Prevention of Murine Lupus Nephritis by Carrier-Dependent Induction of Immunologic Tolerance to Denatured DNA

Abstract. *Four nucleosides were covalently bound to isogeneic mouse immunoglobulin G (IgG) and injected into New Zealand mice. Mice that received the tetranucleoside isogeneic IgG from birth to 5 months of age failed to make antibody to denatured DNA. In contrast, mice that were similarly treated with tetranucleoside bovine serum albumin or tetranucleoside free of carrier produced the same amount of antibody to denatured DNA as did untreated mice of this strain. Mice that were rendered tolerant to denatured DNA by tetranucleoside isogeneic IgG failed to develop the chronic membranous glomerulonephritis that characterizes the renal lesions in animals of this strain.*

One may induce immunologic tolerance by injecting an antigenic determinant (hapten) that is covalently bound to an isologous protein carrier (*1*). The ability to induce tolerance depends on the nature of the carrier protein; isogeneic immunoglobulin G (IgG) is the most effective carrier for the induction of tolerance to a hapten (*2*). Carrier-determined tolerance has been induced in a number of inbred strains of mice and the ease of tolerance induction was independent of the ability of these mice to respond to the antigen (*3*). We now report that carrier-determined tolerance can be applied therapeutically to an autoimmune disease.

New Zealand black mice (NZB) and their first-generation hybrid offspring tend to produce antibodies to nucleic acid antigens, and these antibodies are present in the immune complex–mediated lesions that characterize the murine form of systemic lupus erythematosus (SLE). We tested the principle of carrier-determined tolerance to nucleic acid antigens as follows. We prepared IgG by starch block electrophoresis from serums of young, asymptomatic NZB mice and covalently bound adenosine, guanosine, cytosine, and thymidine to NZB IgG (*4*) to produce a tetranucleoside-IgG conjugate. The nucleosides bind primarily to the ε-amino lysine residues in the purified γ-globulin molecule, a binding site that has been shown to induce tolerance in the dinitrophenol-IgG system (*2*). These nucleotides were selected as haptens because they are not, by themselves, immunogenic and most of the antibody to denatured DNA is directed to them (*5*), and because it is more feasible to bind free nucleosides to a protein carrier than either double- or single-strand DNA. The degree of nucleoside binding to the carrier protein was measured by comparing the optical absorption spectra at wavelengths between 240 and 300 nm with the spectra of the carrier proteins that were similarly treated but free of nucleosides. The ratio of nucleoside molecules to protein molecules was determined, with 10 as the average millimolar extinction at 260 nm. The nucleoside content was 10.5 mole per mole of IgG. We used bovine serum albumin (BSA) as a control carrier protein and bound the nucleosides to BSA in a similar way. The molar nucleoside-BSA ratio was 12.9. Before they were injected, all preparations were cleared of large aggregates by centrifugation at 3000 rev/min for 30 minutes.

Litters of newborn (BW)F_1 mice were divided into four groups: group

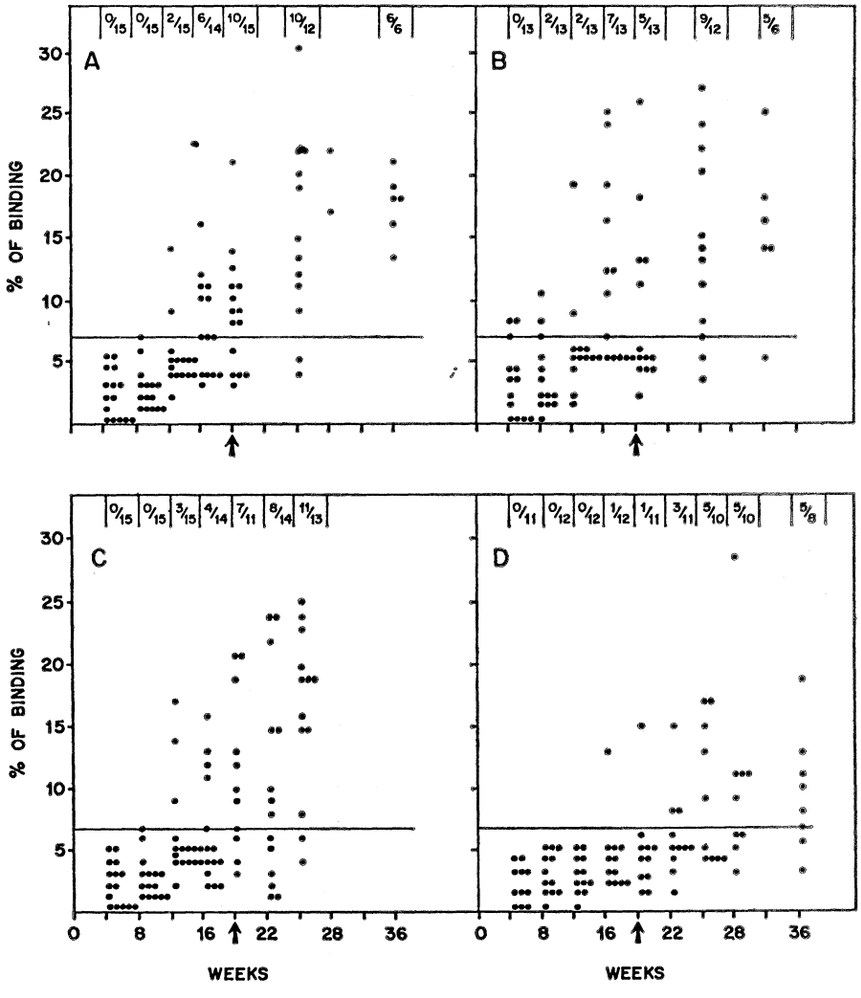

Fig. 1. Carrier-determined immunologic tolerance to denatured DNA (D-DNA). Each square represents the binding of D-DNA by a group of animals. (A) Animals were untreated, (B) animals were treated with the four nucleosides (carrier-free), (C) treated with the nucleosides bound to BSA, and (D) treated with the nucleosides bound to isogeneic IgG. Each point represents the results of a single mouse. The horizontal line represents the upper limit of the normal D-DNA binding. The figures on the upper part of the squares represent the number of animals with positive D-DNA binding over the total number of animals assayed at that time. The arrow represents the arrest of the treatment at 20 weeks of age. The data were analyzed by Student's *t*-test. The geometric mean ± S.E. mean binding on day 20 were: group A, 7.9 (± 1.1); group B, 6.9 (± 1.4); group C, 10.2 (± 2.2); group D, 3.5 (± .8) [mean value of normal serums, 3.3 (± 2.1)]; $P < .001$, for A compared to D; $P > .5$, for A compared to B; $P > .2$, for A compared to C. The sex ratio was the same in all groups.

Table 1. Carrier-determined tolerance to antibody to DNA in (NZB × NZW)F_1 mice. Each ratio represents the number of animals with positive binding of antibody to DNA compared to the total number of animals tested. A binding value of more than 7.5 percent was considered positive. This value was two standard deviations from the mean value for normal serums (3.3 ± 2.1).

Age (weeks)	Uninjected controls		Free nucleosides		BSA nucleosides		NZB IgG nucleosides	
	N-DNA	D-DNA	N-DNA	D-DNA	N-DNA	D-DNA	N-DNA	D-DNA
4	0/15	0/15	0/13	0/13	0/15	0/15	0/12	0/11
8	0/15	0/15	0/13	2/13	0/15	0/15	0/12	0/12
12	0/15	2/15	0/13	2/13	0/15	3/15	0/11	0/12
16	0/15	6/14	0/13	7/13	0/14	4/14	0/11	1/12
20	0/15	10/15	1/13	5/13	0/15	7/11	0/11	1/11
22	1/12	—	2/12	—	0/13	8/14	2/11	3/11
24	1/11	10/12	2/12	9/12	0/12	11/13	1/9	5/10
32	1/6		1/9	—	—	—	3/8	5/8

Table 2. Antigenic specificity of carrier-determined tolerance to D-DNA.

(NZB × NZW) mice No.	Antibody to D-DNA	Antibody to sheep red cells (log_2 titer)
New Zealand black IgG nucleosides		
1	—	8
2	—	9
3	—	9
4	—	9
Bovine serum albumin nucleosides		
5	+	8
6	+	11
7	+	9
8	+	10

A was untreated, group B received a carrier-free mixture of the four nucleosides, group C received the nucleoside-BSA conjugate, and group D was treated with the nucleoside-IgG conjugate. Group D was given 50 μg of nucleoside-IgG per injection and the other groups were given a molar equivalent of nucleoside-BSA or free nucleoside. The animals were injected intraperitoneally on the day of birth and then twice weekly for 4 weeks. Between the ages of 4 and 12 weeks, the mice received the same dose once weekly intravenously; from 12 to 20 weeks, 50 μg was given once a week intraperitoneally. All treatment stopped at 20 weeks of age. During the course of the experiment, the animals were bled once a month from the tail vein, and the serum was analyzed for the antibody to native DNA (N-DNA) and to denatured DNA (D-DNA) (*6*).

At 4 weeks of age none of the mice had antibody to D-DNA or N-DNA. Later, however, the uninjected animals began to form antibody to D-DNA, and by 16 weeks of age approximately 50 percent of the control group showed significant amounts of antibody to D-DNA (Fig. 1A and Table 1). Similarly, approximately 50 percent of the animals injected with either free nucleoside or nucleoside-BSA (Fig. 1, B and C, and Table 1) had significant amounts of antibody to D-DNA at 20 weeks of age. In contrast, animals injected from birth with nucleoside-IgG did not form antibody to D-DNA, and by 20 weeks of age only 1 of the 11 animals in this group had demonstrable levels of antibody to D-DNA. Two weeks later, after treatment was stopped, some animals in this group began to produce antibody to D-DNA. Other animals appeared to remain tolerant up to 9 months of age.

Antibody to N-DNA was detected in only a few animals after 20 weeks of age and no significant differences were observed between the various groups (Table 1).

The antigenic specificity of the tolerance to D-DNA was tested by injecting four recipients of nucleoside-IgG, whose serums were negative at 20 weeks of age, with sheep red blood cells. As controls, animals whose serums were positive, treated with nucleoside-BSA, were injected with sheep red blood cells. The immune response to sheep red blood cells was the same in both groups (Table 2).

To exclude the possibility that the tolerance to D-DNA was due to a feedback mechanism mediated by antibodies to DNA that were present in the nucleoside-IgG preparation, the nucleoside-IgG was tested and found to be negative for antibody activity to both N-DNA and D-DNA.

Examination of autopsy material from the three mice in group D that were still tolerant of D-DNA at 9 months of age revealed no lesions suggestive of murine SLE, and in particular no evidence of membranous glomerulonephritis. Tissues from mice that received nucleoside without carrier or nucleoside-BSA, and uninjected control mice consistently showed lesions typical of murine SLE and 94 percent (17 of 18) of these animals had membranous glomerulonephritis.

The five remaining mice in group D which had broken tolerance to D-DNA and had produced antibody to D-DNA by 9 months of age were killed and examined histologically between 10 and 14 months of age. Pathological evidence of membranous glomerulonephritis was present in only three of these animals, and both mice examined at 14 months of age completely lacked glomerular lesions. Thus, five of the eight (BW)F_1 mice that were tolerant of D-DNA (64 percent) failed to develop glomerulonephritis when examined 4 months after administration of the nucleoside-IgG, and two of these animals had no evidence of glomerular lesions 9 months after their last injection.

The results of this experiment show the following. (i) The administration of nucleosides covalently bound to autologous IgG prevented the formation of antibody to D-DNA in mice that are genetically predisposed to produce this antibody. (ii) Nucleosides similarly bound to BSA or injected without carrier did not prevent the production of antibody to D-DNA in recipient mice. (iii) The tolerant state is antigen specific for the nucleosides used in the experiment and did not extend to an unrelated antigen, sheep red blood cells. (iv) Upon stopping the administration of the nucleoside-IgG, tolerance persisted in some of the recipient mice but others began to produce antibodies to D-DNA in their normal fashion. (v) Of critical importance is the fact that five of the eight mice rendered tolerant of D-DNA failed to develop glomerulonephritis after administration of the nucleoside-IgG was stopped. This is in sharp contrast to the regular development of this lesion in the control animal groups, where 17 of 18 of the mice had lesions characteristic of murine lupus nephritis.

In view of the known difficulty in inducing immunologic tolerance in sensitized animals it would be important to determine whether we can suppress murine SLE in animals that have already formed antibody to D-DNA. If this is feasible, it may provide an initial step toward developing new therapeutic regimens for treatment of SLE in man.

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Vitamin B_{12} and the Megaloblastic Development

Abstract. *In patients with vitamin B_{12} deficiency, phytohemagglutinin-stimulated cultured lymphocytes had little or no thymidylate synthetase activity. Ample activity was found in such lymphocytes from normal individuals and patients with folic acid deficiency or pernicious anemia in remission. It therefore appears that the megaloblastosis that is associated with vitamin B_{12} deficiency is related to low thymidylate synthetase activity.*

In a study of the folate coenzymes in an unusual case of megaloblastic anemia due to defective utilization or transport of vitamin B_{12}, it was found that during relapse although vitamin B_{12} in the serum was high (2000 to 3000 pg/ml) the thymidylate synthetase activity of cultured phytohemagglutinin-stimulated lymphocytes was low. Accordingly we studied this enzyme in patients with vitamin B_{12} deficiency both in relapse and in remission, in patients with folic acid deficiency, and in normal individuals. The enzyme activity was either low or absent only in lymphocytes of patients with vitamin B_{12} deficiency. We assumed that the various biochemical activities of the stimulated human lymphocyte are the same as those that occur in the human pronormoblast. Others have provided cytological and biochemical evidence that deficiency of vitamin B_{12} or folate cause derangement of DNA synthesis in the human lymphocyte (*1*).

The lymphocytes were collected and cultured as follows (*2*): blood (100 ml) was collected from each individual and placed in a heparinized flask. An equal volume of 3 percent high molecular weight dextran was added. In most of the experiments the lymphocytes (50×10^6 to about 100×10^6) were separated from the rest of the leukocytes by a Ficoll (Pharmacia) gradient. The cells were cultured in two portions (60 ml each) in medium 199 (Grand Island Biological Company) in the presence of 0.025 percent of phytohemagglutinin M (Difco) and newborn calf serum (20 percent). The final culture medium contained folic acid (8 ng/ml) and vitamin B_{12} (60 pg/ml). The amount of vitamin B_{12} in the medium is obviously extremely small and that of folic acid is still below the minimum amount needed for appreciable uptake by stimulated lymphocytes (*3*). The cells were incubated at 37°C in an atmosphere containing 5 percent CO_2 (General Electric Hotpack CO_2 incubator). The stimulated lymphocytes were harvested on day 3. A smear was stained and differential cell count made. The cells were counted. Some cells were used for the uptake of radioactive nucleosides, and the rest were lysed and used for protein determination (*4*) and the assay of thymidylate synthetase. The enzyme was assayed by the method of Kammen (*5*). The complete enzyme assay system (*5*) contained 0.1 ml of lysate or water and 0.1 ml of the following: formaldehyde (1 μmole); DL-tetrahydrofolate (0.1 μmole); tris·HCl buffer (*p*H 7.4) (10.0 μmole); magnesium chloride (4.0 μmole); 2-mercaptoethanol (20.0 μmole); and deoxyuridine monophosphate (dUMP) (0.02 μmole) (partly containing [5-^{3}H]-dUMP). Tetrahydrofolic acid was prepared by hydrogenation of folic acid (*6*). Tetrahydrofolic acid is spontaneously converted to the active form in the assay. Thymidylate synthetase activity results in the release of a tritium atom as a proton when thymidylic acid is formed. We made a dilution curve for the enzyme activity by using various dilutions of a strain of *Escherichia coli* (*7*). This established the minimum amount of cell lysate to be tested. The uptake of nucleosides by the stimulated lymphocytes was measured by incubating 10^6 cells (occasionally less) for 1 hour in Hanks buffered solution (*8*) at 37°C with one ^{14}C-labeled nucleoside, either thymidine, deoxyuridine, or uridine (no unlabeled nucleoside was added). At the end of the incubation, the tubes were chilled in ice. The cells were filtered on cellulose acetate membranes (pore size, 5 μm). The residue in the tubes was rinsed onto the membranes with 10 ml of ice-cold balanced saline. The membranes were washed with two 10-ml volumes of ice-cold 5 percent trichloroacetic acid and then placed in scintillation vials and digested overnight with 0.5 ml NCS solubilizer (Amersham/Searle). We measured radioactivity in a liquid scintillation counter (*9*). The nucleoside uptake was expressed as a percentage of the total amount of nucleoside in the incubation medium per 10^7 cells.

Many of the vitamin B_{12} deficient patients were suffering from leukopenia and so the recovery of lymphocytes from their peripheral blood was often reduced (but not by less than 50 percent). However, these lymphocytes did undergo blastogenesis following PHA stimulation. We detected blastogenesis by both morphological criteria and by assaying the uptake of [^{14}C]thymidine by the cells. The uptake of [^{14}C]thymidine in the vitamin B_{12} deficiency group ranged from 3.0 to 14.0 percent with an average of 6.0 ± 3.7 percent S.D.; in the normal group it ranged from 2.6 to 16.0 per-

Table 1. Thymidylate synthetase activity in phytohemagglutinin-stimulated lymphocytes. The enzyme activity is expressed as the uptake of dUMP by 10^7 cells. Two of the six patients with pernicious anemia were studied both in relapse and in remission; the others were studied only in remission.

Condition	Number of individuals	Enzyme activity (100 pmole/hr)	Average activity
Vitamin B_{12} deficiency	8	0.0–2.9	1.1*
Pernicious anemia			
Relapse	2	0.0	
Remission	2	6.8, 6.1	
Relapse			
Remission	4	3.3–12.9	7.1†
Folic acid deficiency	4	7.4–10.5	8.9
Normal	15	4.7–13.8	8.2

* The enzyme activity is the average of the activities of the enzymes from patients with vitamin B_{12} deficiency including the activities of the two patients with pernicious anemia in relapse. † The enzyme activity is the average for the activities of the enzymes from all six patients with pernicious anemia in remission.