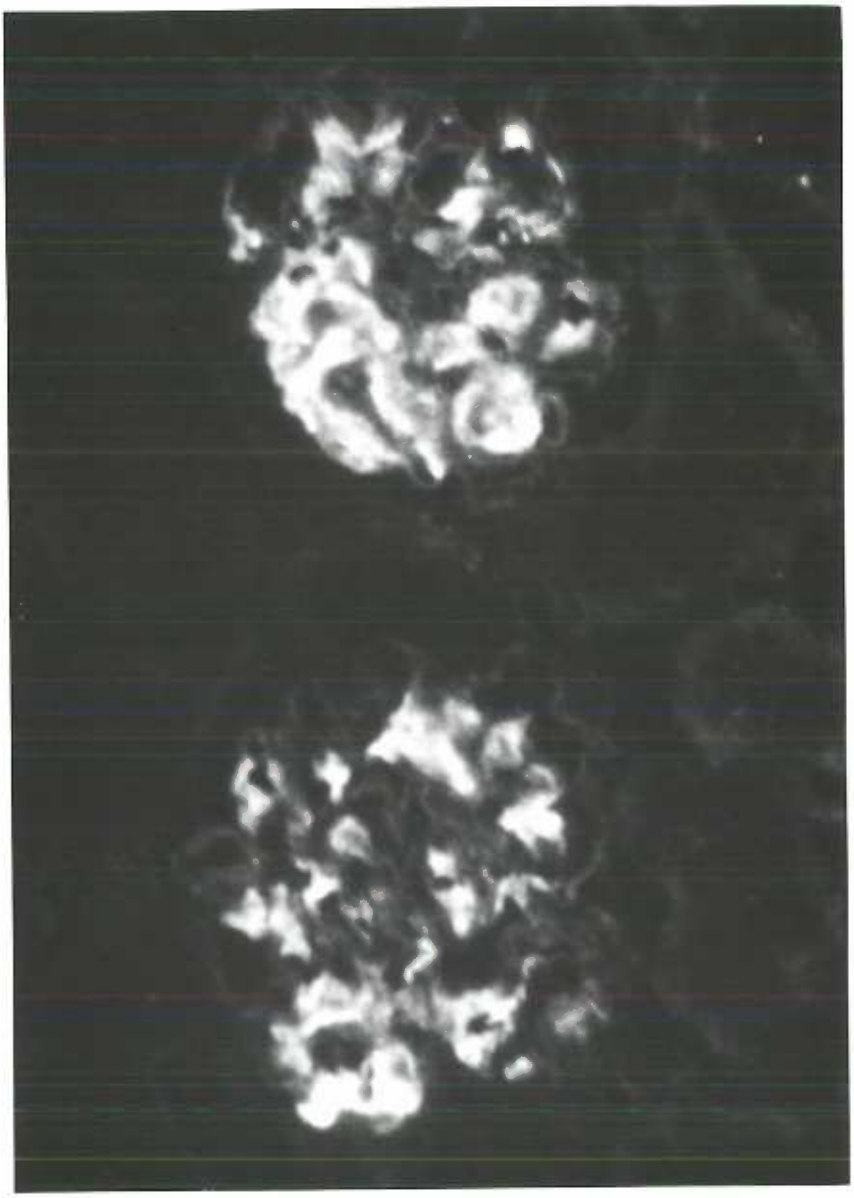

Fig. 11

Figure 12. Glomerulus from a lethally irradiated DBA/2 mouse that received a DBA/2 marrow transplant. The fluorescent staining is limited to several small foci. Most glomeruli from these animals were negative. This illustration depicts the heaviest staining observed in mice that received other than NZB marrow. X 320.

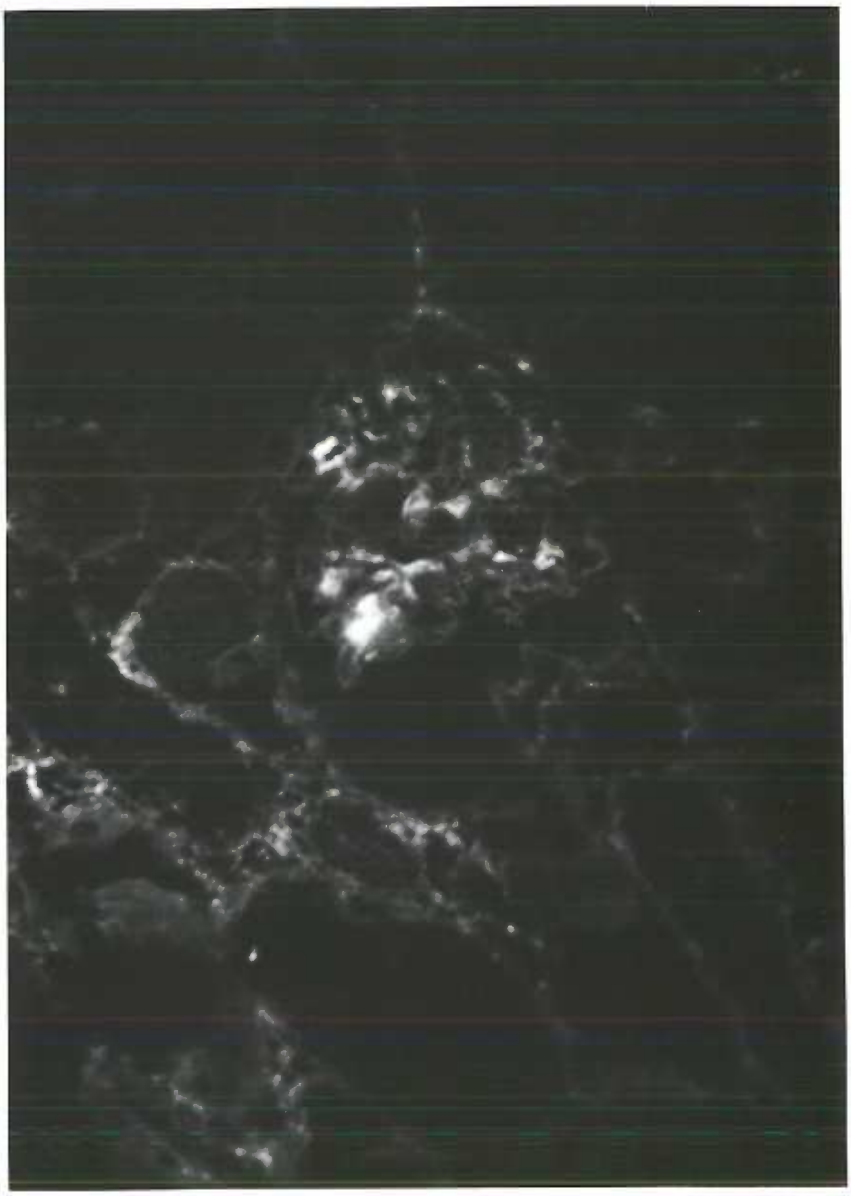

Fig. 12

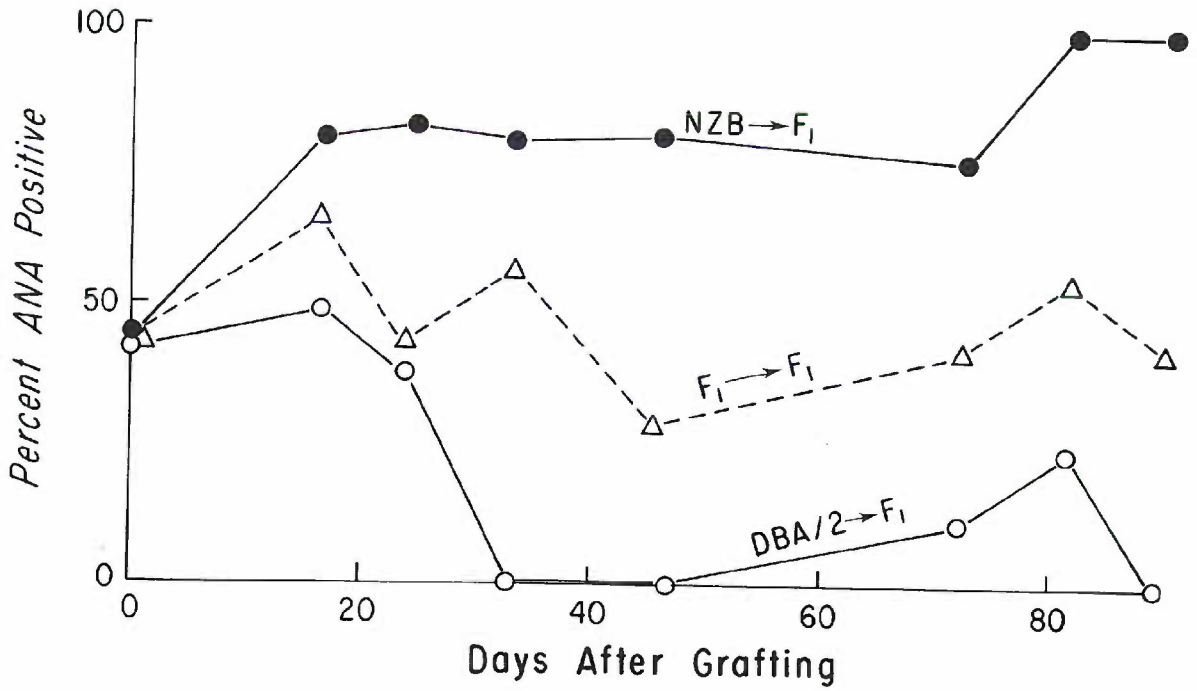

Figure 13. Percent ANA positivity among lethally irradiated (800 rads) 5- to 6-month-old (NZBXDBA/2)F1 hybrid mice injected intravenously with 4 X 10^6 syngeneic F1 or parental NZB or DBA/2 bone marrow cells. Each experimental group consisted of 8 mice.

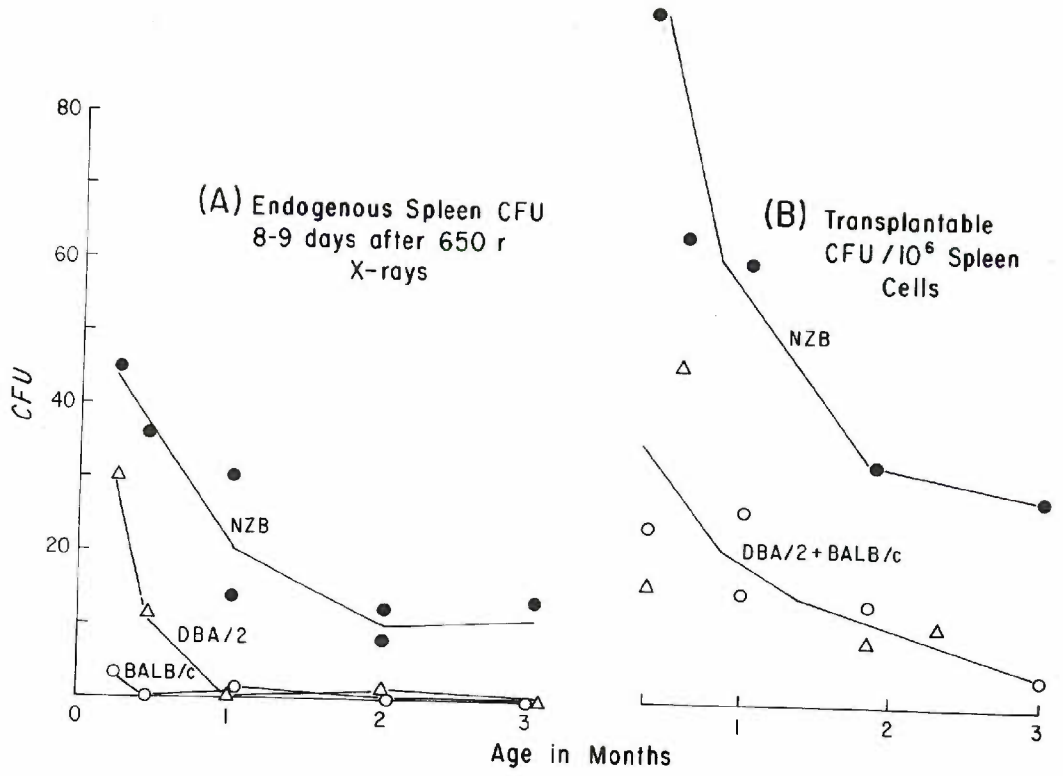

Figure 14. (A) Endogenous spleen colony formation in NZB, BALB/c and DBA/2 strain mice of different ages, 8-9 days following exposure to 650 rads. Each point is the average value for 4-6 mice. (B) Transplantable spleen colonies per 10^6 spleen cells for NZB BALB/c and DBA/2 mice of different ages. Each point represents the average for spleen cell pools from 3 donors injected into 4-6 lethally irradiated syngeneic or H-2 histocompatible allogeneic recipients. Colonies were counted after 8-9 days.