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CHAPTER I

INTRODUCTION AND HISTORY

Anti-nuclear Antibodies in Man

Characterization of Anti-nuclear Antibodies

The basis for the study of autoimmune anti-nuclear antibodies reactive with autologous nuclear materials was provided by Hargraves et al. (1948) in a report which described an unusual cell present in samples of bone marrow derived from patients with systemic lupus erythematosus (SLE). This cell was named the lupus erythematosus (LE) cell. Typical LE cells were not found in fresh samples of bone marrow but appeared in samples of bone marrow after incubation at 37° C. They were described as polymorphonuclear leukocytes containing a large mass in their cytoplasm which stained dark pink to purple with Wright's stain. It was soon demonstrated that LE cell preparations could be obtained also with peripheral blood samples (Fisher and Moyer, 1950; Suksta and Conley, 1951; Moffatt et al., 1950). Further investigations suggested that the only factor contributed by the blood specimen was serum. Leukocytes used in the test could be obtained from the blood of another individual, as well as from other mammalian species (Lee et al., 1951; Carrera et al., 1954; Haserick, 1956). Subsequent investigations

revealed that the serum factor responsible for LE cell formation was associated with the gamma-globulin fraction of serum as shown by moving boundary electrophoresis (Haserick et al., 1950), starch electrophoresis (Holman and Kunkel, 1957), salt fractionation (Haserick and Lewis, 1950), and cellulose column ion exchange (Willkens et al., 1958; Fallet et al., 1958). It was also demonstrated by Fallet and associates (1958) that the LE cell factor was found only in the IgG fraction of the immunoglobulins. Conflicting claims have been made concerning the role of complement in LE cell formation. Formijne and van Soren (1958) reported that complement is required for the formation of LE cells, although this had been denied earlier by Lee et al. (1951).

Following the reports that the serum of SLE patients would induce LE cell formation, Miescher and Fauconnet (1954) showed that the LE factor could be completely removed from SLE sera by absorption with cell nuclei obtained from numerous animal and human tissues and suggested that the LE cell factor was a true autoantibody. The reaction of the LE cell factor and cell nuclei was shown to fix complement (Robbins et al., 1957; Holman and Kunkel, 1957). The serum factor that reacts with cell nuclei was shown to be antigenically similar to gamma-globulin (Holborow et al., 1957; Friou et al., 1958). More recent studies have shown that anti-nuclear antibodies may exist in all classes of immunoglobulins (Barnett et al., 1964) in contrast to the LE cell factor which is found only in the IgG fraction. When deoxyribonucleoprotein (DNP) prepared from cell nuclei of calf thymus was used in absorption tests, the results suggested that the LE cell antibody was probably specific for DNP (Holman and Kunkel, 1957; Friou, 1958). DNP

absorption completely removed the LE cell factor, whereas absorptions with deoxyribonucleic acid (DNA) and histone were ineffective. However, other immunologic tests have shown that the serum of patients with SLE may contain a variety of anti-nuclear antibodies other than the LE cell factor. The following components of cell nuclei have been clearly identified as reacting with different anti-nuclear antibodies: DNP, DNA, and histone (Robbins et al., 1957; Aisenberg, 1959). The predominant methods that have been used to demonstrate the multiplicity of anti-nuclear antibodies in SLE sera are complement fixation, precipitin or agglutinin formation, and fluorescent antibody.

Anti-DNP antibodies have been found in almost all sera from patients with SLE that have been tested with DNP in a complement fixation test (Holman and Kunkel, 1957) or the DNP immunofluorescent spot test (Friou, 1958; Friou et al., 1958; Casals et al., 1964). Sera containing this factor produce the homogeneous type of nuclear immunofluorescence (Lachmann, 1964). Absorption of these sera with DNP will remove the DNP reactive antibodies and a speckled type of nuclear immunofluorescence will sometimes remain (Lachmann and Kunkel, 1961). Anti-DNP antibodies have also been found in the serum of patients with other connective tissue diseases, but the incidence and the titers are much lower than in patients with SLE (Casals et al., 1964).

A smaller percentage of SLE sera have been shown to contain anti-DNA antibodies in addition to anti-DNP antibodies. Anti-DNA antibodies were first detected in complement fixation tests with calf thymus DNA (Holman and Kunkel, 1957; Robbins et al., 1957; Ceppellini et al., 1957; Casals et al., 1963). Absorption experiments have

demonstrated that although DNA will remove the anti-DNA antibody, it will not remove the LE cell promoting factor or the anti-DNP antibody responsible for the homogeneous pattern of nuclear immunofluorescence. Although the complement fixation test has been the most widely used test to detect anti-DNA antibody activity, Casals et al. (1963) found that many SLE sera containing anti-DNA antibodies are anticomplementary and thus unsuitable for the test. They also found that SLE sera containing anti-DNA antibodies produced a "shaggy" pattern of nuclear immunofluorescence which was not produced by sera lacking this antibody. By using antibody obtained from DNA-anti-DNA precipitates, they found that anti-DNA antibody was responsible for the shaggy staining pattern. The authors also suggested that anti-DNA antibodies never occur in sera in the absence of anti-DNP antibodies. The reports of Stollar and Levine (1961) and Stollar, Levine and associates (1962) indicated that single-standed or denatured DNA is superior to double-stranded DNA when used as an antigen in quantitative precipitin or complement fixation tests with anti-DNA antisera. Inhibition of complement fixation was obtained with a wide variety of nucleotides and purine derivatives. The greatest inhibitory activity was obtained with a pentanucleotide. Some sera had antibodies reactive primarily with adenine; others were more reactive with thymidine. Since these results indicated that the anti-DNA antibodies were specific for the purine and pyrimidine bases in DNA, Kunkel and Tan (1964) proposed that this could explain the greater reactivity of sera with single-stranded DNA, due to the exposure of these groups in the single-stranded configuration. DNA from 40 sources has been shown to react with certain SLE sera

(Levine, 1963). Quantitative differences were observed in the extent of these reactions. The differences were ascribed to variation in the specificity of the antibodies and to variation in the DNA preparations with respect to the extent of single-stranded areas on the DNA molecules. Anti-DNA antibodies have been shown to be active in passive cutaneous anaphylaxis (Deicher et al., 1960).

Pathologic States Associated with Anti-nuclear Antibodies

The results of several clinical studies have been interpreted to indicate that anti-nuclear antibodies are not significant in the pathogenesis of SLE. Negative LE cell tests with the blood of SLE patients have been reported by Dubois (1956). Many of the patients who had negative tests had high titers of anti-nuclear antibody activity in their sera as indicated by various immunofluorescent tests (Friou et al., 1958, and Casals et al., 1964). It has also been suggested that anti-nuclear antibodies do not apparently cross the cell membrane into living cells in a biologically active form to react with nuclear antigens (Casals et al., 1964). However, a claim to the contrary without published data has been made in a review by Kunkel and Tan (1964). Beck and Rowell (1963) have reported that newborn infants born of mothers having SLE are usually not affected, even though the LE factor is present in the infant's blood for approximately 7 weeks. The transfusion of human serum containing anti-nuclear antibodies into normal individuals (Bencze et al., 1958) and dogs (Bencze and Ludanyi, 1960) did not result in detectable pathologic changes or disease other than LE cell formation in vitro. These reports suggest that anti-nuclear antibodies

are not involved in the pathogenesis of SLE and are therefore not important. Other reports, however, have stressed the possible significance of specific anti-nuclear antibodies. It has been suggested, for example, that the presence of anti-nuclear antibodies in an individual indicates an abnormality in the immunocompetent cells and a potential cause of disease (Dameshek, 1963). Friou (1958) and Casals et al. (1964) reported a high incidence of anti-DNP (calf thymus) antibodies in the sera of SLE patients. Low titers of antibody activity were also found in a small number of patients with other connective tissue diseases. Although Casals et al. (1964) suggested that there was a general correlation between high titers of anti-DNP antibody activity and acute SLE, it had been shown previously that certain patients may have high titers of antibody activity without showing evidence of acute disease (Friou, 1958; Townes et al., 1963). The possible importance of anti-DNA antibodies in acute SLE has been discussed by Casals and associates (1964) and by others (Seligman and Milgrom, 1957; Kayhoe et al., 1960). In support of the concept that anti-DNA antibodies may be involved in acute disease, Casals et al. (1964) described a study of 37 patients with SLE, of whom only 19 had anti-DNA antibodies, and these were the only patients who were acutely ill or had a history of severe illness before treatment. Anti-DNA antibody activity was not found in the sera of either normal individuals or those with other connective tissue diseases. Considering the observations that serum complement levels are low in patients with acute SLE (Townes et al., 1963) as well as in serum sickness (Germuth, 1953; McClusky et al., 1960; Dixon, 1963), it has been suggested by Casals et al. (1964) that complement

could have been removed by antigen-antibody complexes (possibly DNA-anti-DNA) and "may play a causal role in the production of some of the manifestations of acute disease, glomerulonephritis and vasculitis". Dixon (1963) demonstrated that chronic glomerulonephritis develcped only in animals making small amounts of antibody which allowed soluble antigen-antibody complexes to develop during serum sickness. It was speculated by the author that individuals making a small antibody response could be particularly prone to develop certain pathologic lesions in tissues that do not have any antigenic relationship to the antigen involved.

Anti-nuclear Antibodies in Experimental Animals

Antibodies Induced by Immunizations

A number of claims have been made that immunizations with DNP- or DNA-containing materials induce certain experimental animals to produce antibodies reactive with autologous nuclear constituents or nuclei from other sources. Blix et al. (1954) reported that the sera of rabbits hyperimmunized with crude preparations of DNA from calf thymus or Mycobacterium tuberculosis contained antibodies that reacted in a complement fixation test with the specific material used for immunization, but did not cross-react with other DNA preparations. Phillips and associates (1958) immunized rabbits with 12 injections of Brucella abortus DNA extract containing 25% protein. In gel diffusion the antisera produced precipitin bands that were Feulgen positive, indicating that the precipitate contained DNA. These antisera also showed cross reactions with DNA from calf thymus and salmon sperm. The antibody activity could not be absorbed with whole B. abortus cells or the

residue remaining after chloroform extraction. Successful immunization with DNP was also accomplished by Goodman (1959). He gave 9 rabbits a prolonged series of injections with human liver DNP extract in complete Freund's adjuvant. All rabbits developed antibodies that reacted with both human and rabbit liver DNP extracts. Lachmann (1961) attempted to characterize the LE cell antigen by injecting rabbits with either mouse, human, or guinea pig cell nuclei, calf thymus DNP or DNA, or human sperm. Although the injections were given over a period of several months, none of the animals was positive in the LE cell test. A similar experiment was conducted by Miescher et al. (1961) in which rabbits were given 6 weekly injections of purified nuclear constituents or intact cell nuclei. One-half of the rabbits produced antibodies that reacted with the injected material in precipitin tests. Cell nuclei were suggested as the best antigen for inducing anti-nuclear antibody formation. All antisera were found to be cross-reactive with heterologous preparations of DNP or DNA in precipitin tests. Treatment of the antigen with DNase inhibited precipitin formation. None of the antisera would produce passive cutaneous anaphylaxis with DNA, and only 1 of 29 immunized rabbits had a positive LE cell test. This was, however, the first report of induced LE cell activity in experimental animals. It was also found that human SLE serum would inhibit the reaction of positive rabbit immune sera in immunofluorescent spot tests with DNP. More recent experiments by Heide et al. (1963) have confirmed that rabbits can be induced to produce the LE cell factor. In the latter studies animals were injected repeatedly until they had received a total of 28 mg of either calf thymus DNP, heat denatured DNA (commercial), or

cell nuclei. The antisera reacted with nuclei of various mammals, including rabbits, and LE cells were produced in whole blood. Purified DNA containing less than 0.01% protein has also been used as an antigen in attempts to induce rabbits to produce anti-DNA antibodies (Coulter and Ellem, 1961). The DNA preparations were extracts of either mouse liver or Ehrlich ascites tumor cells. All injections were given intravenously. Although the DNA preparations from both sources induced antibody activity which had the same specificity in complement fixation tests, only the tumor DNA antisera would inhibit tumor growth. This finding suggested that the antisera had differences in specificity. Studies have also been made of the antigenicity of bacterial DNP and DNA, viral DNA, and killed bacteria. Lachman et al. (1941) found that pneumococcus antisera would precipitate with calf thymus nucleic acids. The reaction was inhibited by purine nucleotides, nucleosides, and purine bases but not by pentoses. It has also been reported that sera from rabbits immunized with a total of 1.8 mg of Salmonella typhimurium DNA (0.07% protein and 10% ribonucleic acid) or heat killed cells contained antibodies reactive with DNA (Timakov et al., 1963). The rabbits injected with heat killed bacteria had higher titers of complement fixing antibody than the animals injected with purified DNA. Treatment of the antigens with DNase caused a significant decrease in the titer. Treatment with RNase, trypsin, or chymotrypsin was ineffective. It was concluded that whole cells are more effective in inducing anti-DNA antibody production because the nuclear antigens are not susceptible to serum nucleases. Christain et al. (1964) induced rabbits to produce anti-DNA antibodies during hyperimmunization with a saline suspension

of formalin killed Escherichia coli or S. typhimurium. Immunization with Bacillus subtilis was ineffective. Four of 28 rabbits produced antibodies that would cross-react with various DNAs in complement fixation tests. These antisera also reacted with rabbit DNA, but did not combine with the nuclei of calf thymus or rabbit testis. Absorption of the antisera with the bacterial DNA used for injection did not remove all of the activity for host DNA. Absorption with adenylic acid, however, removed all antibody activity for host and bacterial DNA. Another autoantibody was also produced by the rabbits that developed anti-DNA antibodies, as evidenced by the fact that all of these animals became sensitized to their own gamma-globulin. Levine and associates (1960) and Levine (1963) presented conclusive evidence for the antigenicity of bacteriophage T4 DNA in rabbits. The antisera did not cross react with DNA from E. coli or calf thymus. The authors concluded that the antibodies were specific for T4 DNA and were directed, in part, to the glucosyl moieties unique to this molecule. Recent investigations have also indicated that purine or pyrimidine conjugates with bovine serum albumin (BSA) or other proteins induce the production of anti-DNA antibodies when injected into rabbits. The antisera reacted with both native and heat denatured DNA preparations in complement fixation tests and passive cutaneous anaphylaxis (Butler et al., 1962; Tanenbaum and Beiser, 1963; Plescia et al., 1964; Plescia et al., 1965). The immunogenicity of these preparations appears to be greater than that of native DNA since all sera collected after 3 injections contained antibodies reactive with DNA from several bacterial sources. The antisera also inhibited the transformation capacities of DNA from Diplococcus pneumoniae.

Inhibition tests with various known nucleotides indicated that the hapten was a tetra- to a hexanucleotide. Although most of the evidence in these studies indicates that DNP and DNA are immunogenic in some rabbits, it will be noted that it was necessary, in most experiments, to give numerous immunizations before the animals developed antibody to the injected material.

Spontaneous Production of Anti-nuclear Antibodies by Experimental Animals

In the systematic study to determine the etiology of the hemolytic anemia that occurs in NZB/BL mice, it was observed that some of the animals had pathologic renal lesions similar to those found in SLE patients (Bielschowsky et al., 1959). LE cell tests were performed and 4% of the mice were positive. The author considered that these findings were suggestive of an autoimmune disorder analogous to human SLE. Following this preliminary report, numerous investigations of NZB/BL and NZB/BL hybrids have been made to determine the similarities in serologic and pathologic findings in these strains of mice and in humans with SLE. Norrins and Holmes (1964) reported that 45% of 8-week-old NZB/BL mice have anti-nuclear antibodies reactive with human leukocyte nuclei. The antibody activity could be absorbed with calf thymus DNP. Ultracentrifugation and mercaptoethanol treatment of positive sera indicated that the anti-nuclear antibody activity was in the 7 S class of immunoglobulins. Although there was no apparent difference in the incidence of anti-nuclear antibody activity according to sex, it was found that those mice dying of renal disease had twice the incidence of anti-nuclear antibodies as did those dying of other causes. A somewhat

lower incidence of antibody activity in NZB/BL mice has been reported by Mellors (1965). He found that 8 of 10 mice examined had glomerulonephritis, but only 1 of 15 mice that were 4 to 12 months old had anti-nuclear antibodies reactive with cell nuclei. None of these sera agglutinated DNP coated latex particles. The only apparent difference in these 2 experiments was in the immunofluorescent techniques used. Mellors (1965) prepared his own fluorescent conjugate, whereas Norrins and Holmes (1964) used a commercially prepared fluorescent antibody. Both groups of workers stated that their antisera reacted only with mouse gamma-globulin. Mellors (1965) also reported that: (1) 9 of 12 6-month-old mice had elevated serum 7 S gamma-globulin, (2) 2 of 28 mice had a rheumatoid-like factor in their serum that was sensitive to mercaptoethanol, and (3) gamma-globulin extracted from kidneys showing glomerulonephritis did not combine with mouse erythrocytes, autologous or isologous cell nuclei, or normal, young, isologous mouse kidney.

A higher incidence of anti-nuclear antibody activity has been observed in most F1 hybrids in which one of the parents was an NZB/BL mouse. Helyer and Howie (1961, 1963a) conducted studies of a (NZB/BL x NZY/BL)F1 hybrid strain. They found that 17 of 48 hybrids were positive in LE cell tests. None of the NZY/BL mice was positive. The incidence of positive tests was found to be greater in females, and many of the mice died prematurely from renal failure. Another hybrid, (NZB/BL x NZW/BL)F1 has also been studied extensively. These mice begin to die of renal failure at 2 to 10 months of age (Helyer and Howie, 1963c; Dubois et al., 1965). The incidence of positive LE cell tests

in 6- to 10-month-old mice has been reported to range from 70% to 100% (Helyer and Howie, 1963b, 1963c; Dubois et al., 1965; Channing et al., 1965). The incidence of antibodies reactive with cell nuclei in immunofluorescent techniques is much higher in these hybrids than in NZB/BL mice. Burnet and Holmes (1965) found the incidence to be 81% in sick males and females. The incidence was slightly lower in healthy males and females of the same age as the sick animals. Not all dead mice had anti-nuclear antibody activity in their sera and it was suggested that this finding lent support to the view that anti-nuclear antibodies were probably not responsible for the kidney lesions (Burnet and Holmes, 1965). However, since these experiments did not include serologic observations of the mice when they were younger, it appears that the conclusion is not completely valid.

Naturally occurring anti-nuclear antibody activity has also been found in another F1 hybrid strain of mice which had an NZB/BL mouse as one of the parents. Norrins and Holmes (1964) found that (NZB/BL x C3H)F1 mice spontaneously develop antibodies reactive with the cell nuclei of various mammals.

Anti-nuclear antibodies have also been found to appear spontaneously in some members of other strains of untreated mice. Inbred strains in which this activity has been described include A/HeJ and C57Bl/6J (Shulman et al., 1964), C57 and C3H (Norrins and Holmes, 1964), and C57BR (Holborow et al., 1965). Anti-nuclear antibody activity has also been found in a large percentage of random bred mice, strain HI (Norrins and Holmes, 1964).

The LE cell factor has also been found in other animal species. Lewis et al. (1965) described a systemic disease in 7 dogs in which all animals had kidney lesions similar to those seen in human SLE. All of the animals had the LE cell factor, rheumatoid factor, anti-thyroid autoantibodies, and anti-erythrocyte autoantibodies with hemolytic anemia. Five of the dogs died in renal failure.

Theories of the Etiology of Anti-nuclear Antibody Production

Although numerous theories have been proposed to account for the spontaneous and persistent production of antibodies reactive with nuclear antigens in man and experimental animals, none of them is totally accepted. This could be due to the fact that the inducing agent(s) is(are) unknown, physiologic and genetic variables are not completely understood, and complete knowledge of the mechanisms controlling and inhibiting antigen catabolism and induction of antibody synthesis is lacking.

Some authors consider that microorganisms might be responsible for inducing the production of anti-nuclear antibodies. Bardawil et al. (1958) and Seligman (1958) suggested that heterologous antigenic stimulation from microbial nuclear components may induce anti-nuclear antibody formation. Sbarra et al. (1963) proposed that, following phagocytosis of microorganisms and lysosomal rupture, DNases attack nuclear DNA of the phagocyte leading to the formation of a "disturbed nucleus". The partially degraded nucleus was suggested as being the inducing agent. The authors speculated that if the antibodies could participate in tissue destruction, additional altered antigen would be released and

thus it would not be necessary to assume a need for the continued presence of the microorganism.

Another theory of anti-nuclear antibody formation suggests that it is a normal physiologic process mediated by antibodies which are carriers of normal catabolic components of the host (Boyden, 1964). It was assumed by this investigator that antibodies reactive with autoantigens are normally present in all animals but in undetectable concentrations in most animals. He suggested that the presence of autoantibodies, indicated by positive serologic tests, is the result of an exaggeration of a normal immunologic process.

A possible explanation for the spontaneous production of antibodies that react with nuclear antigens and other body constituents normally accessible to the lymphatic tissues has been developed by F. M. Burnet and others. They suggested that the normal animal does not initiate an immunological response to autologous (self) tissue components in a recognizable form because of the existence of certain normal homeostatic controls. Autoimmunity is explained as being the result of somatic mutation in immunopoietic stem cells which allows subsequent antibody producing cells ("forbidden" clones) to proliferate and express their autoimmune activity (Burnet, 1958). In establishing a working hypothesis, the author assumed that the essential genetic lesion is an increased lability of immunologically competent stem cells to undergo somatic mutation toward resistance to immunologic homeostasis (Holmes et al., 1961). Another possibility might involve a generalized weakness of the homeostatic mechanism in certain animals which could allow "forbidden" clones of antibody forming cells

to proliferate. These clones of cells would be eliminated in the normal animal. In a recent modification, Burnet and Holmes (1964) suggested that the thymus is the site of the primary homeostatic process by which any cells which develop the capacity to react with antigenic determinants present in accessible regions of the body are destroyed or inhibited. If cells are capable of resisting the intrathymic controls and react with accessible antigens in the thymus, it was speculated that germinal centers and plasma cells found in the thymus of NZB/BL mice and some patients with autoimmune diseases would indicate an abnormal condition. The significance of germinal centers in the thymus becomes difficult to interpret in view of the report by Steiger (1965) showing that germinal centers can also be found in the perivascular connective tissues of numerous organs of NZB/BL mice and in the thymus of aged Swiss mice. The Swiss mice were devoid of detectable autoimmunity. Germinal centers are not found in the thymus of A/J mice which have developed anti-DNP antibodies spontaneously (Myers and Friou, 1965). Although the role of the thymus in autoimmunity is thus uncertain, it does appear to be involved in the spontaneous appearance of autoimmune hemolytic anemia in certain mice. Helyer and Howie (1963b) and East and Parrott (1965) reported that neonatal thymectomy of NZB/BL mice causes an earlier appearance of the anemia. Once the autoimmune condition developed, it could not be reversed by thymic grafts from normal CBA/T6 mice. These findings were interpreted as evidence that abnormal clones of immunopoietic cells are not dependent upon the presence of a thymus for their immune functions. If the thymus were the source of the "forbidden" clones, the cells had already left it before

birth. The latter concept has also been supported by Holmes and Burnet (1964).

Other investigators have published data which indicate that the thymus and possibly other lymphoid organs might be involved in both normal and autoimmune processes. Many of the thymic-associated immune functions have been reviewed by Good and associates (1965). Neonatally thymectomized mice are unable to reject homografts and are defective in the capacity to make circulating antibodies following specific antigenic stimulation. Spleen cells derived from thymectomized mice are immunologically inadequate since they fail to induce a graft versus host reaction in normally susceptible hosts. Such cells are also defective in protecting syngenic recipients from radiation death. Neonatally thymectimized mice that have been transplanted with syngenic thymus tissue in the neonatal period develop normally with respect to their growth, development, immunologic capacity, and longevity. Mice not receiving the thymus graft develop a wasting disease and die. Syngenic thymus transplants performed on neonatally thymectomized mice more than 4 to 6 weeks of age fail to restore normal immunologic capacities and the mice die with symptoms that are similar to those seen in untreated thymectomized mice. Although some cells of the thymic graft gain entrance into the host's lymphoid tissues, the primary function of the graft is apparently that of permitting the normal development of the host's own lymphoid cells. By using thymus grafts contained in Millipore chambers, it has been concluded that the thymus elaborates a humoral factor which is necessary for the continued normal function of the immunopoietic tissues of the thymectomized host. In contrast to the failures seen with

thymus grafts, the injection of dispersed cells into thymectomized mice will completely repopulate the host and nearly always reverse the early symptoms of the wasting disease. Studies with thymectomized adult animals also indicate that the thymus participates in the development of immunologic potential far beyond the neonatal period.

Various theories have been advanced concerning the etiology of the post-thymectomy syndrome that develops in young experimental animals. McIntire et al. (1964) found that germ-free mice do not develop the wasting disease after thymectomy and proposed that it must be caused by microorganisms. Other workers have postulated that the syndrome is caused primarily by a lack of self recognition by the immunologically competent cells of the host, and that the disease may represent an experimentally induced autoimmune state. DeVries and associates (1964) studied neonatally thymectomized CBA/Rij and C57BL/Rij mice. The tissue reactions they observed were similar to those found in graft versus host reactions and in SLE patients. Four of 102 mice had renal pathology identical with that seen in SLE patients. A small number of the mice were examined for the production of LE cells. Although typical LE cells were not found, erythrophagocytosis and other phenomena seen in LE cell preparations of SLE patients were observed. Many of the mice had increased numbers of plasma cells and macrophages and this was interpreted to indicate intense immunologic reactivity. It was concluded that infectious disease alone could not explain all of the pathologic findings. Results of the work of Kellum and associates (1965) also support the theory that the post-thymectomy syndrome is partially due to autoimmunity. Groups of adult rabbits were surgically treated

to remove either the thymus, thymus and spleen, thymus and appendix, spleen, or appendix. After 14 to 17 days all of the rabbits, plus unoperated controls, were given 500 r total body irradiation. Some members of all groups became positive in the direct Coomb's test. The members of the thymectomized-appendectomized group showed the highest incidence. One of 10 irradiated controls, but none of the unirradiated controls was found to be positive. The lymph nodes and spleen of the thymectomized-appendectomized group were almost devoid of lymphatic follicles and were predominantly deficient in small lymphocytes. Amyloidosis was also found in most of these animals. It was proposed that the basic defect which leads to runting, wasting, autoimmune processes, and amyloidosis is a deficiency in the amount and organization of the lymphoid tissues, primarily the spleen and nodes. The thymus and appendix in the rabbit appear to be directly involved in the maintenance of immunological homeostasis which prevents the emergence of self-reactive "forbidden" clones of immunopoietic cells. In contrast to these results, studies reported by East and Parrott (1965) involving 63 neonatally thymectomized mice of 3 strains failed to reveal evidence of autoimmunity expressed by the production of anti-nuclear or anti-erythrocyte antibodies.

The production of anti-nuclear antibodies by certain humans has been ascribed to the consequence of genetically transmitted mechanisms which either normally inhibit the proliferation of "forbidden" clones or predispose these individuals to a higher rate of somatic mutations in immunologic stem cells. Holman (1963) compared the family of an SLE patient with a group of control individuals. A high incidence

of clinical connective tissue diseases or serological abnormalities was found in the family of the SLE patient but not in the controls. SLE was not found to be the predominating disease, and 60% of the family were free of clinical disease. Another family study of SLE patients has been reported by Pollak (1964). Anti-nuclear antibodies were found among the relatives in 25 of 43 families. Negative results were obtained with 24 relatives of matched healthy subjects. Burch and Rowell (1963) have suggested that autoimmunity in SLE patients is the result of somatic mutation in stem cells which leads to the development of "forbidden" clones of lymphocytes and plasma cells. Further development of this concept led to the proposal that anti-nuclear antibody production and SLE are confined to individuals with a specific genetic disposition (Burch and Rowell, 1965). It was suggested that the phenotypic expression of SLE requires the occurrence of 3 specific random mutations. Their average rate was proposed to be constant throughout post-natal life and independent of ordinary environmental factors. The mutations were proposed to occur at 3 predisposing X-linked loci in certain stem cells of the lymphoid series. The authors proposed that an interval or latent period between the last somatic mutation and the clinical onset of the disease is usually about 5 years in females and $2\frac{1}{2}$ years in males. They added that the latent period is probably affected by environmental factors such as drugs, infections, or mental stress. All of the factors are suspected of precipitating SLE in some individuals. It was suggested further that during the latent period there is a proliferation of the "forbidden" clones, with concomitant antagonism being mediated through the formation of humoral

antibodies against autoantigens in the "forbidden" clones. The efficiency of the elimination mechanism was proposed to be about twice as high in males as in females.

Genetic factors have also been suggested as being involved in the ability of experimental animals to respond to immunization with certain antigens. In studying induced autoimmune thyroiditis in experimental animals, McMaster et al. (1965) concluded that the incidence of disease is greater in Hartley guinea pigs and C57Bl mice than in strain 13 guinea pigs and Swiss mice. Levine and associates (1963) found that the ability of certain guinea pigs to respond to simple chemical haptens is transmitted as a unigenic Mendelian dominant character. Genetic factors also appear to be involved in the ability of certain strains of mice to respond to synthetic polypeptides (McDevit and Sela, 1965), tetanus toxoid (Ipsen, 1959), and bovine serum albumin (Farr et al., 1963).

The purposes and objectives of the present research were to (1) find a strain of mice in which anti-nuclear antibodies are produced spontaneously, (2) determine the incidence and types of anti-nuclear antibodies produced by these mice, (3) determine if the incidence of anti-nuclear antibody activity is similar or different in males and females, (4) determine if specific immunization of seronegative mice would induce the production of anti-nuclear antibodies, and (5) determine if passive transfer of anti-nuclear antibody production could be accomplished with spleen cells obtained from seropositive donors.

CHAPTER II

MATERIALS AND METHODS

Mice

Strains and Sources

A/J, C57BL/6J, DBA/1J, and AJDF1 (F1 hybrid of female A/J and male DBA/1J) mice were purchased commercially[1]. Young A/J and DBA/1J mice (4 to 6 weeks old) were from production stocks, while 8-month-old A/J, C57BL/6J, and DBA/1J mice had been retired from breeding colonies. AJDF1 mice were obtained when they were 6 months old. Two pair of NZB/B1 mice (brother-sister pairs) were kindly provided by Dr. J. B. Howie of the University of Otago Medical School, North Taieri, New Zealand. Each pair produced a litter. The progeny were weaned at 4 weeks of age and separated according to sex. At 6 weeks of age, they were brother-sister mated. By continuing this process approximately 100 NZB/B1 mice were obtained.

ANZBF1 mice were obtained by mating A/J females with NZB/B1 males. The parental animals were 2 months old. Five males and 20 females were used. One male was placed in a cage with 4 females for 17 days. Each female was then isolated. The progeny, which were all black, were weaned after 4 weeks and separated according to sex.

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[1] R. B. Jackson Memorial Laboratory, Bar Harbor, Maine.

Care and Handling

All of the mice were housed in the same isolated air-conditioned mouse room. They were handled whenever possible with tongs dipped in Amphyl. The diet consisted of Rockland Mouse-Rat diet[2] and autoclaved tap water. No more than 5 animals of the same sex were housed in the same plastic disposable cage[3]. Every 7 days the mice were placed in new cages with fresh sawdust. Animals in each cage were grouped according to strain, date of purchase, age, sex, and an ear punch code number.

Mouse Serum Pools

Five pools of serum from each of 17 inbred strains of mice were obtained commercially[1]. All serum donors were from breeding stocks and were 8 months old. Each pool contained 1 ml serum which was composed of 0.1 ml aliquots from 10 donor mice. Undiluted samples of each serum pool were tested for antibodies which would react with calf thymus DNP spots and peripheral blood leukocyte nuclei.

Serum Samples from Individual Mice

Prior to bleeding, the mice were placed in a 37° C incubator for 10 minutes. The mouse to be bled was then placed inside a 50 cc syringe which was held stationary at approximately a 45° angle from horizontal, with the head of the mouse elevated. Tape was placed over the large open end of the syringe and the mouse's tail was withdrawn. A vein in the anterior ventral surface of the tail was cut with a

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[2]Teklad Incorporated, Monmouth, Illinois.

[3]Aloe Scientific, St. Louis, Missouri.

sterile surgical blade and the blood was collected in capillary tubes[4]. Centrifugation was carried out for 5 minutes. The tubes were then broken above the clot and the portion of the tube containing the clot was discarded. The serum was either used immediately in serologic tests or was stored at -20° C.

Serologic Tests for Anti-nuclear Antibodies

The indirect fluorescent-antibody technique was used for the detection in mouse serum of antibodies reactive with calf and rabbit thymus DNP, purified calf thymus DNA, or leukocyte nuclei.

Calf and Rabbit Thymus DNP

DNP extraction. DNP was extracted from the thymus of a calf and from thymus tissue collected from 10 young adult albino rabbits. The extraction procedure was started within a few hours after the tissue was obtained (Friou, 1958; Friou, 1962). All reagents were kept at 4 to 6° C. Centrifugation was also carried out at this temperature. A 50 gm portion of thymus was cut into small pieces and then added to 100 ml of a solution containing 0.05 M sodium chloride and 0.025 M sodium citrate, pH 7.0. The mixture was triturated in a high-speed blender and then centrifuged at 1,000 g for 30 minutes. The supernatant was discarded. The sediment was suspended in 100 ml of the same solution, and centrifuged as above. The supernatant was discarded and the sediment was washed 3 times with 50 ml of distilled-deionized (D-D) water, pH 7.0. The sediment was then blended for 15 seconds with 240 ml of pH 7.0 D-D water and shaken overnight on a rotary-horizontal

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[4] Clay-Adams, Inc., New York, New York.

shaker at 4 to 6° C. The viscous extract was blended briefly and centrifuged for 30 minutes at 2,000 g. The sediment was discarded. The supernatant was mixed with 5.66 volumes of 0.177 M sodium chloride and incubated at 4 to 6° C for 30 minutes. The precipitate was collected by centrifugation at 2,000 g for 30 minutes and resuspended in 0.15 M sodium chloride. The sediment, collected by centrifugation, was washed with a small volume of pH 7.0 D-D water and recentrifuged. Three-hundred ml of pH 7.0 D-D water was added to the sediment. The mixture was then shaken overnight as before. The white, viscous, opalescent solution was lyophilized in 30 ml volumes and stored at room temperature.

DNP spot test standardization. To determine the optimal amount of DNP to be used in preparing DNP spots, aqueous solutions of both calf and rabbit thymus DNP were prepared containing 5.0, 2.5, 1.25, and 0.675 mg/ml. DNP spots were made by placing 1 drop of each of these solutions on clean, 3 x 1 inch microscope slides. After drying overnight at room temperature, the spots were tested with undiluted mouse and human sera containing anti-DNP antibodies, and with normal sera, using the standard spot test procedure. Five-hundred to 1,000 DNP spot slides were then prepared as needed and stored in microscope slide boxes at -10 to -20° C.

Calf Thymus DNA

Purified calf thymus DNA (0.73% protein)[5] was used to prepare DNA spot slides. Spots were made by applying to formalin-fixed,

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[5]Worthington Biochemical Corp., Freehold, New Jersey.

gelatin coated microscope slides (1 x 3 inch) 2 drops of a solution containing 90 mcg/ml DNA and 10 mcg/ml neutral detergent (Naccanol DB)[6] in pH 7.0 D-D water (Casals et al., 1964). The DNA was stirred overnight at 4 to 6° C, and the detergent solution was then added to provide the correct concentration of both components. The slides were dried at room temperature, fixed in 95% ethanol for 30 minutes, and stored at -20° C in microscope slide boxes.

DNP and DNA Spot Tests

The procedure used in testing mouse sera for the presence of antibodies reactive with DNP or DNA was similar to that described previously (Friou, 1962; Casals et al., 1964). One to 4 known positive and known negative mouse sera were used as controls. Spot test slides were removed from the freezer and allowed to dry thoroughly at room temperature. A circle (inside diameter, 5 to 8 mm) was made on each spot with a black-ink Mark-X-Tech pen[7]. After drying, the ink ring served as a reservoir for serum and conjugate, made the spot easy to identify, and provided contrast in determining the intensity of fluorescence in ultra-violet light. Each slide was numbered for identification with the Mark-X-Tech pen. The area of the spot inside the black ring was completely covered with serum (approximately 0.025 ml). Each slide was placed over moist paper towels in an enclosed stainless-steel container and incubated at room temperature for 30 minutes. The slides were then removed from the container and washed gently with phosphate

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[6] Microbiological Associates, Bethesda, Maryland.

[7] Mark-Tex Corp., New York, New York.

buffered saline (PBS) containing 0.15 M sodium chloride and 0.01 M Na_2HPO_4, pH 7.0. Additional washing was accomplished by placing the slides in a glass tray which was then submerged in 1,000 ml of PBS for 30 minutes at room temperature while the solution was under constant agitation by a magnetic mixer. The slides were then removed and gauze was used to remove excess fluid from the slide outside the ring enclosing each spot. The entire area of each spot inside the black ring was covered with fluorescein-labeled rabbit anti-mouse gamma-globulin conjugate containing 1% BSA (fraction V)[8] and incubated for 30 minutes at room temperature as described above. After the slides were washed in PBS as described previously, excess fluid was removed with gauze, and the DNP spots were blotted gently with filter paper or washed by pouring D-D water over them. DNA spots were mounted with phosphate buffered glycerol, pH 7.0, and covered with a coverslip (1 x 1 inch). Both DNP and DNA spot slides were examined microscopically with an ultra-violet light (Blak-Ray, B-100, 100 watts)[9] in a dark room. The intensity of fluorescence of each spot was graded 3 plus (+++) to negative (-) by comparison with positive and negative controls. An example of the fluorescence obtained with a positive (+++) and a negative (-) serum is shown in Figure 1.

Peripheral Blood Leukocytes

Two ml of mouse (A/J), rabbit, or human blood was drawn directly into a glass syringe containing heparin and 1 ml of 4%

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[8]Nutritional Biochemicals Corp., Cleveland, Ohio.

[9]Ultra-Violet Products, Inc., San Gabriel, California.

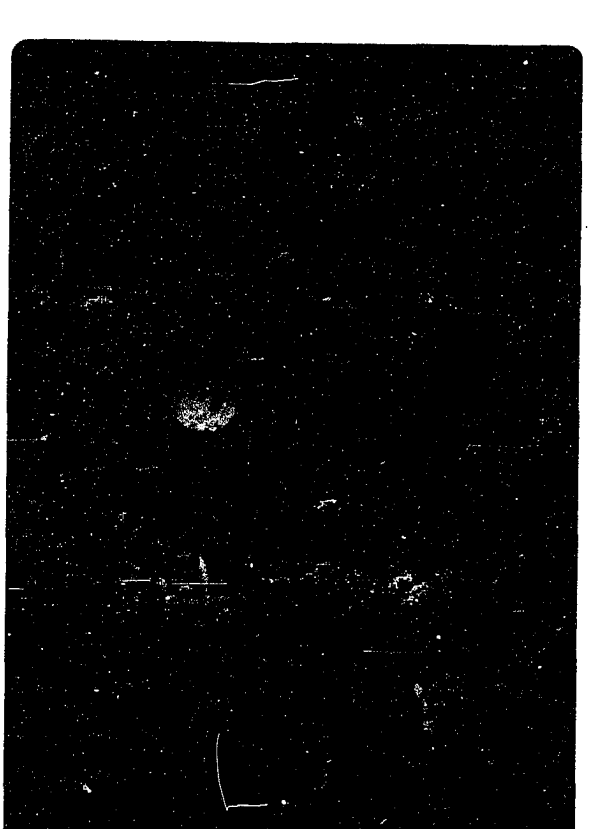

Figure 1. Fluorescence of calf thymus DNP spots after treatment with either serum containing anti-DNP antibodies (+++) or normal serum (-), and fluorescein isothiocyanate labeled rabbit and anti-mouse gamma-globulin conjugate (Lot 1).

polyvinylpyrrolidone (molecular weight, 40,000)[10] (Casals et al., 1963). A blunt needle was attached to the syringe and a tight fitting piece of polyethylene tubing was placed over the needle. The tubing was then closed with a clamp. The syringe was placed in a vertical position at 4 to 6° C with the needle upright. After the erythrocytes had completely sedimented, the syringe was removed from the refrigerator in the upright position, the clamp was removed, and gentle pressure was applied to the plunger. Drops of the leukocyte suspension were placed on gelatinized slides in a moist chamber for 30 minutes at room temperature and then washed in PBS for 30 minutes. The slides were fixed in 95% ethanol for 30 minutes and allowed to air dry. Leukocyte preparations which were not used immediately were discarded. The preparations were covered with undiluted mouse serum (approximately 0.025 ml) and the spot test procedure was followed. After the final washing, the slides were mounted with phosphate buffered glycerol, pH 7.0, and covered with a 1 x 1 inch coverslip. Each slide was examined microscopically within 18 hours for fluorescence of the leukocyte nuclei. The fluorescence was rated +++ to negative (-) by comparison with positive and negative controls. If the slides could not be examined immediately, they were stored at 4 to 6° C. Microscopic observations were made with a Leitz Ortholux microscope equipped with an ultra-violet light source (Osram HBO-200 mercury vapor bulb), a 4 mm UG1 filter, and a Wratten 2A ocular filter.

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[10]Antara Chemicals, New York, New York

Rabbit Anti-mouse Gamma-Globulin

The two antisera used in these experiments were purchased commercially[11]. One preparation (Lot 1) was obtained as a fluorescein isothiocyanate-labeled gamma-globulin fraction of serum from rabbits immunized with purified mouse gamma-globulin. The other preparation (Lot 2) was obtained as whole serum from rabbits that had been immunized with purified mouse gamma-globulin.

Ammonium sulfate fractionation. A crude globulin fraction of the serum was prepared by precipitation with $(NH_4)_2SO_4$ and was labeled with fluorescein isothiocyanate[8] by the method of Coons and Kaplan (1950), as modified by Riggs et al. (1958) and Marshall et al. (1958). The $(NH_4)_2SO_4$ fractionation was carried out at 4 to 6° C. The serum was first diluted with an equal volume of PBS. Saturated $(NH_4)_2SO_4$ was then added dropwise while the mixture was being stirred gently until twice the original volume of serum was added. The mixture was stirred for an additional 30 minutes after the last drop of $(NH_4)_2SO_4$ had been added. The precipitate was collected by centrifugation at 1,000 g for 15 minutes, washed with one-half saturated $(NH_4)_2SO_4$, and again collected by centrifugation. The crude globulins in the precipitate were dissolved in 0.01 M phosphate buffer, pH 7.0, in a volume equal to one-half that of the original serum, and dialyzed against 1,000 ml of volumes of the same buffer for 18 hours, using 3 changes of buffer. The globulin preparation was stored at -20° C.

Protein assay. The protein content of the globulin preparation was determined with a modification of the method of Lowry et al.

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[11] Antibodies Incorporated, Davis, California

(1951). Stock solutions of a rabbit gamma-globulin[8] standard were prepared in PBS to contain 25, 50, 100, and 200 mcg/0.2 ml. These standards were stored at -20° C and were used in each protein determination. A portion of the unknown immune globulin solution was diluted with PBS to contain 25 to 200 mcg of protein per 0.2 ml. A series of tubes was then set up containing 0.72 ml of 1 N NaOH, 0.2 ml of the unknown globulin or standard solution, and 0.28 ml of D-D water. Fifty ml of reagent A (2% Na_2CO_3) were mixed with 1 ml of reagent B (1 gm $NaKC_4H_4O_6 \cdot 4H_2O$, 0.5 gm $CuSO_4.5H_2O$ per 100 ml D-D water) to form reagent C. Reagent C was prepared fresh for each protein determination. Six ml of reagent C was added to each tube. The contents of the tubes were mixed thoroughly and allowed to stand at room temperature for 10 minutes. Six-tenths ml of Folin-Ciocalteau reagent[12] was then added to the contents of each tube. The tubes were incubated for 30 minutes at room temperature for color development. Optical density was read at 500 mμ in a Coleman Universal Model 14 spectrophotometer using a reagent-PBS blank as the zero absorbancy. The standards were plotted on semilog graph paper and the concentration of protein in the unknown globulin solution was determined by reference to the standard curve.

Fluorescent labeling. For fluorescent labeling, a portion of the globulin was diluted with 0.15 M saline and 0.5 M carbonate-bicarbonate buffer, pH 9.0, until the final concentration in the mixture was 10 mg protein/ml and 10% (by volume) of the pH 9.0 buffer. The beaker containing the globulin was placed in an ice bath and its contents were

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[12]Fisher Scientific Co., Fair Lawn, New Jersey.

mixed gently. When the temperature of the globulin solution was 1 to 2° C, fluorescein isothiocyanate[8] (0.05 mg/mg of protein) was added. The beaker containing the gluorescein-globulin mixture was transferred to a refrigerator (4 to 6° C) where mixing was continued for 18 hours. The fluorescent conjugate was then dialyzed against 0.01 M phosphate buffer, pH 7.0, for 18 hours and passed through a column of Amberlite CG 400[12] (Friou, 1962). To prepare the Amberlite column, 4 gm of the resin were washed in 300 ml of 3 N HCl and then in 0.01 M phosphate buffer, pH 7.5, until the pH became 7.5. The resin was then poured into a 10 ml serological pipette equipped with a glass wool plug and allowed to settle by gravity flow. After passage through the column, the conjugate was centrifuged at 10,000 g for 20 minutes and the precipitate was discarded. The conjugate was stored at -20° C in 3 ml volumes.

Standardization of conjugates. Both Lot 1 and Lot 2 conjugates were standardized by testing serial two-fold dilutions against DNP and DNA spots and human leukocytes which had been exposed to undiluted mouse serum under the test conditions described above. Twice the concentration of the highest dilution of conjugate which produced maximum fluorescence with positive sera and no fluorescence with negative sera was used in each test.

Absorption of conjugates. Both conjugates were tested for non-specific staining after absorbing them with an equal volume of A/J mouse serum. For this purpose, a mixture consisting of equal volumes of conjugate and serum was incubated for 30 minutes at room temperature and then centrifuged for 30 minutes at 10,000 g. The

absorbed conjugate was used, along with an unabsorbed control conjugate (diluted to the same final volume), in serologic tests for anti-DNP antibodies.

Specificity of conjugates. Immunoelectrophoresis (Scheidegger, 1955) was used to determine the specificity of Lot 1 and Lot 2 conjugates. Clean glass microscope slides were layered with 2 ml of melted 0.75% Ionagar No. 2[13] in barbital-acetate buffer (pH 8.6; ionic strength 0.05) and cooled in a moist chamber. A slide cutter and plastic rack were used to cut 2 wells and 1 trench in each slide. The wells were filled with A/J or DBA/1J mouse serum. After 10 minutes absorption, the wells were sealed with a small amount of the warm agar-buffer mixture. Electrophoresis was performed for 45 minutes at 4 to 6° C in a Buchler microimmunoelectrophoresis chamber using barbital-acetate buffer (pH 8.6, ionic strength 0.05). The potential was 40 volts (approximately 6 volts/cm). The slides were then removed, the Lot 1 or Lot 2 conjugates were added to the trenches, and the slides were incubated for 48 hours in a moist chamber. They were then examined for specific precipitation patterns.

LE Cell Tests

Blood samples of A/J and ANZBF1 mice were tested in vitro for the formation of LE cells. The technique used was a modification of the clot method (Rosenfeld et al., 1954). Blood was drawn into a capillary tube (micro-hematocrit)[14] from a cut made in a tail vein. The clot was fragmented with a 27-gauge stainless-steel wire and

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[13]Consolidated Laboratories, Inc., Chicago Heights, Illinois.

incubated for 2 hours at 39° C. The tube was then centrifuged for 4 minutes in an International Micro-Capillary centrifuge, Model MB. A smear was made of the leukocyte layer and the cells were stained with Wright's stain. The slides were mounted with Permount[12] and covered with a coverslip (1 x 1 inch). Two to 6 smears were obtained from each blood sample. The entire area of cells on each slide was examined microscopically and a given blood sample was considered negative only when LE cells were not found on any of the slides prepared from a single animal. The blood of A/J mice whose sera did not contain anti-DNP antibodies was used as a control. The slides were coded by a number and the microscopic examinations was made without knowledge of the source of the slide.

Starch Block Electrophoresis

Three serum pools from the A/J strain mice were subjected to electrophoresis in starch blocks (Paigen, 1956). Starch blocks were prepared in plastic trays by mixing 42 gm of purified potato starch powder[12] with 42 ml of barbital buffer (pH 8.6, ionic strength 0.05). A 1/4 inch wide well was prepared and into it was placed a mixture of 0.5 ml serum and 0.5 gm starch. Electrophoresis was carried out for 18 hours at 4 to 6° C using a barbital buffer (pH 8.6, ionic strength 0.05) and a potential of 60 volts per starch block. The serum protein electrophoretic fractions were then located by touching each block with dry Whatman chromatographic filter paper[14] and staining the proteins absorbed into the paper with bromphenol blue. The major serum

[14] W. H. Curtin and Co., Dallas, Texas

protein components in the starch blocks were identified by comparison with the stained preparation. Each portion of the block containing a major serum protein component was removed in 1 cm sections, washed with 2 ml of 0.15 M PBS, and eluted by vacuum filtration. Each sample was then dialyzed against 20% polyvinylpyrrolidone[10] until the volume was reduced to 0.5 ml. Each of the fractions was then tested for its gamma-globulin content with ring tests, using specific rabbit anti-mouse gamma-globulin. The ring tests were done in small bore (4 mm) tubes. Samples of each fraction were layered over undiluted antiserum with a capillary pipette. The tubes were then incubated at room temperature and observed after 30 minutes. Each fraction was also tested for its reactivity with calf thymus DNP and human leukocyte nuclei. Tests were performed with a sample of each fraction, treating each as if it were undiluted serum.

Antigens and Immunizations

A/J, DBA/1J, and AJDF1 mice were used to study the effect of immunization on anti-nuclear antibody production. Injections were made in 0.1 ml volumes using a disposable plastic 1 cc tuberculin syringe and a 1/2 inch, 24 gauge needle[15]. One to 3 injections were given; multiple injections were administered at weekly intervals. Fresh antigen preparations were prepared for each injection series.

Calf Thymus DNP

An aqueous solution of calf thymus DNP was prepared by mixing

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[15]Becton, Dickinson and Company, Rutherford, N. J.

20 mg of lyophilized DNP in 1 ml of D-D water overnight at room temperature.

Calf Thymus DNP in Complete Freund's Adjuvant

An aqueous solution (4 mg/0.1 ml) of calf thymus DNP was added to an equal volume of complete Freund's adjuvant[16] and homogenized in a high speed blender (Omni-Mixer)[17]. Homogenization was carried out for 15 to 30 seconds at room temperature.

Calf Thymus DNA in Complete Freund's Adjuvant

Forty mg of purified DNA (0.73% protein)[5] were dissolved in 1 ml PBS by stirring the solution overnight at 4 to 6° C with a magnetic mixer. A portion of the solution was homogenized with an equal volume of complete Freund's adjuvant. Another portion was heated at 100° C for 15 minutes and then cooled in an ice bath (Stolar and Levine, 1961). The denatured DNA solution was then homogenized for 15 to 30 seconds at room temperature with an equal volume of complete Freund's adjuvant.

Complete Freund's Adjuvant

Complete Freund's adjuvant was mixed with an equal volume of D-D water. The mixture was then homogenized for 15 to 30 seconds at room temperature.

Incomplete Freund's adjuvant. An equal volume of D-D water was mixed with incomplete Freund's adjuvant[16] and the mixture was homogenized for 15 to 30 seconds at room temperature.

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[16] Difco Laboratories, Detroit, Mich.

[17] Ivan Sorvall, Inc., Norwalk, Conn.

BSA

Solutions of BSA, fraction V[8], were prepared in PBS to contain 40 mg/ml, 2 mg/ml, and 20 mcg/ml.

Cell Transfers

A/J mice were used as donors and recipients in studies of the serologic changes in recipient mice following the injection of viable spleen or thymic cell suspensions, or spleen cell lysates.

Spleen or Thymic Cell Suspensions

Viable cell suspensions were obtained by using a modification of the method of Howard and Woodruff (1961). Donor mice were killed by cervical dislocation. Cardiac blood was collected and tested for anti-DNP activity to confirm previous serologic findings. Two spleens or 4 thymuses (unless otherwise stated) were obtained from each group of donor mice. Aseptic techniques were used throughout. Similar organs were pooled in 0.5 ml of Medium 199 tissue culture solution[6] (without antibiotics) and disrupted within a glass homogenizer equipped with a loose fitting piston. The resulting cell suspension was passed through an 80-mesh stainless-steel wire screen. A viable cell count was made by adding 0.1 ml 0.9% trypan blue to 0.1 ml of the suspension and observing the number of unstained mononuclear cells in a hemocytometer (Davis et al., 1958). Suspensions were diluted with Medium 199 to contain 4 to 8 X 10^6 viable mononuclear cells per 0.1 ml.

Cell Lysates

Disrupted cells were prepared by subjecting 0.5 to 1 ml of viable cells (4 to 8 X 10^6 per 0.1 ml of Medium 199) to 3 alternate

cycles of freezing in a carbon dioxide-ethanol bath and thawing at room temperature. The lysate was centrifuged at 1,000 g for 10 minutes and the sediment was examined microscopically for intact cells. No intact cells could be found in 2 counting areas of a hemocytometer.

Injections

Recipient mice were injected intraperitoneally with 0.1 ml volumes of cell preparations using a 1 cc sterile plastic disposable syringe and a 25 gauge needle[15]. Injections were made as soon as possible after the cells and cell lysates were prepared.

Photography

Photomicrographs of LE cells, and also the leukocyte nuclei that had been exposed to mouse serum and Lot 1 or Lot 2 conjugates, were made with a Leica camera and a Leitz Ortholux microscope. Exposures were made with Kodak Panatomic-X and Ektachrome film.

CHAPTER III

RESULTS

Incidence of Anti-nuclear Antibody Activity in Strains of Isogenic Mice

Mouse Strains Tested

Pools of serum obtained from 17 isogenic strains of 8-month-old mice were tested for anti-nuclear antibody. All of the serum donors had been recently removed from commercial breeding stocks (retired breeders). The results, as shown in Table 1, indicated that 4 of the 5 pools of A/J mouse sera contained anti-nuclear antibody activity. The pools of serum from the 16 other strains of mice did not have anti-nuclear antibody activity that could be detected with these tests. Controls were not used in this preliminary survey, as it was not known whether or not mice developed anti-nuclear antibodies spontaneously.

Table 2 contains results of the tests with each of the 5 pools of A/J mouse serum. Pools 1, 2, 3, and 4 showed a ++ or greater reaction with both calf thymus DNP spots and the nuclei of human peripheral blood leukocytes. The pattern of nuclear immunofluorescence observed is demonstrated in Figure 2. The nucleus, the only part of the cell that fluoresced, was homogeneous in appearance. The nuclei of leukocytes treated with pool number 5 serum did not fluoresce.

TABLE 1

MOUSE STRAINS TESTED FOR ANTI-NUCLEAR ANTIBODY ACTIVITY

Strain	Serum pools reactive with:	
	Calf thymus DNP[a]	Leukocyte nuclei[b]
A/J	4/5[c]	4/5
A/HeJ	0/5	0/5
AKR/J	0/5	0/5
C3H/HeJ	0/5	0/5
C57L/J	0/5	0/5
C58/J	0/5	0/5
DBA/1J	0/5	0/5
SWR/J	0/5	0/5
129/J	0/5	0/5
C3HeB/FeJ	0/5	0/5
C57BR/FeJ	0/5	0/5
C57BL/6J	0/5	0/5
C57BL/10J	0/5	0/5
RF/J	0/5	0/5
BALB/cJ	0/5	0/5
SJL/J	0/5	0/5
CBA/J	0/5	0/5

[a]Calf thymus DNP spot test.

[b]Human peripheral blood leukocytes.

[c]Number of serum pools positive/number of serum pools tested.

TABLE 2

ANTI-NUCLEAR ANTIBODY ACTIVITY IN A/J SERUM POOLS

Pool Number	Antigen preparations	
	Calf thymus DNP[a]	Leukocyte nuclei[b]
1	+++	+++
2	+++	+++
3	+++	++
4	++	++
5	-	-

[a]Calf thymus DNP spot test.

[b]Human peripheral blood leukocytes.

Figure 2. Homogeneous immunofluorescent staining pattern of human peripheral blood leukocyte nuclei after treatment with A/J mouse serum and Lot 1 fluorescent conjugate.

Standardization of Antigens in DNP Spot Tests

Calf Thymus DNP Spot Test

Calf thymus DNP spots were prepared from 4 different concentrations of DNP. The spots were treated with both human and mouse sera. Then the appropriate fluorescent conjugate (Lot 1 conjugate, diluted 1:2; anti-human gamma-globulin conjugate, diluted 1:4) was added to determine the minimal concentration of DNP that would yield a maximum (+++) fluorescence with undiluted human and mouse sera containing anti-DNP antibodies, but no fluorescence with undiluted normal sera. It was also of interest to determine which one of these concentrations was optimal for obtaining the highest titer of anti-DNP antibody activity in human sera. All of these criteria were satisfied with spots prepared from a solution containing 1.25 mg/ml of calf thymus DNP (Table 3). Normal human sera number 1 and 2 were negative. The human serum containing anti-DNP activity (DNP 1) had a titer of 1:64. A/J sera number 25 and 36 yielded a maximum fluorescence while the normal sera were negative. As the result of these observations, all calf thymus DNP spots were prepared from a solution containing 1.25 mg/ml of DNP.

Rabbit Thymus DNP Spot Test

A similar procedure was used to determine the optimal amount of rabbit thymus DNP to use in spot tests (Table 4). A 1:2 dilution of Lot 1 conjugate was used. The anti-human gamma-globulin conjugate was used in a 1:4 dilution. Both of the normal human sera (N 1 and N 2) were negative. The highest titer of anti-DNP activity in human serum was obtained in the experiment conducted with DNP spots prepared

TABLE 3

CALF THYMUS DNP SPOT TEST--STANDARDIZATION OF ANTIGEN

Serum[b]	Serum dilution	Fluorescence of serum and conjugate[a] treated spots made from DNP solutions containing:			
		5.0 mg/ml	2.5 mg/ml	1.25 mg/ml	0.675 mg/ml
Human					
N1	Undiluted	-	-	-	-
N2	Undiluted	-	-	-	-
DNP 1	Undiluted	++	+++	+++	++
DNP 1	1:4	+	++	++	+
DNP 1	1:16	-	+	+	+
DNP 1	1:64	-	+	+	-
DNP 1	1:256	-	-	-	-
Mouse					
A/J DNP 25	Undiluted	+	++	+++	+
A/J DNP 36	Undiluted	++	+++	+++	++
A/J N 41	Undiluted	-	-	-	-
DBA/1J N 1	Undiluted	-	-	-	-
DBA/1J N 2	Undiluted	-	-	-	-

[a]Specific anti-human gamma-globulin fluorescent conjugate used with human serum treated spots; Lot 1 conjugate used with mouse serum treated spots.

[b]N = serum containing no anti-DNP antibodies; DNP = serum containing anti-DNP antibodies.

TABLE 4

RABBIT THYMUS DNP SPOT TEST--STANDARDIZATION OF ANTIGEN

Serum[b]	Serum dilution	Fluorescence of serum and conjugate[a] treated spots made from DNP solutions containing			
		5.0 mg/ml	2.5 mg/ml	1.25 mg/ml	0.675 mg/ml
Human					
N 1	Undiluted	-	-	-	-
N 2	Undiluted	-	-	-	-
DNP 1	Undiluted	++	++	+++	++
DNP 1	1:4	++	+	++	+
DNP 1	1:16	-	+	+	+
DNP 1	1:64	-	-	+	-
DNP 1	1:256	-	-	-	-
Mouse					
A/J DNP 25	Undiluted	+	++	+++	++
A/J DNP 36	Undiluted	++	++	+++	++
A/J N 41	Undiluted	-	-	-	-
DBA/1J N 1	Undiluted	-	-	-	-
DBA/1J N 2	Undiluted	-	-	-	-

[a]Specific anti-human gamma-globulin fluorescent conjugate used with human serum treated spots; Lot 1 conjugate used with mouse serum treated spots.

[b]N = serum containing no anti-DNP antibodies; DNP = serum containing anti-DNP antibodies.

from the 1.25 mg/ml solution. A +++ fluorescence was also observed in tests with positive mouse sera and DNP spots prepared from the 1.25 mg/ml solution. The control mouse sera was negative. It was decided that all rabbit thymus DNP spots should be prepared from a solution containing 1.25 mg/ml of DNP.

Standardization of Lot 1 and Lot 2 Fluorescent Conjugates

Conjugate Titration

Both Lot 1 and Lot 2 conjugates were standardized by testing serial two-fold dilutions against calf thymus DNP and DNA spots and human leukocytes which had been exposed to undiluted mouse serum. All sera used in this experiment were selected on the basis of a previous +++ or - result obtained with Lot 1 conjugate diluted 1:2. The results of tests with calf thymus DNP spots (Table 5) indicated that all immunofluorescent tests with this antiserum should be performed with a 1:2 dilution of the conjugate. The results of the test with Lot 2 conjugate indicated that it should be diluted 1:4. Fluorescence was not observed when calf thymus DNA spots were treated with these same sera and the same dilutions of conjugates.

When experiments were performed using human peripheral blood leukocytes, together with the same sera and the same dilutions of conjugates, the results were similar to those seen in Table 5. All subsequent serologic experiments using leukocytes as a source of nuclear antigen were performed with Lot 1 conjugate diluted 1:2 and Lot 2 conjugate diluted 1:4. Sera that could not be shown to contain anti-DNP

TABLE 5

CALF THYMUS DNP SPOT TEST--TITRATION OF LOT 1 AND LOT 2 FLUORESCENT CONJUGATES

Conjugate dilution	Fluorescence of serum and conjugate treated spots							
	Lot 1				Lot 2			
	A/J 42[a]	A/J 48	A/J 2	A/J 3	A/J 25	A/J 36	A/J 41	DBA/1J 1
Undiluted	+++	+++	+	-	+++	+++	-	-
1:2	+++	+++	-	-	+++	+++	-	-
1:4	+++	+++	-	-	+++	+++	-	-
1:8	+	+	-	-	+++	+++	-	-
1:16	-	-	-	-	++	+	-	-
1:32	-	-	-	-	-	-	-	-

[a]Mouse strain and number. All mice were 8 months old.

antibodies with the calf thymus DNP spot test did not produce nuclear fluorescence in this standardization procedure.

Non-specific Staining of Lot 1 and Lot 2 Conjugates

To determine if either of the conjugates could produce non-specific staining of calf thymus DNP spots and the nuclei of human peripheral blood leukocytes, each conjugate was absorbed with an equal volume of undiluted A/J mouse serum. Duplicate calf thymus DNP spots and human leukocyte slides were exposed to the sera of 2 normal A/J mice and 2 A/J sera that contained anti-DNP antibody activity. Following this treatment both absorbed and unabsorbed conjugates were used to complete the test procedure. The results indicated that neither of the conjugates caused non-specific immunofluorescent staining of the DNP spots or human leukocyte nuclei. Absorption with A/J mouse serum eliminated all of the fluorescence of both DNP spots and the nuclei of human leukocytes which had been exposed to mouse sera containing anti-DNP antibody activity. In contrast, fluorescence was observed in samples treated with the unabsorbed sera. All results were negative in the experiment using normal mouse sera plus absorbed and unabsorbed Lot 1 and Lot 2 conjugates.

Specificity of Lot 1 and Lot 2 Conjugates

Both of the conjugates were examined by the immunoelectrophoresis technique to determine their specificity for mouse gamma-globulin. The precipitin pattern that developed after the electrophoretic separation of both A/J and DBA/1J sera and the addition of either Lot 1 or Lot 2 conjugate indicated that the antisera were

specific for mouse gamma-globulin. Only 1 precipitation band developed in each test. The band formed at the cathodic end of the slide and was a long, slightly curved arc, characteristic of gamma-globulin.

Electrophoretic Separation and Isolation of Anti-nuclear Antibody Activity

Eluates of starch blocks containing electrophoretically separated mouse serum protein fractions were tested for their gamma-globulin content and also their reactivity with calf thymus DNP spots and human leukocyte nuclei. The results are shown in Table 6. A/J mouse serum pools 1 and 2 were known to contain anti-nuclear antibody activity and pool 5 was known to be negative (Table 2). Eluates 1 and 2 of each of the sera were expected to contain predominantly gamma-globulin since they were eluted from sections of the starch blocks which were near the origin. The remainder of the eluates were expected to contain other serum proteins. Ring tests performed with each eluate and anti-mouse gamma-globulin (undiluted Lot 1 conjugate) revealed that eluates 1 and 2 of each serum pool contained most of the gamma-globulin. Comparison of anti-nuclear antibody activity with gamma-globulin content in each eluate indicated that the anti-nuclear antibody activity migrated with gamma-globulin under the conditions of this experiment. The eluates from the starch blocks prepared with pool 5 serum did not react in the 2 tests for anti-nuclear antibody activity.

Types of Anti-nuclear Antibody Activities in the Blood of 8-month-old A/J Female Mice (Retired Breeders)

Ten positive and 6 negative mice, selected on the basis of the

TABLE 6

RECOVERY OF ANTI-NUCLEAR ANTIBODY ACTIVITY FROM STARCH BLOCKS AFTER ELECTROPHORESIS OF A/J SERUM POOLS

Pool number	Eluate number[a]	Eluate tested with:		
		Anti-mouse gamma-globulin[b]	Calf thymus DNP[c]	Leukocyte nuclei[d]
1	1	+++	+++	+++
1	2	++	+	+
1	3	+	-	-
1	4	-	-	-
1	5	-	-	-
1	6	-	-	-
1	7	-	-	-
1	8	-	-	-
1	9	-	-	-
2	1	+++	+++	+++
2	2	+++	++	++
2	3	+	+	+
2	4	-	-	-
2	5	-	-	-
2	6	-	-	-
2	7	-	-	-
2	8	-	-	-
2	9	-	-	-
5	1	+++	-	-
5	2	+	-	-
5	3	-	-	-
5	4	-	-	-
5	5	-	-	-
5	6	-	-	-
5	7	-	-	-
5	8	-	-	-

[a]Eluate number corresponds to the number given each 1 cm section of the block from which serum proteins were eluted.

[b]Intensity of ring reaction in ring test.

[c]Calf thymus DNP spot test.

[d]Human peripheral blood leukocytes.

results obtained with the calf thymus DNP spot test, were examined to determine if their anti-nuclear antibody would react with calf thymus DNA spots, induce LE cell formation in vitro, and/or combine with the nuclei of mouse, rabbit, and human leukocytes. These results are shown in Table 7. None of the sera contained anti-DNA antibody activity that could be detected with the calf thymus DNA spot test. Four of the 10 sera which contained anti-DNP antibodies did not produce LE cells. Both mice that had a minimal amount (+) of anti-DNP antibody activity failed to form LE cells and 2 of the 4 that had ++ anti-DNP antibody activity also failed to form LE cells. In contrast, all of the mice that were +++ in the DNP spot test formed LE cells. A typical LE cell is shown in Figure 3. With one exception, mouse 45, the sera of all mice that were determined to have anti-DNP antibody activity also reacted with the nuclei of mouse, rabbit, and human leukocytes. The amount of anti-nuclear antibody activity present in each serum sample, expressed as +++, ++, +, or -, is apparently estimated with about the same sensitivity with each of the immunofluorescent tests since in no case was there a deviation of more than one + with any of the positive serum samples. In contrast, the LE cell test does not appear to be as sensitive as the immunofluorescent tests since it was observed that 4 of 10 mice whose sera contained anti-nuclear antibody activity did not form LE cells. Six sera which were negative by the calf thymus DNP spot test were also negative by all other tests used.

TABLE 7

COMPARISON OF ANTI-NUCLEAR ANTIBODY ACTIVITIES
IN 8-MONTH-OLD A/J FEMALE MICE

Mouse number	Calf thymus DNP spots	Calf thymus DNA spots	LE cells	Mouse LN[a]	Rabbit LN	Human LN
1	+++	-	pos.[b]	+++	+++	+++
12	++	-	pos.	+++	++	++
18	+	-	neg.[c]	+	+	+
26	-	-	neg.	-	-	-
27	-	-	neg.	-	-	-
28	-	-	neg.	-	-	-
34	+++	-	pos.	+++	+++	+++
35	++	-	neg.	+	+	+
37	++	-	pos.	+++	++	++
42	+++	-	pos.	+++	+++	+++
44	-	-	neg.	-	-	-
45	+	-	neg.	-	-	-
46	-	-	neg.	-	-	-
47	-	-	neg.	-	-	-
48	++	-	neg.	++	+	+
61	+++	-	pos.	+++	+++	+++

[a]LN = leukocyte nuclei.

[b]Pos. = positive LE cell preparation.

[c]Neg. = negative LE cell preparation.

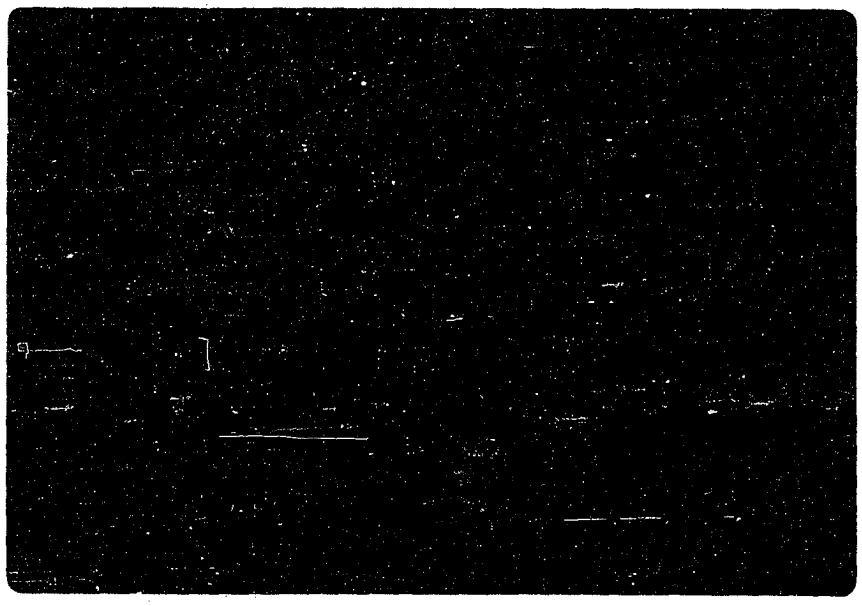

Figure 3. LE cell produced by an 11-month-old mouse of the A/J strain.

Incidence of Anti-nuclear Antibody Activity in Mice

Anti-nuclear Antibodies in A/J Mice

It was of interest to determine what percentage of 8-month-old retired-breeder mice develop anti-nuclear antibody activity spontaneously, whether or not additional mice develop antibody with increasing age, and whether or not mice that become positive in these tests remain positive permanently. It was also hoped that the age at which anti-DNP antibody activity begins to appear in A/J mice could be determined by studying the sera of aging virgin mice.

Fifty 8-month-old A/J females and 50 males that had been retired from breeding stocks were bled at weekly intervals for 4 weeks and their sera were tested for the presence of anti-DNP antibody activity. The results of tests with the sera of the female mice are shown in Table 8. Initially, 34% of the mice had anti-DNP antibody activity in their sera. After 4 weeks, 36% were positive. Two mice that were originally negative (numbers 7 and 24) became positive after 2 weeks. With one exception (number 17), all mice that were originally positive at 8 weeks of age remained positive throughout the 4-week observation period. The results of the studies with the male mice are presented in Table 9. At the beginning of the study, 18% of the animals had anti-nuclear antibody activity. At the end of the observation period, 20% of the mice were positive. None of the positive males became negative during the experimental period, and only 1 negative mouse (number 4) developed anti-DNP antibody activity.

The difference between males and females in the percentage of positive animals is clearly demonstrated in Table 10, which includes

TABLE 8

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY[a]
IN ADULT FEMALE A/J MICE

Mouse number	Age in months				
	8	8 1/4	8 1/2	8 3/4	9
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+	+	+	+	+
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	+	+	++
8	-	-	-	-	-
9	++	++	++	++	++
10	-	-	-	-	-
11	-	-	-	-	-
12	++	++	+++	+++	+++
13	-	-	-	-	-
14	+++	+++	+++	+++	+++
15	-	-	-	-	-
16	-	-	-	-	-
17	+	+	+	-	+
18	+	+	+	+	+
19	++	++	++	++	++
20	-	-	-	-	-
21	-	-	-	-	-
22	+	+	+	+	+
23	+	+	+	+	+
24	-	-	+	+	++
25	+	+	++	++	++
26	-	-	-	-	-
27	-	-	-	-	-
28	+	+	+	+	+
29	-	-	-	-	-
30	-	-	-	-	-
31	+	+	+	+	-
32	++	++	++	+++	++
33	-	-	-	-	-
34	-	-	-	-	-
35	+	+	+	+	++

[a]Determined with calf thymus DNP spot test.

TABLE 8--Continued

Mouse number	Age in months				
	8	8 1/4	8 1/2	8 3/4	9
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	-	-	-
39	-	-	-	-	-
40	-	-	-	-	-
41	-	-	-	-	-
42	++	++	++	+++	+++
43	-	-	-	-	-
44	-	-	-	-	-
45	-	-	-	-	-
46	-	-	-	-	-
47	+	+	+	+	+
48	+	++	++	++	++
49	-	-	-	-	-
50	-	-	-	-	-
Percent positive	34%	34%	38%	36%	36%

[a]Determined with calf thymus DNP spot test.

TABLE 9

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY[a]
IN ADULT MALE A/J MICE

Mouse number	Age in months				
	8	8 1/4	8 1/2	8 3/4	9
1	-	-	-	-	-
2	+++	+++	++	+++	+++
3	-	-	-	-	-
4	-	-	-	-	+
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	++	++	++	++	+
12	-	-	-	-	-
13	+	+	+	+	+
14	+	+	+	+	+
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	+++	+++	+++	+++	+++
20	-	-	-	-	-
21	-	-	-	-	-
22	-	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	-	-
26	-	-	-	-	-
27	-	-	-	-	-
28	-	-	-	-	-
29	+	+	+	+	+
30	-	-	-	-	-
31	-	-	-	-	-
32	-	-	-	-	-
33	-	-	-	-	-
34	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

TABLE 9--Continued

Mouse number	Age in months				
	8	8 1/4	8 1/2	8 3/4	9
35	+	+	++	++	+++
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	-	-	-
39	-	-	-	-	-
40	-	-	-	-	-
41	++	++	+++	+++	+++
42	-	-	-	-	-
43	+	+	+	+	++
44	-	-	-	-	-
45	-	-	-	-	-
46	-	-	-	-	-
47	-	-	-	-	-
48	-	-	-	-	-
49	-	-	-	-	-
50	-	-	-	-	-
Percent positive	18%	18%	18%	18%	20%

[a]Determined with calf thymus DNP spot test.

TABLE 10

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY IN ALL 9-MONTH-OLD A/J MICE EXAMINED

Sex	Number of mice tested	Number of mice positive	Percent positive
Female	505	181	35.8
Male	556	132	23.7

the results seen with the sera of all 9-month-old mice that were tested for anti-DNP antibody activity. It can be concluded that there is a significant difference at the 0.01 confidence level ($t = 3.05$) between the percentage of positive females (35.8%) and the percentage of positive males (27.3%).

Two groups of adult, retired-breeder A/J mice were studied to determine if the incidence of anti-nuclear antibody activity increases with age. In the first study, all mice (females and males) were bled at various intervals during aging and the sera were examined for anti-nuclear antibody activity.

The results with sera obtained by repeated bleedings of aging females are shown in Table 11. Thirty-four percent of the mice had anti-nuclear antibody activity in their sera when they were 8 months of age. When they were $13\frac{1}{2}$ months of age, 63% were positive. It appeared that during the observation period there was a gradual increase in the number of female mice that had anti-DNP antibodies in their sera. A similar trend was observed in the data derived from testing the serum of aging A/J male mice for anti-nuclear antibody activity (Table 12). Although the percentage of positive 8-month-old males appeared to be lower than the percentage of positive 8-month-old females (Table 11), and even though it was demonstrated that there was a significant difference in the percentage of positive females and males at 9 months of age (Table 10), the difference between males and females apparently diminishes as the mice become older. At $13\frac{1}{2}$ months of age there does not appear to be any difference between the percentage of positive males and females (Table 11 and Table 12; $t = 0.45$). It can also be seen that

TABLE 11

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY[a]
IN AGING ADULT FEMALE A/J MICE

Mouse number	Age in months				
	8	9	10 3/4	12 1/2	13 1/2
1	-	-	-	-	-
2	-	-	-	-	+++
3	-	-	-	-	-
4	+	++	++	++	++
5	-	-	-	-	++
6	++	+++	++	+++	+++
7	-	-	-	-	-
8	+	++	+	+	+
9	-	-	-	-	-
10	-	-	-	-	-
11	+	+	++	++	++
12	+++	+++	+++	+++	+++
13	++	++	++	++	+++
14	++	++	++	+	++
15	-	-	-	+++	++
16	++	++	++	+++	+++
17	-	-	+	+	+
18	-	+	+	++	+++
19	-	-	-	+	+
20	+	+	+	+	++
21	-	-	-	-	+
22	-	-	-	-	-
23	-	+	+	+	+
24	-	-	-	-	-
25	-	-	-	-	-
26	+	+	+	+	+
27	-	-	-	-	-
28	-	-	-	-	+
29	-	-	+	+	++
30	-	-	-	-	-
31	+++	+++	+++	+++	+++
32	-	-	-	-	-
33	++	++	++	+++	+++
34	-	-	-	+	-

[a]Determined with calf thymus DNP spot test.

TABLE 11--Continued

Mouse number	Age in months				
	8	9	10 3/4	12 1/2	13 1/2
35	-	-	-	-	-
36	+++	+++	++	++	++
37	-	-	-	-	-
38	-	-	-	-	0
39	+	+	++	+	+
40	+	+	+	+	+
41	-	-	-	-	-
42	-	-	-	-	++
43	-	-	-	-	-
44	-	-	-	-	-
45	+	++	+	+	+
46	-	-	-	-	-
47	-	-	+	+	++
48	-	-	-	++	+++
49	+++	+++	+++	+++	+++
50	-	-	+	+	++
Percent positive	34%	38%	46%	54%	63%

[a]Determined with calf thymus DNP spot test.

0 = animal died between the 12 1/2 and the 13 1/2 month bleedings.

TABLE 12

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY
IN AGING ADULT MALE A/J MICE

Mouse number	Age in months			
	8	10 3/4	12 1/2	13 1/2
1	+	+	-	+
2	-	+	0	0
3	++	++	++	++
4	-	-	-	-
5	-	-	+	+
6	++	+++	+++	+++
7	++	++	++	++
8	-	-	-	-
9	+++	+++	+++	+++
10	-	-	-	-
11	-	-	-	-
12	-	+	-	+
13	-	-	-	+
14	++	+++	+++	++
15	-	-	-	-
16	-	-	-	+
17	-	-	-	-
18	+	+	+	++
19	+	+	+	+
20	+	++	+++	++
21	-	-	+	+
22	-	-	-	-
23	-	-	-	-
24	-	++	+	+
25	-	+	+	+
26	-	+	+	+
27	-	++	+	+
28	-	++	+	++
29	-	-	-	-
30	-	+++	+++	+++
31	-	-	++	++
32	-	-	-	-

[a]Determined with calf thymus DNP spot test.

0 = animal died before 12 1/2 months.

TABLE 12--Continued

Mouse number	Age in months			
	8	10 3/4	12 1/2	13 1/2
33	-	-	-	+
34	-	-	-	+++
35	-	-	-	+
36	-	-	-	+
37	-	-	+	++
38	-	-	-	++
39	-	-	-	+
40	-	-	-	-
41	-	+	+	+
42	-	-	-	+
43	-	-	-	-
44	-	-	-	-
45	-	-	-	-
46	-	-	-	-
47	-	-	-	-
48	-	-	+	+
49	-	-	+	+
50	++	++	++	++
Percent positive	20%	38%	44.9%	67.3%

[a]Determined with calf thymus DNP spot test.

0 = animal died before 12 1/2 months.

mice whose sera showed a ++ or greater reaction at 8 months of age did not demonstrate any decrease in activity with increasing age. No consistent pattern in the spontaneous conversion of mice to positive could be observed. Some mice did not become positive, some developed + reactions and stayed at this level, and others developed +++ reactions and remained at this maximum level.

In the second study, five groups of retired-breeder A/J females and males were bled and all sera were tested for the presence of anti-nuclear antibody activity. All mice in each group were the same age, but each group of mice was bled when they were a different age. Table 13 contains the results obtained with the female mice. The incidence of anti-DNP antibody activity in these mice increased from 34% at 8 months of age to 90% at 23 months of age. The increase was statistically significant ($p = 0.001$). In Table 14 are shown data obtained with sera from the various groups of male mice. The incidence of positive animals in these groups increased from 18% at 8 months of age to 67.3% at $13\frac{1}{2}$ months of age. This increase was also statistically significant ($p = 0.001$).

It was also of interest to determine the age at which A/J mice begin to produce anti-nuclear antibodies spontaneously. In the first study, 48 female virgin mice were bled when they were 1 3/4 months old and at intervals thereafter until they were 19 3/4 months old. All sera were tested for anti-nuclear antibody activity with the calf thymus DNP spot test. The results of this study are contained in Table 15. None of the females developed anti-DNP antibody activity between 1 3/4 and 5 1/2 months of age. When they were 8 1/4 months old, only 3 mice (6.3%)

TABLE 13

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY
IN FEMALE A/J MICE OF VARIOUS AGES

	Age in months				
	8	10	12	13 1/2	23
Total number tested	50	50	49	50	20
Percent positive	34%	44%	63%	66%	90%

TABLE 14

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY
IN MALE A/J MICE OF VARIOUS AGES

	Age in months				
	8	9	10	12 1/2	13 1/2
Total number tested	50	50	50	50	49
Percent positive	18%	24%	40%	54%	67.3%

TABLE 15

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY[a]
IN AGING VIRGIN FEMALE A/J MICE

Mouse number	Age in months							
	1 3/4	2 1/2	3 1/4	4	4 3/4	5 1/2	8 1/4	19 3/4
1	-	-	-	-	-	-	-	0
2	-	-	-	-	-	-	-	+++
3	-	-	-	-	-	-	-	+
4	-	-	-	-	-	-	-	0
5	-	-	-	-	-	-	-	0
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	+++
8	-	-	-	-	-	-	-	0
9	-	-	-	-	-	-	-	+++
10	-	-	-	-	-	-	-	++
11	-	-	-	-	-	-	-	+++
12	-	-	-	-	-	-	++	+++
13	-	-	-	-	-	-	-	0
14	-	-	-	-	-	-	-	+++
15	-	-	-	-	-	-	-	0
16	-	-	-	-	-	-	-	+
17	-	-	-	-	-	-	-	+++
18	-	-	-	-	-	-	-	0
19	-	-	-	-	-	-	-	+++
20	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	+++
22	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	+
26	-	-	-	-	-	-	-	0
27	-	-	-	-	-	-	-	0
28	-	-	-	-	-	-	-	0
29	-	-	-	-	-	-	-	+
30	-	-	-	-	-	-	-	+++
31	-	-	-	-	-	-	-	0
32	-	-	-	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

0 = animal not available.

TABLE 15--Continued

Mouse number	Age in months							
	1 3/4	2 1/2	3 1/4	4	4 3/4	5 1/2	8 1/4	19 3/4
33	-	-	-	-	-	-	-	0
34	-	-	-	-	-	-	-	0
35	-	-	-	-	-	-	-	+++
36	-	-	-	-	-	-	++	++
37	-	-	-	-	-	-	-	+++
38	-	-	-	-	-	-	-	+++
39	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	+
41	-	-	-	-	-	-	-	0
42	-	-	-	-	-	-	+++	0
43	-	-	-	-	-	-	-	0
44	-	-	-	-	-	-	-	0
45	-	-	-	-	-	-	-	+
46	-	-	-	-	-	-	-	0
47	-	-	-	-	-	-	-	++
48	-	-	-	-	-	-	-	+
Percent Positive	0%	0%	0%	0%	0%	0%	6.3%	77.3%

[a]Determined with calf thymus DNP spot test.

0 = animal not available

had become positive. At the end of 19 3/4 months, 76.6% had developed anti-DNP antibody activity. Fifty male mice were also bled at 3-week intervals beginning when they were 1 3/4 months old and continuing until they were 5 1/2 months old. None of the mice developed anti-DNP antibody activity. These animals were not studied beyond this age.

Although the incidence of anti-nuclear antibody activity in 8 1/4-month-old virgin female mice appeared to be lower than that of the 8- to 9-month-old retired-breeder A/J females, the data in Table 16 indicate that the observed increase in incidence of anti-DNP antibody activity in virgin females between 5 1/2 and 19 3/4 months of age is statistically significant ($p = 0.001$). Observations were also made on sera collected from groups of virgin A/J male mice of various ages (Table 17). Although none of the males in the 5 1/2-month-old group had anti-DNP antibody activity, the incidence in the 8 1/4-month-old group was 10%. The incidence increased to 31.7% in the 10-month-old group. The increase between 5 1/2 and 10 months of age is statistically significant ($p = 0.001$). These data also indicate that the spontaneous appearance of anti-nuclear antibody activity in the virgin male mice occurred at about the same rate as it did in retired-breeder males. This can be seen by comparing the data in this table with that in Table 14.

Although it was shown in Table 7 that 8-month-old A/J mice apparently did not produce anti-DNA antibodies spontaneously, it was of interest to determine if older mice would develop antibody. For this purpose, 50 female mice were bled periodically and their sera tested for both anti-DNP and anti-DNA antibodies. All of the mice

TABLE 16

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY IN VIRGIN FEMALE A/J MICE OF VARIOUS AGES

	Age in months		
	5 1/2	8 1/4	19 3/4
Total number tested	50	40	30
Percent positive	0%	12.5%	76.6%

TABLE 17

INCIDENCE OF ANTI-DNP ANTIBODY ACTIVITY IN VIRGIN MALE A/J MICE OF VARIOUS AGES

	Age in months		
	5 1/2	8 1/4	10
Total number tested	50	40	41
Percent positive	0%	10%	31.7%

were retired breeders. The results of these tests are presented in Table 18. Although these mice were found to have an increase in incidence of anti-DNP antibody activity with increasing age, similar to that seen in the earlier experiments with female mice (Tables 11 and 13), they showed no anti-DNA antibody activity until they were 23 months old. However, tests were not performed on these mice between 12 and 23 months of age. At 23 months of age, 6 of 19 mice (31.6%) had anti-DNA antibodies detected with the DNA spot test and 17 of 19 mice (89.4%) had anti-DNP antibody in their sera.

Incidence of Anti-nuclear Antibody Activity in Other Isogenic Strains of Mice

Anti-nuclear antibodies in aging DBA/1J mice. Previous tests with pooled sera (Table 1) suggested that DBA/1J mice did not develop anti-DNP antibodies spontaneously before they were 8 months old. Since it was desired to use such a strain as a control in certain experiments, a group of retired-breeder DBA/1J mice of various ages was obtained and their individual sera were tested for anti-nuclear antibody activity. The results of these tests are shown in Table 19. Due to the nature of the results, the data were pooled and not separated according to sex. It can be seen that none of the sera had anti-nuclear antibody activity that could be detected with calf thymus DNP, DNA, or the nuclei of human peripheral blood leukocytes. It would appear that mice of this strain do not develop the autoimmune condition spontaneously, at least not before they are 18 months of age.

Anti-nuclear antibodies in aging AJDF1 mice. The incidence of anti-nuclear antibodies in aging virgin AJDF1 mice is shown in Table 20.

TABLE 18

INCIDENCE OF ANTI-DNP AND ANTI-DNA ANTIBODY ACTIVITIES IN AGING FEMALE A/J MICE

Mouse number	Age in months							
	8		10		12		23	
	DNP[a]	DNA[b]	DNP	DNA	DNP	DNA	DNP	DNA
1	-	-	-	-	-	-	0	0
2	-	-	-	-	-	-	+	+
3	+	-	+	-	++	-	+	+
4	-	-	-	-	-	-	+++	-
5	-	-	-	-	+	-	+	++
6	-	-	-	-	-	-	+	-
7	-	-	-	-	-	-	++	-
8	+	-	++	-	+++	-	+	+
9	-	-	-	-	++	-	+++	-
10	-	-	-	-	+	-	++	-
11	-	-	-	-	+++	-	0	0
12	-	-	-	-	-	-	0	0
13	-	-	+	-	+	-	0	0
14	+	-	+	-	++	-	0	0
15	-	-	-	-	-	-	0	0
16	-	-	-	-	++	-	-	-
17	+	-	-	-	+	-	0	0
18	+	-	+	-	++	-	+	-
19	-	-	-	-	-	-	+++	-
20	+	-	+	-	++	-	0	0
21	-	-	-	-	+++	-	++	-
22	++	-	++	-	++	-	++	+
23	-	-	+	-	++	-	0	0
24	-	-	+	-	++	-	0	0
25	-	-	-	-	+	-	++	-
26	-	-	-	-	-	-	0	0
27	+++	-	+++	-	+++	-	0	0
28	+++	-	++	-	+++	-	0	0
29	+	-	++	-	++	-	0	0
30	-	-	-	-	+	-	0	0

[a]DNP = calf thymus DNP spot test.

[b]DNA = calf thymus DNA spot test.

0 = animal not available.

TABLE 18--Continued

Mouse number	Age in months							
	8		10		12		13	
	DNP[a]	DNA[b]	DNP	DNA	DNP	DNA	DNP	DNA
31	-	-	-	-	+	-	0	0
32	-	-	-	-	+	-	-	-
33	-	-	-	-	-	-	+	+
34	-	-	+	-	++	-	0	0
35	-	-	+	-	++	-	0	0
36	-	-	-	-	-	-	++	-
37	-	-	-	-	-	-	0	0
38	-	-	+	-	+	-	0	0
39	-	-	++	-	+++	-	0	0
40	+	-	+	-	++	-	0	0
41	-	-	-	-	-	-	0	0
42	+	-	++	-	+++	-	0	0
43	+	-	++	-	+	-	0	0
44	-	-	-	-	-	-	0	0
45	-	-	-	-	-	-	++	-
46	+	-	+	-	+	-	+	-
47	-	-	-	-	-	-	0	0
48	-	-	-	-	-	-	0	0
49	++	-	+	-	+++	-	0	0
50	+++	-	+++	-	+++	-	0	0
Percent positive	32%	0%	44%	0%	66%	0%	89.4%	31.6%

[a]DNP = calf thymus DNP spot test.

[b]DNA = calf thymus DNA spot test.

0 = animal not available.

TABLE 19

INCIDENCE OF ANTI-NUCLEAR ANTIBODY ACTIVITY IN DBA/1J MICE OF VARIOUS AGES[a]

Age in months	Test	Intensity of fluorescence		
		+++	++	+
8	Calf thymus DNP spots	0/106[b]	0/106	0/106
8	Human leukocytes[c]	0/106	0/106	0/106
18	Calf thymus DNP spots	0/50	0/50	0/50
18	Calf thymus DNA spots	0/50	0/50	0/50

[a]Pooled data for both sexes.

[b]Number of sera positive/number of sera tested.

[c]Human peripheral blood leukocytes.

TABLE 20

INCIDENCE OF ANTI-NUCLEAR ANTIBODY ACTIVITY IN AJDF1 MICE OF VARIOUS AGES[a]

Age in months	Test	Intensity of fluorescence		
		+++	++	+
9	Calf thymus DNP spots	0/82[b]	0/82	0/82
9	Calf thymus DNA spots	0/82	0/82	0/82
16	Calf thymus DNP spots	0/80	0/80	0/80
16	Calf thymus DNA spots	0/80	0/80	0/80

[a] Pooled data for both sexes.

[b] Number of sera positive/number of sera tested.

These F1 hybrid mice were the progeny of A/J females and DBA/1J males. None of the sera contained detectable anti-nuclear antibody activity.

Anti-nuclear antibodies in aging ANZBF1 mice. These hybrid mice were obtained by mating A/J females with NZB/B1 males. Groups of the hybrid males and females were bled and their sera, or bloods, were examined for the presence of anti-nuclear antibodies. The results of these experiments are contained in Table 21. It can be seen that 14 of 20 females and 5 of 19 males had developed anti-DNP antibodies spontaneously by the time they were 3 1/2 months old. These sera were not examined for anti-DNA antibodies. Although not all mice were bled when they were 5 1/2 months old, sera from all of the 9 females that were bled and tested for anti-DNP antibody activity were positive. All but 1 of the 9 males tested had anti-DNP antibody activity. It can also be seen that all of the females that had anti-DNP antibodies also had anti-DNA antibodies. None of the mice was found to have anti-DNA antibody only. Sera from 6 of 9 male mice had anti-DNA antibody activity. One of the 3 males that did not develop anti-DNA antibodies by 5 1/2 months of age was the only one that was not positive in tests with calf thymus DNP. Although these results indicate that anti-nuclear antibodies were present in most of the hybrid mice at 5 1/2 months of age, only 1 female and none of the males produced LE cells in vitro. Certain of the mice that were examined when they were 3 1/2 months old were also bled when they were 11 months old. It can be seen that all females (9 of 9) had anti-nuclear antibody activity detectable with both DNP and DNA spot tests. However, sera from only 5 of 8 males had anti-DNP antibodies and only 3 of these 5

TABLE 21

INCIDENCE OF ANTI-NUCLEAR ANTIBODY ACTIVITY
IN AGING ANZBF1 MICE

Mouse number	Sex	Age in months					
		3 1/2	5 1/2			11	
		CT[a] DNP	CT DNP	CT[b] DNA	LE cells	CT DNP	CT DNA
1	female	-	0	0	0	+++	++
2		+	0	0	0	++	+
3		++	+++	+	neg.	0	0
4		-	+++	+++	neg.	0	0
5		+	++	+	neg.	0	0
6		+	+	++	neg.	0	0
7		-	+	++	neg.	0	0
8		+	0	0	0	++	++
9		-	0	0	0	+++	+
10		+	0	0	0	++	++
11		+	0	0	0	+++	++
12		++	+++	+++	pos.	0	0
13		++	+++	+++	neg.	0	0
14		-	0	0	0	++	++
15		+	0	0	0	++	+
16		+	0	0	0	+	+++
17		++	0	0	0	0	0
18		-	0	0	0	0	0
26		-	+	+	neg.	0	0
27		+++	+	+++	neg.	0	0

[a] CT DNP = calf thymus DNP spot test.

[b] CT DNA = calf thymus DNA spot test.

0 = test not done.

Neg. = negative LE cell preparation.

Pos. = positive LE cell preparation.

TABLE 21--Continued

Mouse number	Sex	Age in months					
		3 1/2	5 1/2			11	
		CT[a] DNP	CT DNP	CT[b] DNA	LE cells	CT DNP	CT DNA
10	male	-	+	+	neg.	0	0
11		-	+	+	neg.	0	0
12		+	+	+	neg.	0	0
13		-	0	0	0	0	0
14		-	+	-	neg.	0	0
15		-	+	-	neg.	0	0
16		+	+	+	neg.	0	0
17		+++	+	+	neg.	0	0
18		-	-	-	neg.	0	0
23		-	0	0	0	0	0
25		+	0	0	0	+++	++
26		-	0	0	0	+	-
27		+	0	0	0	-	-
30		-	0	0	0	-	-
31		-	0	0	0	+++	++
32		-	0	0	0	-	-
33		-	0	0	0	+	-
34		-	0	0	0	+	+
35		-	0	0	0	0	0

[a]CT DNP = calf thymus DNP spot test.

[b]CT DNA = calf thymus DNA spot test.

0 = test not done.

Neg. = negative LE cell preparation.

Pos. = positive LE cell preparation.

sera also had anti-DNA activity.

Anti-nuclear antibodies in C57BL/6J mice. The incidence of anti-nuclear antibody activity in the sera of C57BL/6J retired breeders is shown in Table 22. These mice were 8 months of age and were to be used as negative controls in later experiments. It was assumed that their sera would be negative, since in earlier studies pooled sera from mice of this strain did not contain detectable anti-nuclear antibody activity (Table 1). However, the results of the present study, obtained with tests using individual samples, revealed that C57BL/6J mice do develop autoimmune anti-nuclear antibodies spontaneously. As the result of this finding, the idea of using mice of this strain as a negative control was abandoned, and the DBA/1J strain was selected as a negative control strain.

Immunization Studies

Since it was established that A/J mice produce anti-nuclear antibodies spontaneously and that DBA/1J mice do not, it was of interest to determine if members of either of these strains could be induced to produce anti-nuclear antibodies following immunization with various nuclear and non-nuclear substances.

Injection of 4 1/2- and 8-month-old A/J Mice with Denatured Calf Thymus DNA or DNP Complete Freund's Adjuvant

In Table 23 are data showing the immunologic responses that were observed following the immunization of 4 1/2-month-old virgin females or 8-month-old retired-breeder females. None of the 4 1/2-month-old mice had anti-nuclear antibody activity in their sera

TABLE 22

INCIDENCE OF ANTI-NUCLEAR ANTIBODY ACTIVITY
IN 8-MONTH-OLD C57BL/6J MICE

Test	Intensity of fluorescence			
	+++	++	+	-
Calf thymus LNP spots	6/100[a]	9/100	10/100	75/100
Calf thymus DNA spots	0/100	0/100	0/100	100/100

[a]Number of reactive sera/total number tested.

TABLE 23

SEROLOGIC RESULTS FOLLOWING IMMUNIZATION OF FEMALE A/J MICE WITH 2 WEEKLY INJECTIONS OF CALF THYMUS DNP OR DNA IN COMPLETE FREUND'S ADJUVANT

Age	Mouse number	Antigen injected	Results of serologic tests							
			Before injection		After 1 injection		After 2 injections			
			DNP[a]	DNA[b]	DNP	DNA	DNP	DNA	HLN[c]	LE[d] cells
4½ mo.	67	DNP	-	-	-	-	-	-	-	neg.
	68	DNP	-	-	-	-	-	-	-	neg.
	69	DNP	-	-	-	-	-	-	-	neg.
	70	DNP	-	-	-	-	-	-	-	neg.
	71	DNP	-	-	-	-	-	-	-	neg.
	72	DNP	-	-	-	-	-	-	-	neg.
4½ mo.	61	DNA	-	-	-	-	-	-	-	neg.
	62	DNA	-	-	-	-	-	-	-	neg.
	63	DNA	-	-	-	-	-	-	-	neg.
	64	DNA	-	-	-	-	-	-	-	neg.
	65	DNA	-	-	-	-	-	-	-	neg.
	66	DNA	-	-	-	-	-	-	-	neg.
4½ mo.	55	none	-	-	-	-	-	-	-	neg.
	56	none	-	-	-	-	-	-	-	neg.
	57	none	-	-	-	-	-	-	-	neg.
	58	none	-	-	-	-	-	-	-	neg.
	59	none	-	-	-	-	-	-	-	neg.
	60	none	-	-	-	-	-	-	-	neg.

[a] Calf thymus DNP spot test.

[b] Calf thymus DNA spot test.

[c] HLN = human leukocyte nuclei.

[d] LE cell test; neg. = negative LE cell preparation, pos. = positive LE cell preparation.

TABLE 23--Continued

Age	Mouse number	Antigen injected	Results of serologic tests							
			Before injection		After 1 injection		After 2 injections			
			DNP[a]	DNA[b]	DNP	DNA	DNP	DNA	HLN[c]	LE[d] cells
8 mo.	4	DNP	+	-	++	-	++	-	++	pos.
	5	DNP	++	-	+	-	+	-	+	pos.
	6	DNP	+++	-	++	-	+++	-	+++	pos.
	7	DNP	+	-	+	-	+++	-	+++	pos.
8 mo.	12	DNA	++	-	+	-	+	-	+	pos.
	14	DNA	++	-	+	-	0	0	0	0
	15	DNA	+++	-	++	-	++	-	+++	pos.
	17	DNA	+	-	-	-	+	-	+	pos.
8 mo.	44	DNP	-	-	-	-	++	-	+	pos.
	47	DNP	-	-	+++	-	+++	-	+++	pos.
	51	DNP	-	-	-	-	+	-	+	pos.
	52	DNP	-	-	-	-	++	-	++	pos.
	53	DNP	-	-	-	-	0	0	0	0
8 mo.	3	DNA	-	-	+	-	++	-	+	pos.
	13	DNA	-	-	-	-	+	-	+	pos.
	5	DNA	-	-	+	-	+++	-	+++	pos.
	41	DNA	-	-	-	-	++	-	++	pos.
	50	DNA	-	-	+	-	++	-	+++	pos.
8 mo.	1	none	-	-	-	-	-	-	-	neg.
	2	none	-	-	-	-	-	-	-	neg.
	3	none	-	-	-	-	-	-	-	neg.
	5	none	-	-	-	-	-	-	-	neg.
	6	none	-	-	-	-	-	-	-	neg.
	7	none	-	-	-	-	-	-	-	neg.

[a]Calf thymus DNP spot test.

[b]Calf thymus DNA spot test.

[c]HLN = human leukocyte nuclei.

[d]LE cell test; neg. = negative LE cell preparation, pos. = positive LE cell preparation.

0 = animal died.

before immunization. The older mice that were to be immunized were divided into 2 groups. One group was composed of mice that had developed anti-nuclear antibody activity spontaneously. The other group was negative before immunization. Six negative controls were used with each age group. One week after the first injection of antigen, all mice were bled, and each was given a second injection of the appropriate antigen. One week later the mice were bled again and all sera and blood were examined for anti-nuclear antibody activity. None of the mice of either age group produced anti-DNA antibody. None of the 4 1/2-month-old mice was induced to produce anti-nuclear antibodies as the result of the injections. It also seemed apparent that immunizations did not alter the pre-injection status of positive mice. In contrast to the younger mice, the negative 8-month-old mice that were immunized produced anti-DNP antibodies. Although not all of these mice produced anti-DNP antibody after 1 injection of either antigen, they all developed anti-DNP antibody after the second injection. All positive mice also produced LE cells. The uninjected control mice, which were bled and tested along with the experimental animals, did not develop anti-nuclear antibody activity during the course of the experiment.

Injection of 8-month-old Seronegative A/J Mice with Various Substances

Groups of seronegative male and female mice were injected with the antigens indicated in Table 24. All of the mice were retired breeders. All mice were injected on the same day. One week later, they were bled and then all, except those that were injected with BSA, were injected a second time with the appropriate material. A second bleeding

TABLE 24

SEROLOGIC RESULTS FOLLOWING IMMUNIZATION OF 8-MONTH-OLD A/J MICE LACKING ANTI-DNP ANTIBODY ACTIVITY

Antigen	Sex	Sera reactive with calf (CT) and rabbit (RT) thymus DNP spots after:			
		1 injection		2 injections	
		CT DNP	RT DNP	CT DNP	RT DNP
CT DNP	male	4/6[a]	4/6	4/6	4/6
CT DNP	female	3/6	3/6	4/6	4/6
CT DNP in complete Freund's adjuvant	male	4/6	4/6	4/6	4/6
CT DNP in complete Freund's adjuvant	female	3/6	3/6	6/6	6/6
CT DNA in complete Freund's adjuvant	male	5/6	5/6	5/6	5/6
CT DNA in complete Freund's adjuvant	female	0/5	0/5	3/5	3/5
Denatured CT DNA in complete Freund's adjuvant	male	4/5	4/5	3/5	3/5
Denatured CT DNA in complete Freund's adjuvant	female	2/5	2/5	3/5	3/5
Complete Freund's adjuvant	male	4/6	4/6	4/6	4/6
Complete Freund's adjuvant	female	4/6	4/6	4/6	4/6
Incomplete Freund's adjuvant	male	0/6	0/6	0/6	0/6
Incomplete Freund's adjuvant	female	0/6	0/6	0/6	0/6
BSA, 40 mg[b]	female	2/5	2/5	4/5	4/5
BSA, 2 mg[b]	female	2/4	2/4	2/4	2/4
BSA, 20 mcg[b]	female	2/4	2/4	2/4	2/4
Control, no injection	male	0/16	0/16	0/16	0/16
Control, no injection	female	0/16	0/16	0/16	0/16

[a]Number of positive mice/total number of mice immunized.

[b]Only 1 injection of BSA was given; animals were bled when those in all other groups were bled.

was performed 1 week later. All of the sera were examined for anti-DNP antibody activity with the calf and rabbit thymus DNP spot tests. It was found that the serum of all mice that were induced to produce antibodies that reacted with the calf thymus DNP spots also contained antibodies that reacted with the rabbit thymus DNP spots. In contrast, none of the uninjected control mice developed anti-DNP antibody. A portion of the mice in each group had anti-DNP antibodies in their sera after 1 immunization, with the exception of the females that were injected with calf thymus DNA in complete Freund's adjuvant, and both males and females that were injected with incomplete Freund's adjuvant. The serologic results obtained with sera collected after the second injection indicated that the number of positive mice either remained the same or increased. The only exception to this observation was the group of male mice that was injected with denatured calf thymus DNA in complete Freund's adjuvant. Four of 5 of these mice produced anti-DNP antibodies after 1 injection but only 3 of the 4 were positive after the second injection. In only 1 group did all the mice produce anti-DNP antibodies. This was the group composed of females that were injected with calf thymus DNP in complete Freund's adjuvant. The groups of mice that were given 2 injections of incomplete Freund's adjuvant did not become positive. Complete Freund's adjuvant, however, either alone or in combinations with other antigens, appeared to be as effective as any of the other antigens injected. No sex differences could be detected.

Injection of 8-month-old Seronegative DBA/1J Mice with Various Substances

Groups of 8-month-old retired-breeder DBA/1J mice were injected

with the same antigen preparations used with the A/J mice in the preceding experiment. It had been shown previously that aging DBA/1J did not produce anti-DNP antibodies spontaneously (Table 19). It can be concluded from the results shown in Table 25 that these mice were not induced to produce anti-DNP antibodies under the experimental conditions used. In another experiment, 6 male and 6 female seronegative DBA/1J mice that were 20 months old were given 2 injections of calf thymus DNP in complete Freund's adjuvant 1 week apart. Sera were obtained 1 week after each injection. Each animal was bled before it was given the second injection. None of these sera contained detectable anti-DNP antibodies. It can be concluded from the results of these experiments that DBA/1J mice do not produce anti-DNP antibodies spontaneously and they cannot be induced to produce them after specific or non-specific immunizations.

Cell Transfer Studies

Transfer of Anti-DNP Antibody Production to 4-month-old A/J Mice with Spleen Cells from Isogenic Donors

In a preliminary experiment, male and female mice were injected with spleen cells derived from positive or negative 11-month-old donors. All recipients lacked anti-DNP antibody activity before cell transfer. Each animal was bled on a weekly schedule for 7 weeks after the cell transfer. The results obtained with female recipients are shown in Table 26. Four of 6 females injected with viable cells from positive females had anti-DNP antibody activity in their sera 1 week after cell transfer. In only 1 of these mice (number 5) did the

TABLE 25

SEROLOGIC RESULTS FOLLOWING IMMUNIZATION OF 8-MONTH-OLD DBA/1J MICE LACKING ANTI-DNP ANTIBODY ACTIVITY

Antigen	Sex	Sera reactive with calf (CT) and rabbit (RT) thymus DNP spots after:			
		1 injection		2 injections	
		CT DNP	RT DNP	CT DNP	RT DNP
CT DNP	male	0/6[a]	0/6	0/6	0/6
CT DNP	female	0/6	0/6	0/6	0/6
CT DNP in complete Freund's adjuvant	male	0/6	0/6	0/6	0/6
CT DNP in complete Freund's adjuvant	female	0/6	0/6	0/6	0/6
CT DNA in complete Freund's adjuvant	male	0/6	0/6	0/6	0/6
CT DNA in complete Freund's adjuvant	female	0/6	0/6	0/6	0/6
Denatured CT DNA in complete Freund's adjuvant	male	0/6	0/6	0/6	0/6
Denatured CT DNA in complete Freund's adjuvant	female	0/6	0/6	0/6	0/6
Complete Freund's adjuvant	male	0/6	0/6	0/6	0/6
Complete Freund's adjuvant	female	0/6	0/6	0/6	0/6
Incomplete Freund's adjuvant	male	0/6	0/6	0/6	0/6
Incomplete Freund's adjuvant	female	0/6	0/6	0/6	0/6
BSA, 40 mg[b]	female	0/6	0/6	0/6	0/6
BSA, 2 mg[b]	female	0/6	0/6	0/6	0/6
BSA, 20 mcg[b]	female	0/6	0/6	0/6	0/6
Control, no injection	male	0/12	0/12	0/12	0/12
Control, no injection	female	0/12	0/12	0/12	0/12

[a] Number of positive mice/total number of mice immunized.

[b] Only 1 injection of BSA was given; animals were bled when those in all other groups were bled.

TABLE 26

ANTI-DNP ANTIBODY ACTIVITY IN 4-MONTH-OLD FEMALE A/J MICE AFTER INJECTION OF SPLEEN CELLS DERIVED FROM POSITIVE OR NEGATIVE 11-MONTH-OLD ISOGENIC DONORS

Material injected	Number of recipient	Anti-DNP antibody activity[a] in recipients							
		Before transfer	Weeks after transfer						
			1	2	3	4	5	6	7
Positive viable female cells[b]	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	+++	++	++	++	+++	+++	+++
	4	-	++	+	+	+	++	++	++
	5	-	++	+	-	-	-	-	-
	6	-	+++	++	+	+	++	++	++
Negative viable female cells[c]	7	-	-	-	-	-	-	-	-
	8	-	++	+	+	+	+	++	++
	9	-	-	-	-	-	+	+	++
	10	-	-	-	-	+	+	+	+
	11	-	-	-	-	-	-	-	-
	12	-	+++	+++	+++	+++	+++	+++	+++
Negative lysed female cells	13	-	++	-	-	-	-	-	-
	14	-	+++	++	++	+++	++	+++	++
	15	-	-	-	-	-	-	-	-
	16	-	+++	++	++	+++	+++	+++	++
	17	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-
Medium 199	19	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

[b]6.6×10^6 viable mononuclear cells.

[c]7.0×10^6 viable mononuclear cells.

anti-DNP antibody level decrease with time. Tests with the serum of mouse number 1 in this group were negative for 3 weeks following the spleen cell injection, but all sera collected after the third week were positive. Mouse number 2 remained negative during the entire observation period. Two of the 6 female mice injected with spleen cells from negative animals demonstrated anti-DNP antibodies in their sera 1 week later. These animals remained positive. Two other mice in this group, numbers 9 and 10, became positive at the fifth and fourth weeks respectively. Of the 6 females injected with the lysate of the negative spleen cell suspension, 3 had anti-DNP antibody activity in their sera 1 week later. Two of these mice (number 14 and number 16) remained positive while number 13 reverted to negative. None of the control mice injected with Medium 199 developed anti-DNP antibody activity during the experimental period.

The serologic changes seen in the sera of male recipients are shown in Table 27. It can be seen that the results obtained with the animals which were injected with spleen cells obtained from positive males were similar to those seen with female recipients (Table 26). Three of the males had anti-DNP antibody in their sera 1 week after cell transfer and 2 others became positive by the second week. Three of these 5 mice (numbers 1, 2, and 6) remained positive. Number 3 became negative after the second week and finally became consistently positive at the fifth week. Number 4 reverted to negative at the third week. Number 5 was negative throughout. None of the males injected with viable cells from negative donors developed anti-DNP antibodies and only one of the group injected with a lysate of these cells became

TABLE 27

ANTI-DNP ANTIBODY ACTIVITY IN 4-MONTH-OLD MALE A/J MICE AFTER INJECTION OF SPLEEN CELLS DERIVED FROM POSITIVE OR NEGATIVE 11-MONTH-OLD ISOGENIC DONORS

Material injected	Number of recipient	Anti-DNP antibody activity[a] in recipients							
		Before transfer	Weeks after transfer						
			1	2	3	4	5	6	7
Positive viable male cells[b]	1	-	++	+++	++	+	++	+++	++
	2	-	+++	++	++	+++	++	+++	+++
	3	-	-	+	-	-	+	+	+
	4	-	+	+	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	+++	+	+	+	+	+
	7	-	-	-	-	-	-	-	-
Negative viable male cells[c]	8	-	-	0	0	0	0	0	0
	9	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	0	0	0
	12	-	-	-	-	-	-	-	-
Negative lysed male cells	13	-	+++	++	+++	++	++	+	+
	14	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-
	17	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-
Medium 199	19	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-

[a] Determined with calf thymus DNP spot test.

[b] 7.8×10^6 viable mononuclear cells.

[c] 7.2×10^6 viable mononuclear cells.

0 = animal died.

positive. The controls that were injected with Medium 199 did not produce anti-DNP antibodies during the experimental period.

In further study of the ability to transfer antibody production to DNP with spleen cells, groups of 4-month-old female A/J mice were injected with a suspension of viable spleen cells derived from 9-month-old positive or negative female donors, or a lysate of 1 of these preparations. The results of this experiment are shown in Table 28. None of the recipient's sera, obtained the day before injection, contained anti-DNP antibodies. The results obtained with mice injected with viable positive cells, viable negative cells, or lysed negative cells appeared to be similar to those seen in earlier experiments (Table 22) with 11-month-old donors. Certain mice in each group had anti-nuclear antibody activity in their sera 1 week after transfer, some became positive in subsequent weeks, and some remained negative throughout the experiment. Seven mice were injected with Medium 199. None of these mice had anti-DNP antibody activity in their sera.

Transfer of Anti-DNP Antibody Production to 7-week-old A/J Mice with Spleen Cells from Isogenic Donors

Groups of 6 female and 6 male mice (7 weeks old) were injected with a suspension of viable or lysed spleen cells from positive or negative 11-month-old donors. As can be seen in Table 29, DNP antibodies could not be detected in any of the recipients. Identical results were also obtained with the male recipients.

Injection of Seropositive 9-month-old A/J Mice with Thymus Cells Derived from 4-week-old Isogenic Donors

Female and male retired-breeder mice that had developed anti-DNP

TABLE 28

ANTI-DNP ANTIBODY ACTIVITY IN 4-MONTH-OLD FEMALE A/J MICE AFTER INJECTION OF SPLEEN CELLS DERIVED FROM POSITIVE OR NEGATIVE 9-MONTH-OLD ISOGENIC FEMALE DONORS

Material injected	Number of recipient	Anti-DNP antibody activity[a] in recipients						
		Before transfer	Weeks after transfer					
			1	2	3	4	5	6
5.4×10^6 viable positive cells	1	-	-	+	+++	+++	+++	+++
	2	-	++	+++	+++	+++	+++	+++
	3	-	-	-	-	-	-	-
	4	-	-	++	++	+	+	+
	5	-	+	+	+	+	++	++
lysed positive cells	6	-	+	-	-	-	-	-
	7	-	-	+	+	+++	+++	+++
	8	-	-	-	-	-	-	-
	9	-	+	+++	+++	+++	+++	+++
	10	-	-	++	+	-	-	-
6.1×10^6 viable negative cells	11	-	-	-	+	+++	+++	++
	12	-	-	-	-	-	-	-
	13	-	+	+	++	++	++	++
	14	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-
lysed negative cells	16	-	-	-	-	-	-	-
	17	-	+++	+++	++	++	++	++
	18	-	-	-	-	-	-	-
	19	-	-	-	-	-	-	-
	20	-	+	+	+	+	+	+
Medium 199	21	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-
	27	-	-	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

TABLE 29

ANTI-DNP ANTIBODY ACTIVITY IN 7-WEEK-OLD FEMALE A/J MICE AFTER INJECTION OF SPLEEN CELLS DERIVED FROM POSITIVE OR NEGATIVE 11-MONTH-OLD ISOGENIC DONORS

Material injected	Number of recipient	Anti-DNP antibody activity[a] in recipients							
		Before transfer	Weeks after transfer						
			1	2	3	4	5	6	7
Positive viable female cells[b]	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
Negative viable female cells[c]	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-
Negative lysed female cells	13	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-
	17	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-
Medium 199	19	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-

[a] Determined with calf thymus DNP spot test.

[b] 7.0×10^6 viable mononuclear cells.

[c] 7.1×10^6 viable mononuclear cells.

antibodies spontaneously were injected with a suspension of viable female or male thymus cells. All of the recipients had +++ levels of anti-DNP antibody activity initially. The sera used to make this determination were obtained from the mice the day before they were to be injected. Post-injection bleedings were made at 1 and 2 weeks. It will be seen (Table 30) that a significant decrease in the serum anti-DNP antibody activity occurred during the test period (compared with controls) in female mice numbers 6, 18, 32, and 35 and in male mice numbers 10, 13, and 14. In general, the anti-DNP antibody level decreased in 73% of the recipients. Nine female and 11 male seropositive controls (uninjected) showed no significant alteration in serum antibody level over a 4-week observation period (Table 31).

Injection of Seropositive 9-month-old A/J Female Mice with Thymus Cells Derived from 7-week-old Isogenic Donors

Another experiment was performed in which 9-month-old seropositive (+++) retired-breeder mice were injected with thymus cells derived from young mice. The donors of the thymuses were 7 weeks old instead of 4 weeks old as in the previous experiment. The results are shown in Table 32. Three recipient mice, number 29, 36, and 46, were observed to have a decrease in anti-DNP antibody activity during the observation period. In contrast, mice numbers 28 and 38 showed no significant consistent change in the level of their anti-DNP antibody activity.

Injection of Seropositive 18-month-old A/J Mice with Thymus Cells Derived from 4-week-old Isogenic Donors

It seemed of interest to determine if anti-DNP antibody activity

TABLE 30

ANTI-DNP ANTIBODY ACTIVITY IN SEROPOSITIVE 9-MONTH-OLD A/J MICE INJECTED WITH ISOGENIC THYMUS CELLS DERIVED FROM 4-WEEK-OLD DONORS

Mouse number	Sex	Anti-DNP activity[a] in recipients		
		Before transfer	Weeks after transfer	
			1	2
3	female[b]	+++	+++	+++
6	female	+++	-	-
18	female	+++	-	-
30	female	+++	++	++
32	female	+++	+	+
35	female	+++	+	+
3	male[c]	+++	+	++
9	male	+++	+	++
10	male	+++	-	-
13	male	+++	+	+
14	male	+++	+	+

[a]Determined with calf thymus DNP spot test.

[b]All females injected with 4.7×10^6 cells from female donors.

[c]All males injected with 4.4×10^6 cells from male donors.

TABLE 31

CONSISTENCY OF ANTI-DNP ANTIBODY ACTIVITY IN SERA OF SEROPOSITIVE 9-MONTH-OLD A/J MICE[a]

Mouse number	Sex	Weeks on which sera were collected			
		1	2	3	4
4	female	+++	+++	+++	+++
22		+++	+++	+++	+++
25		+++	+++	+++	+++
29		+++	+++	+++	+++
31		+++	++	+++	+++
36		+++	+++	++	++
38		+++	+++	++	+++
49		+++	++	+++	+++
61		+++	+++	+++	+++
8	male	+++	+++	+++	+++
14		+++	+++	++	+++
20		+++	+++	++	+++
31		+++	+++	+++	+++
66		+++	+++	+++	+++
68		+++	+++	+++	+++
73		+++	+++	+++	+++
83		+++	+++	++	+++
92		+++	+++	+++	+++
93		+++	+++	+++	+++
107		+++	+++	+++	+++

[a]Determined with calf thymus DNP spot test.

TABLE 32

ANTI-DNP ANTIBODY ACTIVITY IN SEROPOSITIVE 9-MONTH-OLD A/J FEMALE MICE INJECTED WITH 6.8×10^6 THYMUS CELLS DERIVED FROM 7-WEEK-OLD ISOGENIC-FEMALE DONORS

Mouse number	Anti-DNP activity[a] in recipients						
	Before transfer	Weeks after transfer					
		1	2	3	4	5	6
28	+++	++	+	+	++	+++	+++
29	+++	+	-	-	-	-	-
36	+++	+	-	-	-	-	-
38	+++	+++	+++	+++	+++	+++	+++
46	+++	-	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

in older A/J mice (retired breeders) would decrease after the injection of thymus cells from young mice. The results of this experiment are presented in Table 33. Of the 19 female recipients used, 8 of them (numbers 3, 9, 11, 13, 17, 19, 35, and 37) appeared to have decreased antibody activity by 1 to 3 weeks after transfer. Two of these animals died before the end of the experiment. The remaining 6 animals all eventually became negative for anti-DNP antibody. In contrast, it can be seen that 4 of the mice (numbers 7, 21, 30, and 38) were not permanently effected by the injection of thymus cells.

Injection of Seronegative 9-month-old A/J Mice with Isogenic Thymus or Spleen Cells and Immunization with Calf Thymus DNP

Since it was observed that the injection of thymus cells into seropositive mice apparently interferes with the continued production of anti-DNP antibodies, it seemed important to determine if the injection of thymus cells into seronegative mice could prevent them from being induced to produce anti-DNP antibody following specific immunization. The results of this study are shown in Table 34. Groups of female and male retired-breeder A/J mice were injected with viable suspensions of thymus or spleen cells, or Medium 199. Two and 3 weeks later all mice were bled and their sera were examined for the presence of anti-DNP antibody activity with the calf thymus DNP spot test. Immediately after all of the mice were bled on the third week they were given an injection of calf thymus DNP in complete Freund's adjuvant. One week later they were bled and reimmunized. One week later they were bled again. None of the sera obtained on the second or third week after the cell suspensions were injected contained anti-DNP antibodies. None

TABLE 33

ANTI-DNP ANTIBODY ACTIVITY IN SEROPOSITIVE 18-MONTH-OLD FEMALE A/J MICE INJECTED WITH 7.0×10^6 ISOGENIC THYMUS CELLS DERIVED FROM 4-WEEK-OLD FEMALE DONORS

Mouse number	Anti-DNP activity[a] in recipients						
	Before transfer	Weeks after transfer					
		1	2	3	4	5	6
2	+++	+	-	-	-	-	-
7	+++	++	+	++	0	0	0
9	+++	+	-	-	-	-	-
11	+++	+	-	0	0	0	0
12	+++	++	-	-	-	-	-
17	+++	+++	+	0	0	0	0
19	+++	+	-	-	-	-	-
21	+++	++	++	+	+++	+++	+++
30	+++	++	++	+	+++	+++	+++
35	+++	+	-	-	-	-	-
37	+++	++	+	+	-	-	-
38	+++	+++	++	++	+++	+++	+++

[a]Determined with calf thymus DNP spot test.

0 = animal died.

TABLE 34

ANTI-DNP ANTIBODY ACTIVITY IN 9-MONTH-OLD A/J MICE INJECTED WITH ISOGENIC THYMUS OR SPLEEN CELLS AND IMMUNIZED WITH CALF THYMUS DNP IN COMPLETE FREUND'S ADJUVANT

Material injected	Number of recipient	Sex	Anti-DNP activity[a] in recipients				
			Before transfer	Weeks after cell transfer		Weeks after DNP immunizations[b]	
				2	3	1	2
4.7×10^6 female thymus cells	9	female	-	-	-	-	-
	10	female	-	-	-	-	-
	20	female	-	-	-	-	-
	24	female	-	-	-	-	-
7.4×10^6 female spleen cells	2	female	-	-	-	-	-
	3	female	-	-	-	-	-
	24	female	-	-	-	-	-
	27	female	-	-	-	-	-
	29	female	-	-	-	-	-
4.2×10^6 male thymus cells	8	male	-	-	-	-	-
	11	male	-	-	-	-	-
	15	male	-	-	-	-	-
	16	male	-	-	-	-	-
5.3×10^6 male spleen cells	26	male	-	-	-	-	-
	27	male	-	-	-	-	-
	28	male	-	-	-	-	-
	30	male	-	-	-	-	-
	31	male	-	-	-	-	-

[a]Determined with calf thymus DNP spot test.

[b]Two injections given; the first, 3 weeks after cell transfer, the second, 1 week later.

TABLE 34--Continued

Material injected	Number of recipient	Sex	Anti-DNP activity[a] in recipients				
			Before transfer	Weeks after cell transfer		Weeks after DNP immunizations[b]	
				2	3	1	2
Medium 199 control	37	female	-	-	-	++	+++
	38	female	-	-	-	+++	+++
	41	female	-	-	-	-	+
	43	female	-	-	-	+++	+++
Medium 199 control	38	male	-	-	-	+++	+++
	42	male	-	-	-	-	-
	43	male	-	-	-	+++	++
	44	male	-	-	-	+	+

[a]Determined with calf thymus DNP spot test.

[b]Two injections given; the first, 3 weeks after cell transfer, the second, 1 week later.

of the mice that received an injection of viable thymus or spleen cells was induced to produce anti-DNP antibodies following immunization with DNP. However, the control mice, injected only with Medium 199, were induced to produce anti-DNP antibodies. Six other controls were not injected. None of these mice became positive spontaneously.

CHAPTER IV

DISCUSSION

The original intent of these studies was to find a strain of isogenic mice that produce anti-nuclear antibody spontaneously. Five pools of serum from each of 17 strains of retired-breeder mice were examined with the indirect immunofluorescent technique for gamma-globulin reactive with calf thymus DNP spots and the nuclei of human peripheral blood leukocytes. The DNP spots were not standardized for serologic tests with mouse serum. They were prepared from a solution containing 1.5 mg/ml of calf thymus DNP. This concentration had been previously determined to be optimal for the detection of anti-DNP antibodies in human SLE sera (Friou, 1964). Positive serologic tests with DNP and leukocyte nuclei were produced only by serum pools from A/J mice (Table 1). Four of the 5 pools of A/J mouse serum were positive in each test. The results obtained with each pool of serum were approximately the same (Table 2). It would appear that the serum of at least 1 donor mouse in each of the positive pools contained gamma-globulin reactive with nuclear material. Although the serum pools from other strains did not contain anti-nuclear factor detectable with these tests, the possibility exists that it could have been present in one or more donor's sera used to make the pools. However, through dilution with negative sera, it would have been undetected with these serologic

tests (Friou and Teague, 1964). All cell nuclei showing immunofluorescence after treatment with serum containing anti-nuclear factor were homogeneous in appearance. This type of pattern has been suggested to be the most predominant one produced by human SLE sera.

Although it was demonstrated that some pools of serum from A/J mice contained anti-nuclear factor activity detectable by the experimental methods used, it was necessary to determine the optimum amounts of DNP and fluorescent anti-mouse gamma-globulin conjugate to be used in serologic tests. The standardization procedure developed for use with human SLE sera was followed (Friou, 1964) and serum samples from individual mice were used. Human serum was also used since it had been used in the original DNP spot test standardization. It was found that calf thymus DNP spots prepared from a solution containing 1.25 mg/ml of DNP gave the desired results (Table 3). This concentration was the lowest in which (1) normal human and mouse sera were negative, (2) a high titer was obtained with a positive human serum, and (3) positive mouse sera gave the greatest intensity of fluorescence. Results of rabbit thymus DNP standardization indicated that a 1.25 mg/ml solution should be utilized to prepare all DNP spots (Table 4).

Experiments concerned with standardization of Lot 1 and Lot 2 fluorescent conjugates indicated that all experiments with both DNP spots (Table 5) and leukocytes should be made using a 1:2 dilution of Lot 1 conjugate and a 1:4 dilution of Lot 2 conjugate.

Non-specific fluorescent staining can be a critical factor in the evaluation of results of immunofluorescent experiments. Absorption of each fluorescent conjugate with A/J mouse serum removed all staining

activity of the conjugates for calf thymus DNP and DNA spots and the nuclei of human peripheral blood leukocytes. These data suggest that fluorescence of serum- and conjugate-treated spots or leukocyte nuclei is the result of the interaction of mouse anti-nuclear factor and antigen and fluorescent rabbit anti-mouse gamma-globulin. Immunoelectrophoresis experiments with mouse sera and Lot 1 and Lot 2 conjugates suggest that both conjugates contained antibody that precipitated only with mouse gamma-globulin.

Serologic tests with starch block eluates after electrophoresis of positive and negative serum pools revealed that the A/J mouse anti-nuclear factor migrated only with the gamma-globulin fraction of mouse serum (Table 6). These results, and the demonstration that the conjugates reacted only with mouse gamma-globulin in immunoelectrophoresis, suggested that anti-nuclear factor in A/J mice is specific antibody. Additional experiments were not performed to determine in which class or classes of the immunoglobulins anti-nuclear antibodies exist. Experiments with human SLE sera have revealed that human anti-nuclear antibody migrates with gamma-globulin in starch block electrophoresis (Holman and Kunkel, 1957) and reacts with specific rabbit anti-human gamma-globulin antibody (Friou, 1958, 1962). More refined experiments have shown that anti-nuclear antibodies may be found in all of the human immunoglobulins (Barnett et al., 1964).

Since most human SLE sera contain anti-DNP antibody and some also have anti-DNA antibody activity, it was of interest to determine if a similar situation prevailed with A/J mice. Individual 8-month-old females were selected on the basis of having a positive or negative

result in the calf thymus DNP spot test (Table 7). Although this was a preliminary experiment, it was found that none of the sera contained antibodies reactive with DNA. Some, but not all mice that were found to have anti-DNP antibody, were positive in the LE cell test. Similar observations have been made in studies with the blood of humans with SLE. A higher percentage of SLE patients are positive in the spot test than in the LE cell test (Friou, 1958; Casals et al., 1964). It has been suggested that negative LE cell tests in SLE patients result, in some instances, from the presence of an inhibitor of LE cell formation in the serum of these patients (Friou et al., 1958). The level of anti-DNP antibody could also be an important factor in LE cell formation by A/J mice. A positive LE cell test was obtained with all mice that were +++ in the DNP spot test. None of the mice which were + or - in the spot test were positive in the LE cell test. The ++ mice were variable in the LE cell test. Since it could be considered that immunofluorescent tests are generally more sensitive than phagocytic tests, it is possible that mice which had low levels of DNP antibody did not have enough antibody to effect the production of detectable LE cells. Although the role of complement in LE cell formation by human SLE sera is controversial, inadequate levels of complement could possibly prevent detectable LE cell formation. However, since all mice with high levels of anti-DNP antibody were positive in the LE cell test, this possibility seems to be remote.

The LE cells appeared to be morphologically similar to human LE cells stained with Wright's stain. As shown in Fig. 3, the nucleus of the polymorphonuclear leukocyte appears to be displaced to a

peripheral location in the cell and apparently surrounds a large, ingested inclusion, probably of nuclear origin. All typical inclusions appeared to be homogeneous and were stained dark pink to light purple. Granular inclusions were not seen. Non-phagocytized, extracellular, homogeneous material was observed routinely in positive LE cell preparations, but not in negative preparations. Rosettes were observed occasionally. They were composed of two or more polymorphonuclear leukocytes which surrounded a homogeneous body. In addition to the reaction with autologous nuclear material, almost all sera containing anti-DNP antibody produced a homogeneous immunofluorescence with the nuclei of mouse, rabbit, and human peripheral blood leukocytes. The lack of species specificity of this factor is similar to the heteroreactivity of human anti-DNP antibody (Friou et al., 1958; Miescher et al., 1961).

The spontaneous appearance of certain anti-nuclear antibodies that react with both heterologous and autologous antigens does not appear to be unique to A/J mice. Similar serologic reactions have been reported to occur in NZB/BL (Bielschowsky et al., 1959), F1 hybrid progeny of NZB/BL and certain other strains of mice (Helyer and Howie, 1961, 1963a, 1963c), C57BL/6J (Friou and Teague, 1964), C57BL and C3H (Norrins and Holmes, 1964), and C57BR mice (Holborow et al., 1965). Anti-nuclear antibody has also been reported to occur in an outbred strain of mice (Norrins and Holmes, 1964) and in dogs (Lewis et al., 1965).

In the initial studies to determine the incidence of anti-DNP antibody activity in retired-breeder A/J mice, it was found that at 8

months of age, 34% of 50 females (Table 8) and 18% of 50 males (Table 9) were positive. The incidence did not increase remarkably during the next 4 weeks. Inspection of these data also indicates that, with one exception, all animals remained positive once they had begun producing anti-DNP antibody. In general, the only change in the estimate of anti-DNP antibody activity (expressed by +++, ++, +, or -) was that of an increase. These data suggest that between 8 and 9 months of age the incidence of anti-DNP antibody activity is greater in females. Data presented in Table 9 confirm this observation. Accumulated serologic data for all untreated 9-month-old mice tested showed the incidence to be 35.8% in females and 23.7% in males. The difference in the incidence in females and males is significant at the 0.01 confidence level ($t = 3.05$). Although the incidence of anti-DNP antibody did not increase between 8 and 9 months of age, experiments with other groups of A/J mice indicated that there is a general tendency for negative retired-breeder A/J mice to develop anti-DNP antibody with increasing age. The incidence of anti-DNP antibody in one group of females increased from 34% at 8 months of age to 63% by 13 1/2 months of age. A similar trend was observed in a group of males (Table 12). It appears that the rate of spontaneous conversion is greater in aging males after 8 months of age, since by 13 1/2 months of age the incidence is approximately the same in both sexes. Only 1 female and 2 males reverted to negative during this period. Therefore, it is assumed that these data reflect a tendency of A/J mice to acquire the ability to produce anti-DNP antibody in the absence of experimental immunization. Additional experiments with other groups of female A/J mice bled at various ages

showed the incidence increases from 34% at 8 months of age to 90% at 20 months of age. This change is highly significant ($p = 0.001$). Experiments with groups of males bled at different ages revealed the same statistically significant trend.

Virgin female and male A/J mice were bled at intervals and their sera were tested for anti-DNP antibody activity. None of the females (Table 15) or males were positive between 1 3/4 and 5 1/2 months of age. At 8 1/4 months of age, 3 of 48 females (6.3%) were positive. By 19 3/4 months of age, 73% of the females were positive. Statistical analysis of serologic data derived from other groups of virgin females (Table 16) and males (Table 17) revealed that the increase in incidence in each sex is significant. Since the percentage of positive 8-month-old virgins (male and female) is lower than the percentage of positive retired-breeder animals of the same approximate age, it appears that the events which allow A/J mice to produce anti-DNP antibody develop at a slower rate in virgin mice. This difference could be due to variables that are inherent in the physiologic and/or environmental conditions of breeder and virgin colonies of mice.

Preliminary studies with the sera of 8-month-old A/J retired breeder mice indicated that anti-DNA antibody was not present in these sera. Additional experiments with the sera of individual A/J retired breeders (female) obtained from bleedings at various intervals during aging indicated that some of these mice eventually gained the capacity to produce anti-DNA antibody (Table 18). None of these animals produced anti-DNA antibody before 12 months of age, but by the time they were 23 months old, 31.6% of the mice were positive in the DNA spot test. The

percentage of these females that had serum anti-DNP antibody at each age tested was very similar to the percentages determined for various age groups of other female A/J mice (Table 13). It would thus appear that some A/J mice develop anti-DNA antibody spontaneously in the absence of experimental immunizations. The rate of spontaneous conversion is apparently delayed in comparison with the rate of conversion to anti-DNP antibody production.

Serologic studies with the serum of individual retired-breeder DBA/1J mice were also made. Anti-DNP antibody was not produced by these mice up to 18 months of age. Members of this strain were therefore selected for the production of F1 hybrids with A/J females, and for controls in immunization experiments.

AJDF1 hybrids (virgins) did not have anti-nuclear antibody in their sera at 8 and 16 months of age. Studies with older hybrids were not performed, therefore, complete analysis of these data cannot be made. However, if it is assumed that anti-nuclear antibody never appeared in the sera of these hybrids during the remainder of their natural life span, the data could then be interpreted to mean that the capacity for anti-DNP antibody production is transmitted by genetic factors, possibly as a recessive character.

In contrast, some ANZBF1 hybrids (virgins) were found to be positive in tests for anti-nuclear antibody. These animals were the progeny of A/J females and NZB/BL males. The incidence of anti-DNP antibody was found to be 49% at 3 1/2 months of age. This figure is very close to the 45% incidence reported to be standard for adult NZB/BL mice (Norrins and Holmes, 1964). Since A/J mice (virgins) did

not show positive tests until after 5 1/2 months of age, and the incidence is not as high in 8-month-old A/J retired breeders as in these hybrids, it appears that the ANZBF1 hybrid could be a valuable strain for studying variables of anti-nuclear antibody production and pathologic conditions that could be associated with the autoimmune state. Although only small numbers of males and females were tested when the animals were 5 1/2 months old, all females (9 of 9) and most males (8 of 9) had antibody reactive with DNP, and most also had anti-DNA antibody. These incidence levels did not change in older animals (11 months old). Since Norrins and Holmes (1964) suggest that the antigen specificity of the antibody produced by NZB/BL mice is for DNP, it appears that the ANZBF1 hybrid's ability to produce anti-DNA antibody was inherited from A/J mice. The results of the present study afford no support for this hypothesis, however, since NZB/BL mice were not examined serologically for anti-DNA antibody. The finding that only one 5 1/2-month-old animal was positive in the LE cell test cannot be explained. LE cell tests have been reported to be positive with both parental strains (Bielschowsky et al., 1959; Friou and League, 1964).

Anti-DNP antibody was also found in the sera of certain 8-month-old C57BL/6J mice (retired breeders). However, tests with pooled sera from mice of this strain were negative. If anti-DNP antibody was present in the serum pools of these mice it apparently was diluted by the sera from negative animals such as to be below the level of sensitivity of the immunofluorescent tests.

The ability of A/J mice to produce anti-DNP antibody, either spontaneously or after immunization, is apparently related to their age

and to their physiologic and genetic status. It was shown that anti-DNP antibody does not appear in the sera of virgin animals before 5 1/2 months of age, and that the incidence of antibody in 8-month-old animals is lower in virgins than in retired breeders. Injections of DNP or DNA into 4 1/2-month-old animals did not induce the production of detectable anti-nuclear antibody (Table 23). Similar treatment of 8-month-old retired breeders which had not developed anti-DNP antibody spontaneously resulted in the formation of antibody reactive with autologous leukocyte nuclei (LE cell formation), calf thymus DNP, and the nuclei of human leukocytes. Uninjected controls did not produce anti-DNP antibody during the experimental period. These data suggest that the ability of A/J mice to produce antibody reactive with both autologous and heterologous DNP could possibly result from failure of tolerance mechanisms during the aging process, a theory proposed by Burnet (1958) to explain autoimmunity in general. Immunization of mice that were producing anti-DNP antibody as the result of spontaneous conversion did not result in the appearance of anti-DNA antibody. Since anti-DNA antibody does not appear spontaneously in A/J mice until after 5 1/2 months of age, it appears that the potential to respond immunologically to DNP is distinct from the potential to respond to DNA, and that tolerance to DNP is lost first.

Since, in the latter experiments, both DNP and DNA were combined with complete Freund's adjuvant for injection, additional experiments were performed in an attempt to clarify the role of the adjuvant in the production of anti-DNP antibody (Table 24). Each serum was tested for antibody activity for both calf thymus and rabbit thymus

DNP. All sera that gave positive reactions with calf thymus DNP were also positive with rabbit thymus DNP. Thus, the anti-DNP antibody which appeared following the immunizations was apparently not directed against an antigenic component unique to calf thymus DNP. The antibody appears to have the same type of auto- and hetero-reactivity as that which appears spontaneously in both A/J mice and human SLE. Although anti-DNP antibody was induced in 8 of 19 mice (males and females) after injections of aqueous solutions of DNP, injections of complete Freund's adjuvant alone into other animals produced a similar serologic response. Animals injected with incomplete Freund's adjuvant did not develop anti-DNP antibody. Thus, these experiments did not completely identify the antigen or antigens to which these mice responded. Immunologic responses could have been directed against one or more of the following: (1) the DNP of the mycobacteria in the adjuvant, (2) the calf thymus DNP, (3) trace amounts of protein in the DNA preparations, (4) DNP which could have been a contaminant in BSA, or (5) autologous DNP released from damaged tissue either during granuloma formation or by circulating antigen-antibody (BSA-anti-BSA) complexes.

Further evidence that the ability of mice to produce antinuclear antibody might be due to a characteristic that exists only in certain strains was derived from immunization studies with DBA/1J retired-breeder mice that were 8 and 20 months old. None of the mice in these groups produced anti-DNP antibody after either specific or non-specific immunizations.

The transfer of lymphoid cells of the spleen and lymph nodes

to normal animals has been used extensively to demonstrate that such tissues contain cells with immunologic memory or potential for both immediate and delayed immunologic responses to various antigens. However, only recently has a spontaneously occurring autoimmune state been transferred with viable lymphoid cells. Holmes et al. (1961) reported that 4- to 6-week-old NZB/BL mice developed a fatal autoimmune hemolytic anemia after being injected with viable spleen cells derived from Coomb's positive adult NZB/BL mice. Although it had been established that NZB/BL mice also develop anti-nuclear antibody spontaneously, this parameter was not measured in either the donors or the recipients. However, in the present study, certain experiments with A/J mice established that recipients of spleen cells from retired breeders developed anti-DNP antibody activity. This activity was produced by most 4-month-old recipients (Tables 26, 27, and 28). The presence or absence of anti-DNP antibody in the sera of the retired-breeder donors did not appear to be a factor which determined the type of serologic response exhibited by the recipients. More than one-half of the recipients in most groups became positive within 2 weeks after the injection of cells or homogenates derived from donors that had serum anti-DNP antibody activity. The continued presence of this antibody in most recipients for the duration of the experimental period (7 weeks) suggests that the majority of the anti-DNP antibody detected in the serologic tests was derived from active antibody synthesis in the recipient rather than from passively transferred antibody. Additional evidence for this concept was the observation that many animals injected with spleen cells obtained from mice which did

not have detectable anti-DNP antibody in their sera also developed anti-DNP antibody. Lysates of cells from seropositive or seronegative donors appeared to be as effective as viable cells in converting the 4-month-old recipients.

The age of the recipient apparently influences the synthesis of anti-DNP antibody since it was found the 7-week-old mice did not have anti-DNP antibody in their sera following the injection of viable cells from seropositive or seronegative retired breeders. Homogenates were also ineffective. These data indicate that the younger recipients did not provide a suitable environment for the synthesis of anti-DNP antibody.

The possibility that the appearance of anti-DNP antibody in the sera of the A/J recipients could have been due to an immune response directed against nuclear materials present in the cell free or viable cell suspensions appears remote in view of the finding that 9-month-old recipients did not develop anti-DNP antibody following the injection of viable thymus or spleen cells from 7-week-old donors (Table 34). The lack of anti-DNP antibody production by immunized 4 1/2-month-old A/J mice adds additional support to this concept.

Although the injection of homogenates of spleen cells from Coomb's positive NZB/BL mice did not induce Coomb's negative mice to produce autoantibody (Holmes et al., 1961), other investigators have reported specific antibody production by non-immune animals or lymphoid cells after treatment with cell-free lymphoid material from immunized animals. Mannick and Egdahl (1962) found that an ribonucleic acid (RNA) extract of immune rabbit lymphoid cells, when incubated

in vitro with normal rabbit lymphoid cells and then injected into normal animals, induced the recipients to develop the specific homograft immunity possessed by the donor. Treatment of the extract with RNase destroyed its transfer capacity. RNA from normal animals was incapable of transfer. "Immune RNA" injected intravenously without prior incubation with cells in vitro was also ineffective (Mannick, 1964). RNA-treated cells were effective in inducing a homograft immunity when given up to 48 hours after skin transplantation (Mannick, 1964), suggesting that both antigen and specific RNA could be involved in the immune response. Additional experiments revealed that ribosomal sites for RNA could be completely saturated with donor RNA, and that this prevented the appearance of specific immunity in the same test system used in previous experiments (Mannick and Egdahl, 1964).

RNA, purified from immune mouse spleens, has also been reported to induce certain normal mouse spleen cells to produce sheep erythrocyte hemolysins in vitro (Cohen and Parks, 1964). Antibody was not produced when the RNA extract was treated with RNase. Friedman (1964) performed similar experiments and obtained essentially the same results. In addition, he found that the RNA extract did not contain detectable antigen and that pretreatment of the prospective donors with Actinomycin-D inhibited the induction of antibody formation by subsequently extracted RNA. This was interpreted by the author to indicate that the production of specific new RNA is essential for antibody formation by the non-immune animal, but it is not known whether the RNA extracts contained an antibody coding mechanism, an immunogenic antigen-nucleic acid complex, or an antibody precursor which may have induced immune

activity by non-immune cells.

Although Weiler (1964) reported that the injection of homogenates of mouse immune spleen cells did not induce normal isogenic animals to produce anti-phage antibody, Friedman (1963) found that specific anti-bacterial antibody was produced by normal rabbits following the intravenous injection of crude preparations of nuclei, mitochondria, DNP, or RNA from immune rabbit spleen.

A RNase sensitive filtrate of immune rat lymphoid cells has been reported to induce specific antibody synthesis by normal rat lymph node cells in tissue culture (Fishman, 1961) and in Millipore chambers placed in the peritoneal cavity of normal animals (Fishman and Adler, 1963). Studies with tritiated cytidine revealed that RNA becomes localized intracellularly in both lymphocytes and macrophages. Lymphocytes surrounding macrophages contained more RNA than did isolated lymphocytes (Fishman and Adler, 1963). Aronson (1963) has reported observing tritium-labeled RNA within cytoplasmic bridges formed between macrophages. Observations of electron micrographs of spleen and lymph nodes of immune rabbits have also revealed cytoplasmic bridge formation between macrophages and plasma cells, as well as the passage through them of particles which appeared to be ribosomes (Schoenberg et al., 1964). Experiments tritium-labeled RNA also indicate that the RNA of lymphoid cells has a long half life (Miller, 1964). The author found that restimulation of immune rats did not increase the amount of stable RNA in plasma cells. He suggested that a single molecule of messenger RNA might serve repeatedly as a template for a large amount of specific coded antibody protein. Campbell and Garvey (1961)

have suggested that antigens are catabolized immediately by the host and are retained for long periods of time as fragments which are intimately associated with RNA of lymphoid cells. They speculated that the incomplete breakdown of antigens and persistence of molecular fragments indicates the existence of linkages in "foreign" molecules which are not completely susceptible to total degradation by host cellular enzymes. This lack of complete antigen catabolism was suggested to be the stimulus for antibody formation. The authors proposed that, under these conditions, even autologous antigens could become immunogenic if they developed a "foreign" configuration, or if abnormal intracellular enzyme caused incomplete breakdown of a potentially immunogenic agent. Thus, the ability to induce animals or isolated lymphoid cells to produce specific antibody following treatment with cell-free material appears to be well established under certain conditions. Although specific RNA appears to be involved in the process, other factors have not yet been excluded.

Burnet and Holmes (1964) have proposed that the thymus is the primary organ involved in the homeostasis mechanism which inhibits the expression of autoimmunity. The present experiments with A/J mice appear to confirm this hypothesis, and also indicate that the spleen has a similar function. A temporary decrease in the level of anti-DNP antibody was observed in one group of mice following the injection of thymus cells from young A/J mice (Table 30). Other experiments indicated that the disappearance of anti-DNP antibody from the sera of mice treated with thymus cells was not transient. Most members of two groups of mice remained negative for a 6-week period (Tables 32 and 33).

Experiments were not performed to determine whether anti-DNP antibody would eventually reappear in the sera of these mice. Other experiments indicated that both the thymus and the spleen of young A/J mice have the capacity to influence anti-DNP antibody synthesis. When sero-negative, retired-breeder mice were injected with suspensions of viable thymus or spleen cells from 7-week-old A/J mice, and were subsequently immunized with calf thymus DNP, none of them produced anti-DNP antibody. In contrast, controls that did not receive a prior injection of thymus or spleen cells produced anti-DNP antibody after immunization. These findings indicate that certain thymus and spleen cells of young A/J mice revived the normal homeostatic mechanisms in old recipients.

The thymus is known to influence normal immunologic responses in both neonatal and adult animals (reviewed by Good et al., 1965) by hormones, by the seeding of cells to the spleen and lymph nodes to influence immune responses in situ, or by allowing cells from other lymphoid organs to gain entrance into the thymus and become immunologically competent (Miller, 1962). Suggestions have also been advanced that the post-thymectomy wasting syndrome in certain strains of mice (DeVries et al., 1964) and in rabbits (Sutherland et al., 1965; Kellum et al., 1965) could be partially the result of autoimmune processes. The studies with rabbits indicated that the appendix and the spleen also contribute to the theoretical homeostasis mechanisms. However, since other investigators (East and Parrott, 1965) insist that autoimmunity did not occur in three strains of neonatally thymectomized mice, it is possible that only certain strains of animals are

susceptible to demonstrable autoimmunity following thymectomy.

With respect to A/J mice, it appears that these animals possess an inherent trait which allows aging animals to produce auto-reactive anti-DNP and anti-DNA antibodies. The absence of this type of anti-nuclear antibody in several other strains of mice suggests that the ability to produce this antibody could be governed by genetic mechanisms. Preliminary experiments with F1 hybrids indicated that genetic transmission could involve a recessive trait.

Cells capable of anti-DNP antibody production were not present in the spleen of 7-week-old mice, but they were present in 8-month or older animals. Thus, these specific cells appear during aging and could arise by somatic mutation, as proposed by Burnet (1958). A mechanism of immunologic homeostasis whose function is to inhibit the biological activity of cells capable of autoimmune responses (Burnet, 1958, 1962, 1963, 1964, 1965) is apparently functional in A/J mice. It apparently decreases in effectiveness with increasing age. Although 4 1/2-month-old mice could not be induced to produce anti-DNP antibody by immunization, indicating that the inhibitor system was functionally intact in these animals, the injection of 4-month-old animals with viable cells or homogenates resulted in anti-DNP antibody production. The existence or induction of cells capable of resisting homeostatic controls could have been responsible for this response. Alternatively, the injection of dispersed spleen cells into the peritoneal cavity could have diminished the effectiveness of the inhibitor system long enough to allow cells to escape permanently its influence.

The nature of the factor or factors in the cell lysates

responsible for the appearance of antibody in the recipients is unknown. Treatment of the material with DNase, RNase, and proteinases might indicate whether any of these substances is involved. Since it appears that the host response following the injection of the spleen cell homogenates was not an immune response directed against the nuclear materials contained therein, other biologic mechanisms must be responsible for the appearance of anti-DNP antibody under the experimental conditions used in the present study.

CHAPTER V

SUMMARY

Immunofluorescent techniques revealed the presence of gamma-globulin reactive with calf thymus DNP spots and the nuclei of human peripheral blood leukocytes in serum pools of 8-month-old retired-breeder A/J mice. Similar serologic tests with serum pools from 16 other strains of mice were negative. The factor in the serum pools migrated with gamma-globulin in zone electrophoresis and reacted with specific rabbit anti-mouse gamma-globulin.

Serologic studies of sera of individual 8-month-old A/J mice (retired breeders) revealed anti-nuclear factor reactive with DNP, but not with calf thymus DNA, was present only in certain animals. These data indicated that the factor is probably a specific antibody. Most sera containing anti-DNP antibody also reacted with nuclei of mouse (A/J), rabbit, or human leukocytes. Most mice that had anti-DNP antibody produced LE cells.

Young virgin A/J mice did not produce anti-DNP antibody before 5 1/2 months of age, and the incidence in these mice at 8 1/2 months of age (females, 12.5% positive; males, 10% positive) appeared to be lower than that of 8-month-old retired breeders (females, 34% positive; males, 20% positive).

A statistically significant sexual difference in the incidence

of anti-DNP antibody was found in 9-month-old A/J retired breeders (females, 35.8% positive; males, 23.7% positive). The incidence of anti-DNP antibody in each sex was found to increase significantly with age, irrespective of whether the animals were virgins or retired breeders, and the sexual difference seemed to disappear with age.

Anti-DNP antibody, but not anti-DNA antibody, was also found in the serum of 25% of C57BL/6J mice (8-month-old retired breeders). However, it was not produced by either AJDF1 hybrids up to 16 months of age or DBA/1J retired breeders up to 18 months of age.

Although anti-DNA antibody was not detected in the sera of 8- to 18-month-old A/J retired breeders, 31.6% had anti-DNA antibody at 23 months of age. In contrast, most ANZBF1 hybrids had both anti-DNP and anti-DNA antibody at 5 1/2 months of age.

Immunization with calf thymus DNP or DNA in complete Freund's adjuvant did not induce 4-month-old virgin A/J mice to produce anti-nuclear antibody. Similar treatment of seronegative 8-month-old retired breeders induced the formation of antibody reactive with both calf and rabbit thymus DNP and autologous nuclei (LE cells), but not with DNA. Non-specific immunization or complete Freund's adjuvant alone was also effective in inducing the production of anti-DNP antibody. Untreated seronegative controls did not develop anti-DNP antibody spontaneously during the experimental periods.

Age and other factors apparently influenced the appearance of anti-DNP antibody in A/J mice that were injected with viable spleen cells or lysates derived from seropositive or seronegative isogenic donors. Some 4-month-old recipients developed anti-DNP antibody, but

none of the 7-week-old recipients became seropositive. When viable thymus cells from 4-week-old A/J donors were transferred to groups of seropositive isogenic recipients (retired breeders), anti-DNP antibody decreased or disappeared from most recipients. Untreated seropositive controls did not show this variation. Seronegative 9-month-old recipients of thymus or spleen cells from 4-week-old donors did not develop anti-DNP antibody, and it could not be induced in these animals by specific immunization.

These observations support the hypothesis that in A/J mice (1) autoimmunity to DNP and DNA occurs as a result of failure of normal homeostatic mechanisms during aging, (2) cells responsible for this control are found in the thymus and spleen of young mice, and (3) the ability of mice to produce anti-nuclear antibody is apparently transmitted by genetic mechanisms.

BIBLIOGRAPHY

Aisenberg, A. C. 1959 Studies on the mechanism of the lupus erythematosus (LE) phenomenon. J. Clin. Invest. 38:325-333.

Aronson, M. 1963 Bridge formation and cytoplasmic flow between phagocytic cells. J. Exp. Med. 118:1083-1088.

Bardawil, W. A., Toy, B. L., Galins, N., and Bayles, T. B. 1958 Disseminated lupus erythematosus, scleroderma, and dermatomyositis as a manifestation of sensitization to DNA-protein. I. An immunohistochemical approach. Am. J. Pathol. 34:607-629.

Barnett, E. V., Condemi, J. J., Leddy, J. P., and Vaughan, J. H. 1964 $Gamma_2$, $gamma_{1A}$, and $gamma_{1M}$ antinuclear factors in human sera. J. Clin. Invest. 43:1104-1115.

Beck, J. S. and Rowell, N. R. 1963 Transplacental passage of antinuclear antibody. Lancet 1:134-136.

Bencze, G., Cserhati, I., Kovacs, J., and Tiboldi, F. 1958 Production of LE cells in vivo by transfusion of systemic lupus erythematosus plasma. Ann. Rheum. Dis. 17:426-428.

Bencze, G. and Ludanyi, M. 1960 Production of lupus erythematosus (LE) cells in the dog by the transfusion of systemic lupus erythematosus plasma. Ann. Rheum. Dis. 19:48-51.

Bielschowsky, M., Helyer, B. J., and Howie, J. B. 1959 Spontaneous hemolytic anaemia in mice of the NZB/BL strain. Proc. Univ. Otago Med. School 37:9-11.

Blix, V., Iland, C. N., and Stacey, M. 1954 The serological activity of deoxypentosenucleic acids. Brit. J. Exp. Pathol. 35:241-251.

Boyden, S. 1964 Autoimmunity and inflammation. Nature 201:200-201.

Burch, P. R. J. and Rowell, N. R. 1963 Autoimmunity. Etiological aspects of chronic discoid and systemic lupus erythematosus, systemic sclerosis, and Hashimoto's thyroiditis. Some immunological applications. Lancet 2:505-513.

Burch, P. R. S. and Rowell, N. R. 1965 Systemic lupus erythematosus. Etiologic aspects--theory. Am. J. Med. 38:793-801.

Burnet, M. 1958 The clonal selection theory of acquired immunity. Vanderbilt University Press, Nashville.

Burnet, M. 1962 Autoimmune disease--experimental and clinical. Proc. Roy. Soc. Med. 55:619-626.

Burnet, M. 1963 Experimental production of auto-antibodies or of auto-immune disease. Brit. Med. Bull. 19:245-250.

Burnet, M. 1964 Pathology of the thymus with special reference to auto-immune disease. Northwest Med. 63:599-601.

Burnet, M. 1965 Somatic mutation and chronic disease. Brit. Med. J. 1:338-342.

Burnet, F. M. and Holmes, M. C. 1964 Thymic changes in the mouse strain NZB in relation to the autoimmune state. J. Pathol. Bacteriol. 88:229-241.

Burnet, F. M. and Holmes, M. C. 1965 The natural history of the NZB/NZWF1 hybrid mouse. A laboratory model of systemic lupus erythematosus. Aust. Ann. Med. 14:185-191.

Butler, V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S., and Bendich, A. 1962 Purine-specific antibodies which react with deoxyribonucleic acid. Proc. Nat. Acad. Sci. U. S. 48:1597-1602.

Campbell, D. H. and Garvey, J. S. 1961 The fate of foreign antigen and speculations as to its role in immune mechanisms. Lab. Invest. 10:1126-1150.

Carrera, A. E., Reid, M. V., and Kurnick, N. B. 1954 Differences in susceptibility of polymorphonuclear leukocytes from several species to alteration by systemic lupus erythematosus serum: application to a more sensitive LE phenomenon test. Blood 9:1165-1171.

Casals, S. P., Friou, G. J., and Myers, L. L. 1964 Significance of antibody to DNA in systemic lupus erythematosus. Arth. Rheum. 7:379-390.

Casals, S. P., Friou, G. J., and Teague, P. O. 1963 Specific nuclear reaction pattern of antibody to DNA in lupus erythematosus sera. J. Lab. Clin. Med. 62:625-631.

Cepellini, R., Polli, E., and Calada, F. 1957 A DNA-reacting factor in the serum of a patient with disseminated lupus erythematosus. Proc. Soc. Exp. Biol. Med. 96:572-574.

Channing, A. A., Kasuga, T., Horowitz, R. E., Dubois, E. L., and Demopoulos, H. B. 1965 An ultrastructural study of spontaneous lupus nephritis in the NZB/BL-NZW mouse. Am. J. Pathol. 47: 677-694.

Christain, C. L., DeSimone, A. R., and Abruzzo, J. L. 1964 Anti-DNA antibodies in hyperimmunized rabbits. J. Exp. Med. 121: 309-321.

Cohen, E. P. and Parks, J. J. 1964 Antibody production by nonimmune spleen cells incubated with RNA from immunized mice. Science 144:1-2.

Coons, A. H. and Kaplan, M. H. 1950 Localization of antigen in tissue cells. II. Improvement in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91:1-13.

Coulter, J. S. and Ellem, K. A. O. 1961 Antigenicity of deoxyribonucleic acids from mouse liver and from the Ehrlich ascites tumor. Nature 190:550-551.

Dameshek, W. 1963 Genetic studies of systemic lupus erythematosus. Arth. Rheum. 6:513-523.

Davis, E. V., Glover, F. L., and McLimans, W. F. 1958 Proliferation of human amnion cells (FL strain) in sumerged culture. Proc. Soc. Exp. Biol. Med. 97:454-456.

Deicher, H. R. G., Holman, H. R., Kunkel, H. G., and Ovary, Z. 1960 Passive cutaneous anaphylaxis reactions with an SLE serum factor and isolated DNA. J. Immunol. 84:106-111.

DeVries, M. J., VanPutten, L. M., Balner, H., and VanBekkum, D. W. 1964 Lésions suggérant une réactivite auto-immune chez des souris atteintes de la "runt disease" aprés thymectomie néonatle. Rev. Franc. Études Clin. Biol. 9:381-397.

Dixon, F. J. 1963 Experimental models in systemic lupus erythematosus. Arth. Rheum. 6:490-512.

Dubois, E. L. 1956 Systemic lupus erythematosus: recent advances in its diagnosis and treatment. Ann. Int. Med. 45:163-184.

Dubois, E. L., Demopoulos, H. B., and Barrett, H. 1965 NZB/NZW mice as a model of systemic lupus erythematosus (SLE). J. Am. Med. Assoc. 192:555.

East, J. and Parrott, D. M. V. 1965 The role of the thymus in autoimmune disease. Acta Allergologica 20:227-234.

Fallet, G. H., Lospalluto, J., and Ziff, M. 1958 Chromatographic and electrophoretic studies of the LE factor. Arth. Rheum. 1:419-425.

Farr, R. S., Grey, H. M., Dickinson, W., and Rosenstein, D. H. 1963 Genetic influences on anti-bovine albumin (BSA) produced in mice. Fed. Proc. 22:265.

Fisher, G. S. and Moyer, J. B. 1950 Hematologic phenomena as a test for acute disseminated lupus erythematosus. Grace Hosp. (Detroit) Bull. 28:3-10.

Fishman, M. 1961 Antibody formation in vitro. J. Exp. Med. 114: 837-856.

Fishman, M. and Alder, F. D. 1963 Antibody formation initiated in vitro. II. Antibody synthesis in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. J. Exp. Med. 117:595-602.

Formijne, P. and van Soren, F. 1958 Negative LE cell phenomenon in true systemic lupus erythematosus. Lancet 2:1206-1209.

Friedman, H. 1963 Appearance of anti-bacterial agglutinins in X-irradiated rabbits receiving immune spleen nucleoproteins. Experientia 19:537-538.

Friedman, H. 1964 Acquisition of antibody plaque forming activity by normal mouse spleen cells treated in vitro with RNA extracted from immune donor spleens. Biochem. Biophys. Res. Comm. 17: 272-277.

Friou, G. J. 1958 The significance of the lupus globulin-nucleoprotein reaction. Ann. Int. Med. 49:866-875.

Friou, G. J. 1962 Fluorescent spot test for anti-nuclear antibodies. Arth. Rheum. 5:407-410.

Friou, G. J., Finch, S. C., and Detre, K. D. 1958 Interaction of nuclei and globulin. J. Immunol. 80:324-327.

Friou, G. J. and Teague, P. O. 1964 Spontaneous autoimmunity in mice: antibodies to nucleoprotein in strain A/J. Science 143: 1333-1334.

Germuth, F. G., Jr. 1953 A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. J. Exp. Med. 97:257-282.

Good, R. A., Peterson, R. D. A., Martinez, C., Sutherland, D. E. R., Kellum, M. J., and Finstad, J. 1965 The thymus in immunobiology: with special reference to autoimmune disease. Ann. N. Y. Acad. Sci. 124:73-94

Goodman, H. C. 1959 Antibodies to nucleoprotein extracts in patients with lupus erythematosus and rabbits immunized with nucleoprotein extracts. Clin. Res. 7:265.

Hargraves, M. M., Richmond, H., and Morton, R. 1948 Presentation of two bone marrow elements: the tart cell and the LE cell. Proc. Staff Meet. Mayo Clin. 23:25-28.

Haserick, J. R. 1956 Evaluation of three diagnostic procedures for lupus erythematosus. Ann. Int. Med. 44:497-502.

Haserick, J. R. and Lewis, L. A. 1950 Blood factor in acute disseminated lupus erythematosus. II. Introduction of specific antibodies against LE factor. Blood 5:718-722.

Haserick, J. R., Lewis, L. A., and Bartz, D. W. 1950 Blood factor in disseminated lupus erythematosus. I. Determination of gamma globulin as specific plasma fraction. Am. J. Med. Sci. 219:660-663.

Heide, G., Leicher, H., and Hartmann, F. 1963 Die erzewgung antinucleärer antikörper im tierversuch. Untersuchungen zur immunologie des lupus erythematodes visceralis. Teutsch. Arch. Klin. Med. 209:144-165.

Helyer, B. J. and Howie, J. B. 1961 Positive lupus erythematosus tests in a crossbred strain of mice NZB/BL-NZY/BL. Proc. Univ. Otago Med. School 39:17-18.

Helyer, B. J. and Howie, J. B. 1963a Spontaneous auto-immune disease in NZB/BL mice. Brit. J. Hemat. 9:119-132.

Helyer, B. J. and Howie, J. B. 1963b The thymus and autoimmune disease. Lancet 2:1026-1029.

Helyer, B. J. and Howie, J. B. 1963c Renal disease associated with positive lupus erythematosus tests in a crossbred strain of mice. Nature 197:197.

Holborow, E. J., Barnes, R. D. S., and Tuffrey, M. 1965 A new red-cell autoantibody in NZB mice. Nature 207:601-604.

Holborow, E. J., Weir, D. M., and Johnson, G. D. A. 1957 A serum factor in lupus erythematosus with affinity for tissue nuclei. Brit. Med. J. 2:732-734.

Holman, H. R. 1963 Genetic studies of systemic lupus erythematosus. Arth. Rheum. 6:513-523.

Holman, H. R. and Kunkel, H. G. 1957 Affinity between LE serum factor and cell nuclei and nucleoprotein. Science 126:162-163.

Holmes, M. C. and Burnet, F. M. 1964 Experimental studies of thymic function in NZB mice and the F1 hybrids with C3H. Aust. J. Exp. Biol. Med. Sci. 42:589-600.

Holmes, M. C., Gorrie, J., and Burnet, F. M. 1961 Transmission by splenic cells of an autoimmune disease occurring spontaneously in mice. Lancet 2:638-639.

Howard, J. G. and Woodruff, M. F. A. 1961 Effect of the graft-versus-host reaction on the immunological responsiveness of the mouse. Proc. Roy. Soc., B. 154:532-539.

Ipsen, J. 1959 Differences in primary and secondary immunizability of inbred mice strains. J. Immunol. 83:448-457.

Kayhoe, D. E., Nasow, J. P., and Bozicevich, J. 1960 Clinical evaluation of the DNA bentonite floculation test for SLE. N. Eng. J. Med. 263:5-10.

Kellum, M. J., Sutherland, D. E. R., Eckert, E., Peterson, R. D. A., and Good, R. A. 1965 Wasting disease, coombs-positivity, and amyloidosis in rabbits subjected to central lymphoid tissue extirpation and irradiation. Int. Arch. Allergy 27:6-26.

Kunkel, H. G. and Tan, E. M. 1964 Autoantibodies and disease. Adv. in Immunol. 4:351-395.

Lachman, D., Mudd, S., Sevag, M. G., Smolens, J., and Wiener, M. 1941 The serological reactivity of nucleic acid. J. Immunol. 40:1-20.

Lachmann, P. J. 1961 An attempt to characterize the lupus erythematosus cell antigen. Immunology 4:153-163.

Lachmann, P. J. 1964 Reactivity of anti-nuclear factors with DNA-nucleoproteins. Ann. Rheum. Dis. 23:311-318.

Lachmann, P. J. and Kunkel, H. G. 1961 Correlation of antinuclear antibodies and nuclear staining patterns. Lancet 2:436-437.

Lee, S. L., Michael, S. R., and Vural, I. L. 1951 The LE (lupus erythematosus) cell. Am. J. Med. 10:446-451.

Levine, B. B., Ojeda, A., and Benacerraf, B. 1963 Studies on artificial antigens. III. The genetic control of the immune response to hapten-poly-l-lysine conjugates in guinea pigs. J. Exp. Med. 118:953-957.

Levine, L. 1963 DNA antibodies. Arth. Rheum. 6:542-557.

Levine, L., Murakami, W. T., VanVunakis, H., and Grossman, L. 1960 Specific antibodies to thermally denatured deoxyribonucleic acid of phage T4. Proc. Nat. Acad. Sci. U. S. 46:1038-1043.

Lewis, R. M., Schwartz, R., and Henry, W. B. 1965 Canine systemic lupus erythematosus. Blood 25:143-160.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951 Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265-271.

Mannick, J. A. 1964 Transfer of "adoptive" immunity to homografts by RNA: a preliminary report. Surgery 56:249-254.

Mannick, J. A., and Egdahl, R. H. 1962 Ribonucleic acid in "transformation" of lymphoid cells. Science 137:976-977.

Mannick, J. A. and Egdahl, R. H. 1964 Inhibition of transplantation immunity by ribonucleic acid. Dis. Chest 46:327-330.

Marshall, J. D., Jr., Eveland, W. C., and Smith, C. W. 1958 Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technique with a modification of its application. Proc. Soc. Exp. Biol. Med. 98:898-900.

McCluskey, R. T., Benacerraf, F., Potter, J. L., and Miller, F. 1960 The pathologic effects of intravenously administered soluble antigen-antibody complexes. J. Exp. Med. 3:181-194.

McDevitt, H. O. and Sela, M. 1965 Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic antigens in two strains of inbred mice. J. Exp. Med. 122:517-531.

McIntire, K. R., Sell, S., and Miller, J. F. A. P. 1964 Pathogenesis of the post-neonatal thymectomy wasting syndrome. Nature 204:151-155.

McMaster, P. R. B., Lerner, E. M., and Mueller, P. S. 1965 Genetic influence on experimental allergic thyroiditis in guinea pigs. Science 147:157-158.

Mellors, R. C. 1965 Autoimmune disease in NZB/BL mice. I. Pathology and pathogenesis of a model system of spontaneous glomerulonephritis. J. Exp. Med. 122:25-40.

Miescher, P. and Fauconnet, M. 1954 L'absorption du facteur LE par des noyaux cellulaires isolés. Experientia 10:252-253.

Miescher, P., Cooper, N. S., and Benacerraf, B. 1961 Experimental production of antinuclear antibodies. J. Immunol. 85:27-36.

Miller, J. F. A. P. 1962 Immunological significance of the thymus of the adult mouse. Nature 195:1318-1319.

Miller, J. J. 1964 An autoradiographic study of the stability of plasma cell ribonucleic acid in rats. J. Immunol. 93:250-254.

Moffatt, T. W., Barnes, S. S., and Weiss, R. S. 1950 The induction of the LE cell (Hargraves) in normal peripheral blood. J. Invest. Dermatol. 14:153-156.

Myers, L. and Friou, G. J. 1965 Histopathology of A/J mice: a laboratory model with spontaneous occurrence of anti-nucleoprotein antibody. Arth. Rheum. 8:459.

Norrins, L. C. and Holmes, M. C. 1964 Antinuclear factor in mice. J. Immunol. 93:148-154.

Paigen, K. 1956 Convenient starch electrophoresis apparatus. Analyt. Chem. 28:284-286.

Phillips, J. H., Braun, W., and Plescia, O. J. 1958 Immunochemical study of a bacterial DNA. J. Am. Chem. Soc. 80:2710-2714.

Plescia, O. J., Braun, W., and Placzuk, N. C. 1964 Production of antibodies to denatured DNA. Proc. Nat. Acad. Sci. U. S. 52:279-285.

Plescia, O. J., Placzuk, N. C., Braun, W., and Cora-Figueroa, E. 1965 Antibodies to DNA and a synthetic polydeoxyribonucleotide produced by oligodeoxyribonucleotides. Science 148: 1102-1103.

Pollak, V. E. 1964 Antinuclear antibodies in families of patients with systemic lupus erythematosus. N. Eng. J. Med. 271: 165-171.

Riggs, J. L., Siewald, R. J., Burckhalter, J., Downs, C. M., and Metcalf, T. G. 1958 Isothiocyanate compounds as fluorescent labeling agents for immune serum. Am. J. Pathol. 34: 1081-1097.

Robbins, W. C., Holman, R. H., Deicher, H., and Kunkel, H. G. 1957 Complement fixation with cell nuclei and DNA in lupus erythematosus. Proc. Soc. Exp. Biol. Med. 96:575-579.

Rosenfeld, S., Swiller, A. I., and Morrison, M. 1954 Simple method of demonstrating the LE cell by finger puncture. J. Am. Med. Assoc. 155:568-569.

Sbarra, A. J., Bardawil, W. A., and Shirley, W. 1963 Relationship between etiology, LE cell phenomenon, and antinuclear antibody in disseminated lupus erythematosus: a hypothesis. Nature 198:159-161.

Scheidegger, J. J. 1955 Une micro-methode de l'immunoelectrophorese. Int. Arch. Allergy 7:103-110.

Schoenberg, M. D., Mumaw, V. R., Moore, R. D., and Weisberger, A. S. 1964 Cytoplasmic interaction between macrophages and lymphocytic cells in antibody synthesis. Science 143:964-965.

Seligman, M. 1958 Etudes immunologiques sur le lupus erythemateux dissemine. Rev. Franc. Etudes Clin. Biol. 3:558-565.

Seligman, M. and Milgrom, F. 1957 Mise en evidence par la fixation du complement de la réaction entre desoxyribonucléique et sérum de malades atteints de lupus érythémateux disséminé. Compt. Rend. Acad. 245:1472-1475.

Shulman, L. E., Gumpel, J. M., D'Angelo, W. A., Souhami, R. L., Stevens, M. B., Townes, A. S., and Masi, A. 1964 Antinuclear factor in inbred strains of mice: the possible role of environmental influence. Arth. Rheum. 7:753.

Steiger, R. 1965 Pathogenesis of thymic changes in NZB mice with hemolytic anemia. J. Exp. Med. 122:929-942.

Stollar, D. and Levine, L. 1961 Antibodies to denatured deoxyribonucleic acid in a lupus erythematosus serum. J. Immunol. 87:477-484.

Stollar, D., Levine, L., Lehrer, H. I., and Van Vanakis, H. 1962 The antigenic determinants of denatured DNA reactive with lupus erythematosus serum. Proc. Nat. Acad. Sci. U. S. 48:874-880.

Suksta, A. and Conley, C. L. 1951 Some observations on the LE cell. J. Lab. Clin. Med. 37:597-602.

Sutherland, D. E. R., Archer, O. K., Peterson, R. D. A., Eckert, E., and Good, R. A. 1965 Development of "autoimmune processes" in rabbits after neonatal removal of central lymphoid tissue. Lancet 1:130-133.

Tanenbaum, S. W. and Beiser, S. M. 1963 Pyrimidine-specific antibodies which react with deoxyribonucleic acid (DNA). Proc. Nat. Acad. Sci. 49:662-668.

Timakov, V. D., Skuvronskaya, A. G., Borisova, N. B., and Zamchuk, L. A. 1963 Antigenic properties of deoxyribonucleic acid of S. typhimurium No. 70. Fed. Proc. Trans. Supp. 22:T1028-T1032.

Townes, A. S., Stewart, C. R., and Osler, C. B. 1963 Immunologic studies of systemic lupus erythematosus. II. Variations of nucleoprotein-reactive gamma globulin and hemolytic serum complement levels with disease activity. Bull. Johns Hopkins Hosp 112:202-219.

Weiler, E. 1964 Delayed antibody synthesis in mice after transfer of immune peritoneal fluid cells. Immunology 7:197-[illegible].

Willkens, R. F., Dreschler, M., and Larson, T. E. 1958 Partial purification of the lupus erythematosus cell promoting factor. Proc. Soc. Exp. Biol. Med. 99:645-648.