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Immunological regulation of spontaneous antibodies to DNA and RNA

III. EARLY EFFECTS OF NEONATAL THYMECTOMY AND SPLENECTOMY

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Summary. NZB/NZW F_1 (B/W) mice were subjected to sham surgery or neonatal thymectomy and/or splenectomy and studied for immunoglobulin class of antibodies to double stranded DNA and polyadenylic acid (Poly A) at 1 and 2 months of age. These antibodies occur spontaneously during the course of autoimmune disease in B/W mice.

The serum from sham-operated female mice bound DNA predominantly in the 7S fraction, whereas serum from sham-operated male mice bound DNA primarily in the 19S fraction. Antibodies to Poly A were exclusively 19S in both sexes. Thymectomy of male B/W mice caused an increase in 7S and 19S antibodies to DNA at the ages studied, while it increased 19S antibodies to DNA in female mice only at 1 month of age. Thymectomy increased the 19S antibodies to Poly A in both sexes. Splenectomy had similar effects in males and females. It reduced both the 19S and 7S responses to DNA and the 19S response to Poly A at 1 month. By the second postoperative month, both 7S anti-DNA and 19S anti-Poly A antibody responses had recovered. Combined thymectomy and splenectomy of male B/W mice produced a disproportionate increase in 7S antibodies to DNA, while the procedures resulted in a decline in 7S and 19S antibodies to DNA in female B/W mice.

These results suggest that the newborn (B/W) thymus and spleen contain regulatory cells exerting different controlling influences on spontaneous antibodies to DNA and Poly A. Moreover, they suggest that the male thymus exerts a suppressor influence while the female thymus exerts primarily a helper effect.

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INTRODUCTION

Animal models such as New Zealand Black mice are experimental tools for the study of autoimmunity. NZB/NZW F_1 (B/W) mice spontaneously produce antibodies to nucleic acids and develop autoimmune disease associated with immune complex glomerulonephritis (Howie & Helyer, 1968). Genetic, immunologic, and viral factors are all implicated in pathogenesis (Talal, 1970). C-type viruses are abundant in B/W mice (Mellors & Huang, 1966).

The immunological abnormalities of B/W mice suggest a premature development of immunological competence which is probably manifest at birth (Evans, Williamson & Irvine, 1968). There follows a progressive decline in T-cell function with loss of suppressor T cells and inability to induce T-cell tolerance to certain antigens (Staples & Talal, 1969).

Anti-nucleic acid antibodies are a hallmark of both murine and human lupus. In an earlier communication, we reported the sequential and predictable appearance of antibodies to DNA and RNA (polyadenylic acid, Poly A) in B/W mice (Papoian, Pillarisetty & Talal, 1976). Antibodies to DNA were detected in both IgM and IgG as early as 4 weeks of age. There was an earlier commitment to IgG antibodies in female mice, which was manifest first for DNA and later for Poly A. We suggested that a disordered regulatory mechanism was responsible for these findings, probably related to the status of helper and suppressor T cells.

MATERIALS AND METHODS

Mice

Mice used in these experiments were derived from our colony at the University of California Vivarium, San Francisco, and were maintained at the Fort Miley Veterans Administration Hospital. B/W mice were obtained from the mating of NZB females to NZW males. They were bled by orbital venous plexus puncture at 1 and 2 months postoperatively. Blood was allowed to clot at room temperature for 1 h and left at 4° overnight. Serum was separated by centrifugation at 1000 *g* for 15 min. Between seven and eighteen mice were studied in various experimental groups (Tables 1 and 2).

Operative Techniques

Animals underwent surgical procedures or sham-operations when 2–3 days of age. They were anaesthetized by placing them on crushed ice for 1–3 min.

(a) *Controlled suction thymectomy.* Thymectomy was carried out under a dissecting microscope at a magnification of 1·6. At this magnification, the entire mediastinal cavity was in the field of vision. The sternal incision was extended from the manubrium down to the sixth rib, permitting visualization of both upper and lower poles of the thymic lobes. The lobes were gently mobilized by disrupting vascular and connective tissue attachments. To achieve adequate control of suction intensity, a 3-mm hole was made in a 5-cm tuberculin syringe barrel which was interspersed between the curved Pasteur pipette and the rubber tubing connecting it to a vacuum outlet. Suction intensity was low. Each thymic lobe was engaged by the pipette at the lower pole and gently teased off. The entire removal process was visualized under the microscope. The mediastinal cavity was free from obvious thymus remnants.

(b) *Splenectomy.* An incision was made in the left upper quadrant of the abdomen. The spleen was mobilized and gently removed. Vascular bundles were cauterized. The abdominal wall was sutured using 6–0 silk.

(c) *Sham procedure.* Control mice were sham-operated by performing the full procedure, but without disturbing the thymus gland or the spleen.

Experimental mice were distinguished from the sham-operated animals by tail clipping. To minimize the likelihood of maternal neglect or cannibalization, the surgical incision and maternal nasal orifices were painted with parlodion-gentian violet solution before returning the operated mice to their mothers. No antibiotics were administered at any time.

Fractionation of serum

Serum samples from all thymectomized, splenectomized, thymectomized-splenectomized or sham-operated mice were pooled separately. Pooled serum samples, 200 μl were subjected to ultracentrifugation in a 10–35% sucrose density gradient (0·15 M NaCl, pH 8·0) as previously described (Talal & Pillarisetty, 1975). Three proteins of known sedimentation constants were run in a companion gradient and served as reference markers. Gradients were collected dropwise into forty fractions. Peak fractions were tested for immunoglobulin content. In the 19S region (fractions 10–20) only μ-chain could be detected by Ouchterlony analysis, while in the 7S region (fractions 20–30) γ-chain, but not μ-chain, was present. Each fraction was analysed for antibody to DNA and RNA. To determine the specificity of binding, monospecific rabbit anti-mouse μ-chain and anti-mouse γ-chain were added to peak fractions from the 19S and 7S regions respectively. Anti-μ inhibited DNA binding between 77–100%, while anti-γ inhibited binding between 66–78%. Addition of goat anti-mouse albumin to these gradient fractions had no inhibitory effect.

Anti-DNA and anti-RNA assays

Antibodies to DNA and to polyadenylic acid (Poly A) were measured by cellulose ester filter radioimmunoassay (Attias, Sylvester & Talal, 1973). The nucleic acids were double-stranded ^{3}H-KB cell DNA (from Electronucleonic, Inc., Bethesda, Maryland)

and ^{3}H-labelled polyriboadenylic acid (from Miles Laboratories, Elkhart, Indiana). The latter is a synthetic single-stranded RNA. The radioactive antigens were incubated with 10 μl of undiluted/decomplemented serum from individual animals or with 50 μl of each fraction for 30 min at 37° followed by an overnight incubation at 4°. The antigen-antibody mixture was then diluted with buffer and passed over a cellulose ester filter under suction. The filters were placed in counting vials and covered with 10 ml of Liquifluor-toluene scintillation medium. Radioactivity was determined in a Packard liquid scintillation counter. The results are expressed as corrected c.p.m. retained on the filter, a value which is directly related to concentration of serum added (Attias, Sylvester & Talal, 1973).

Calculation of 7S: 19S antibody binding

The radioactive binding profiles for DNA revealed clear distribution into 7S and 19S peaks of activity after sucrose gradient fractionation. The radioactivity representing the fractionated 7S and 19S peaks within a single gradient were added and compared for total binding activity. Results with these fractionated sera were generally comparable to results with unfractionated whole serum.

Reproducibility and analysis of data

To determine the reproducibility of our filter radioimmunoassay and the variations seen in age-matched mice, we have analysed data from sham-operated females (6 months old) studied in various experimental protocols over a two year period. There were five sham-operated female groups, with twelve to eighteen per group. The variation from the mean for the total anti-DNA binding in the 19S region ranged from 3–20%, while in the 7S region the range was between 1 and 28%. In the present communication, a variation of 33% or more from the age and sex-matched, sham-operated control was required before a result was considered significant.

Quantitative determination of immunoglobulins

Single radial immunodiffusion technique was used for quantitative determination of IgM, IgG1 and IgG2 immunoglobulin classes in serum samples from

Table 1. Total radioactivity (corrected c.p.m.) representing fractionated 19S and 7S serum peaks in NZB/NZW F_1 female mice

	DNA				Poly A			
	1 Month		2 Months		1 Month		2 Months	
Procedure (no. of mice)	19S	7S	19S	7S	19S	7S	19S	7S
Sham-operated (15)	211	555	442	719	146	0	340	0
Neonatal Thx (18)	407*	512	362	569	158	0	533*	0
Neonatal Splx (14)	119*	267*	74*	634	72*	0	242	0
Neonatal Thx/Splx (7)	72*	663	160*	389*	70*	0	133*	0

* A greater than 33% variation from the value obtained in sham-operated control mice.

Table 2. Total radioactivity (corrected c.p.m.) representing fractionated 19S and 7S serum peaks in NZB/NZW F_1 male mice

	DNA				Poly A			
	1 Month		2 Months		1 Month		2 Months	
Procedure (no. of mice)	19S	7S	19S	7S	19S	7S	19S	7S
Sham-operated (10)	110	48	291	311	124	0	206	0
Neonatal Thx (17)	305*	247*	399*	413*	301*	0	405*	0
Neonatal Splx (11)	55*	66	97*	399	25*	0	158	0
Neonatal Thx/Splx (12)	162*	327*	332	750*	80	0	116*	0

* A greater than 33% variation from the value obtained in sham-operated control mice.

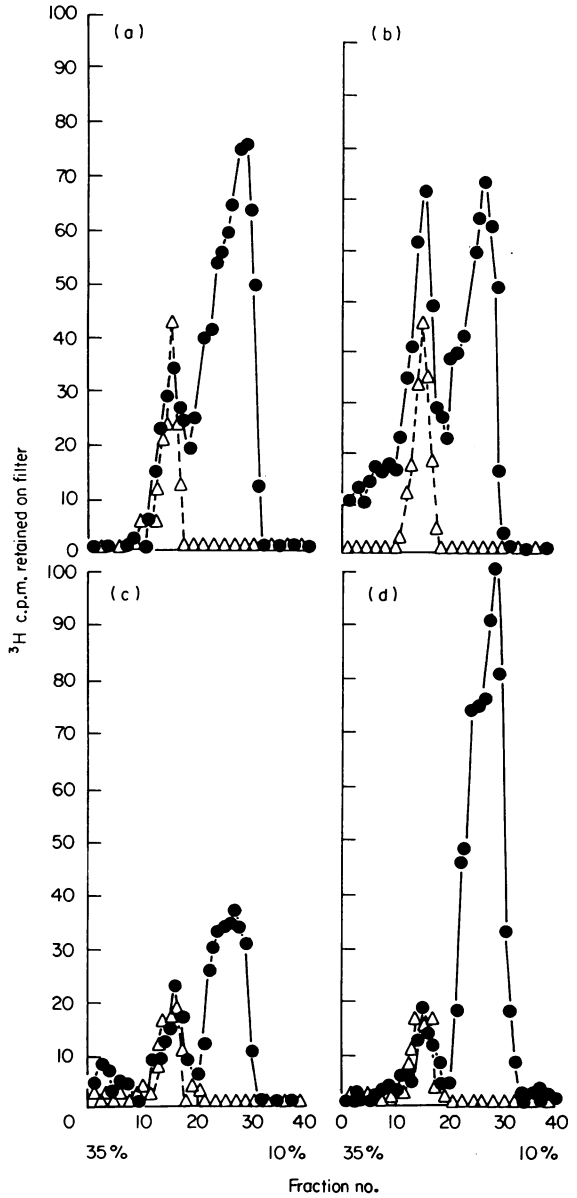

Figure 1. Distribution of antibodies to ^{3}H-labelled DNA (●) and ^{3}H-labelled Poly A (△) determined by sucrose gradient ultracentrifugation and filter radioimmunoassay in 1-month-old B/W female mice who were sham-operated (a); thymectomized (c); splenectomized (c) or thymectomized and splenectomized (d) at 2–3 days of age.

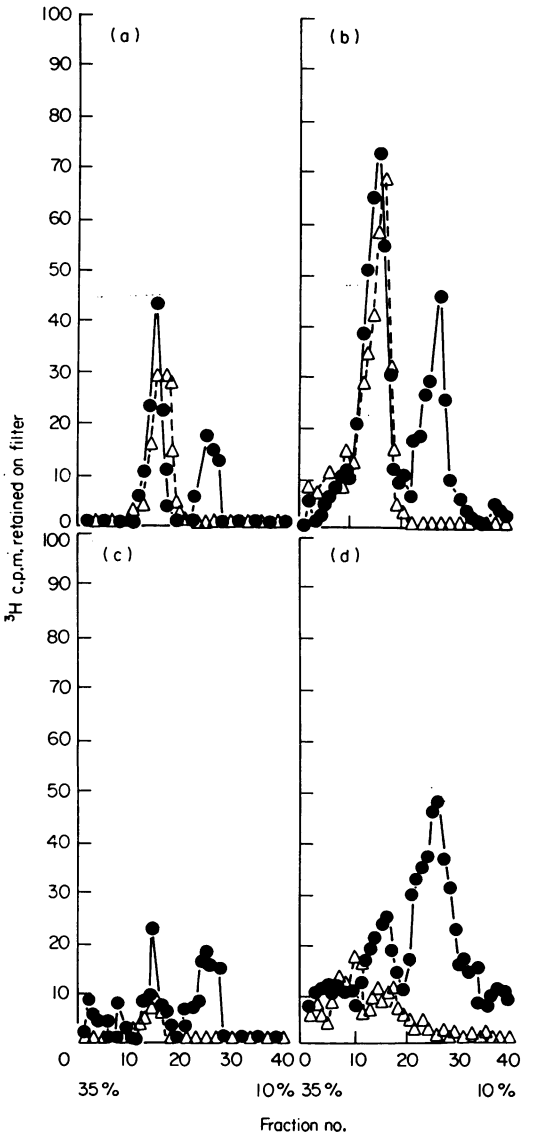

Figure 2. Distribution of antibodies to ^{3}H-labelled DNA (●) and ^{3}H-labelled Poly A (△) determined by sucrose gradient ultra-centrifugation and filter radioimmunoassay in 1-month-old B/W male mice who were sham-operated (a); thymectomized(b); splenectomized (c) or thymectomized and splenectomized (d) at 2–3 days of age.

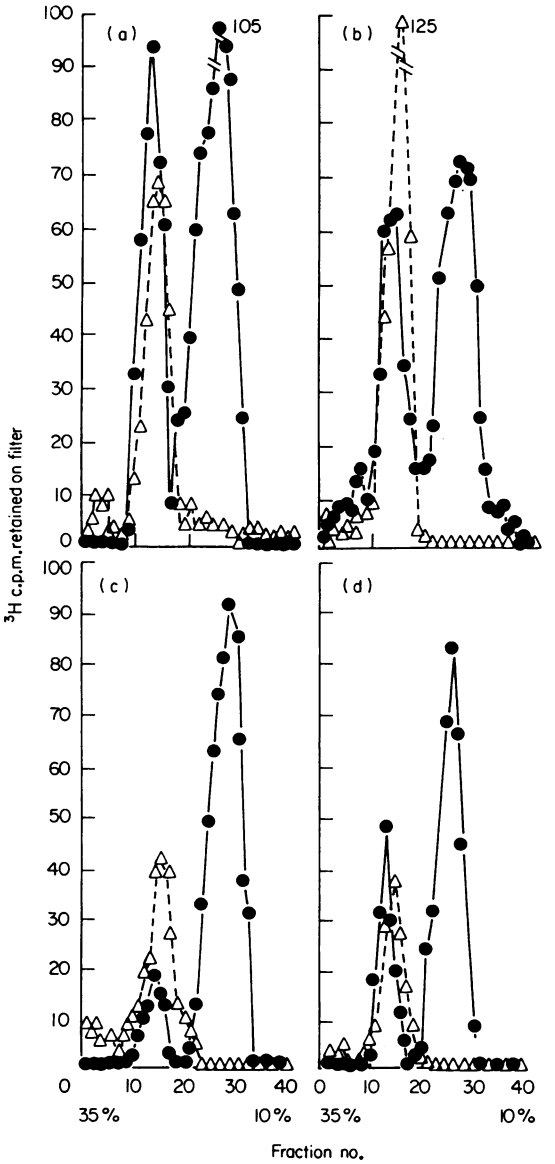

Figure 3. Distribution of antibodies to ^{3}H-labelled DNA (●) and ^{3}H-labelled Poly A (△) determined by sucrose gradient ultracentrifugation and filter radioimmunoassay in 2-months-old B/W female mice who were sham-operated (a); thymectomized (b); splenectomized (c) or thymectomized and splenectomized (d) at 2–3 days of age.

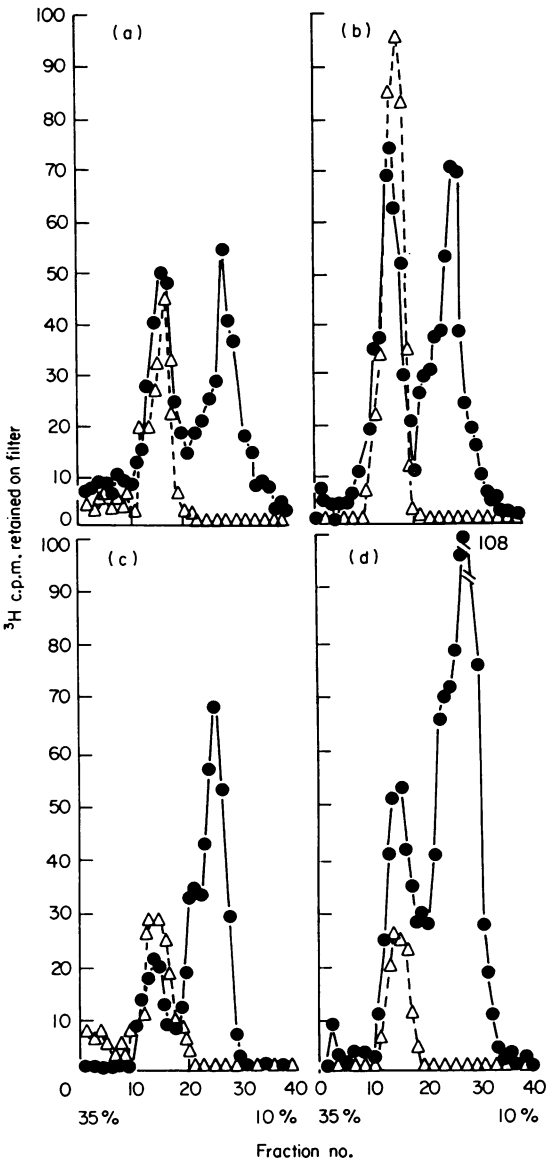

Figure 4. Distribution of antibodies to ^{3}H-labelled DNA (●) and ^{3}H-labelled Poly A (△) determined by sucrose gradient ultracentrifugation and filter radioimmunoassay in 2-months-old B/W male mice who were sham-operated (a); thymectomized (b); splenectomized (c) or thymectomized and splenectomized (d) at 2–3 days of age.

sham-operated, thymectomized, splenectomized, and mice subjected to combined surgical ablation. There was no demonstrable difference in immunoglobulin concentration in experimental and control animals.

RESULTS

Development of antibodies to DNA and Poly A

Sera from 1 month old female or male B/W mice subjected to neonatal sham thymectomy or splenectomy at 2–3 days of age showed significant sex differences in immunoglobulin class response to DNA. Female mice bound DNA primarily by 7S antibodies, whereas male mice bound DNA primarily by 19S antibodies (Figs 1a, 2a, and Tables 1 and 2). The amount of bound DNA increased between 1 and 2 months of age in both sexes. Gradient profiles of the 2-month sera revealed increases in both 7S and 19S antibodies (Figs 3a, 4a, and Tables 1 and 2).

In contrast to DNA, Poly A was bound exclusively by 19S antibodies in both sexes (Tables 1 and 2). Binding was again higher at 2 months.

Effect of neonatal thymectomy on the development of antibodies to DNA and Poly A

In neonatally thymectomized female B/W mice, only 19S anti-DNA antibodies were increased, and only at 1 month of age (Figs 1b, 5 and Table 1). By contrast, thymectomy of male B/W mice increased the immune response to DNA proportionately in 19S and 7S antibodies at both 1 and 2 months (Figs 2b, 6 and Table 2).

Neonatal thymectomy produced a rise in the amount of Poly A bound by male, but not by female, mice at 1 month. The response remained 19S. The augmentation persisted at 2 months when there was a rise in female mice as well (Figs 3b, 4b, and Tables 1 and 2).

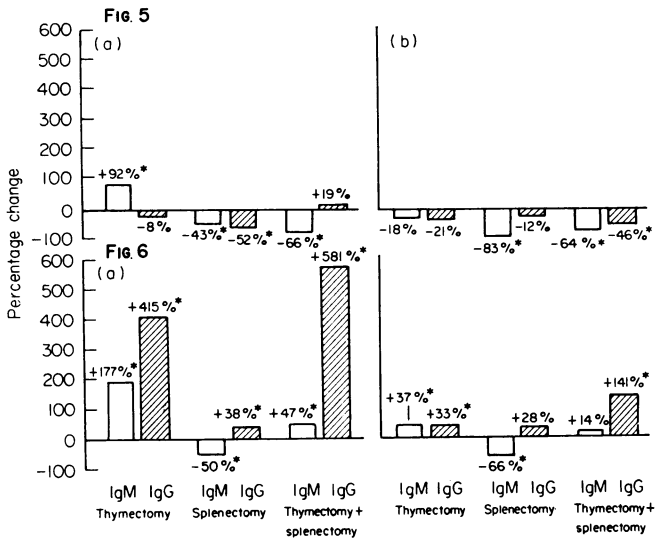

Figure 5. Percentage change in IgM and IgG anti-DNA binding activity of surgically manipulated female B/W mice compared to sham-operated female B/W mice, calculated as follows:

$$\frac{\text{experimental c.p.m.} - \text{sham c.p.m.}}{\text{sham c.p.m.}} \times 100 = \text{per cent change c.p.m.}$$

(a) 1 month; (b) 2 months.

Figure 6. Percentage change in IgM and IgG anti-DNA binding activity of surgically manipulated male B/W mice compared to sham-operated male B/W mice, calculated as follows:

$$\frac{\text{experimental c.p.m.} - \text{sham c.p.m.}}{\text{sham c.p.m.}} \times 100 = \text{per cent change c.p.m.}$$

(a) 1 month; (b) 2 months.

Effect of neonatal splenectomy on the development of antibodies to DNA and Poly A

Neonatal splenectomy caused a depression of antibodies to DNA and Poly A. The gradient profile in 1-month-old female B/W mice showed decreased 19S and 7S antibodies to DNA while in males the decline was confined to 19S antibodies. Poly A declined significantly in both sexes at 1 month (Figs 1c and 2c). At 2 months, males and females showed 7S peaks of antibodies to DNA similar to sham-operated controls, although there was a persistent depression in 19S anti-DNA activity (Figs 3c, 4c, 5, 6 and Tables 1 and 2).

Although the expected 19S antibody to Poly A was virtually absent 1 month post-splenectomy, it was present at 2 months. Poly A binding was similar to that in sham-operated controls. No 7S antibody to Poly A was present (Figs 3c, 4c, and Tables 1 and 2).

Effect of combined neonatal thymectomy and splenectomy on the development of antibodies to DNA and Poly A

One-month-old female mice subjected to combined neonatal thymectomy and splenectomy had depressed 19S antibodies, while 7S antibodies to DNA were unaffected (Figs 1d, 5 and Table 2). Thymectomized and splenectomized males at 1 month had a modest increase in 19S and a substantial increase in 7S antibodies to DNA (Figs 2d, 6 and Table 2). This gave the males a female-type pattern in which most of the DNA was bound in the 7S region.

At 2 months, female mice maintained the decline in 19S anti-DNA response, and also had a concomitant decline in 7S antibodies to DNA (Figs 3d, 5). Male mice, on the other hand, had the highest 7S antibodies to DNA seen in any group (Figs 4d and 6).

Poly A binding in thymectomized-splenectomized mice resembled the pattern seen in splenectomized mice. Poly A binding was almost undetectable at 1 month (Fig. 1d and 2d), but clearly seen in the 19S region at 2 months (Fig. 3d and 4d).

DISCUSSION

This study demonstrates that the spontaneous production of antibodies to DNA and Poly A in B/W mice can be influenced by neonatal removal of thymus and spleen. It suggests that cells or factors in these lymphoid organs may regulate these autoantibody responses, albeit in a defective manner.

We have interpreted our results in terms of a regulatory equilibrium between helper and suppressor T cells, in accordance with current concepts of immunological regulation (Katz & Benacerraf, 1972; Gershon, 1975). This interpretation may be over-simplified and is not intended to exclude other important controlling factors such as quantity and organ-localization of antigen, feedback regulation by antibody, and the influence of viruses (Talal, 1976).

In male B/W mice, antibodies to DNA were primarily 19S and increased significantly between 1 and 2 months of age. Thymectomy resulted in an increase in both 19S and 7S antibodies. By contrast, splenectomy caused a depression of 19S but enhanced 7S antibodies to DNA at 1 month. At 2 months, the 19S response remained depressed, while the 7S response was similar to the level in control mice. Combined thymectomy and splenectomy led to a large increase in 7S antibodies. These results suggest that the neonatal male thymus expresses a suppressor function which is eliminated following thymectomy. The spleen, in addition to producing antibodies to nucleic acids, may also suppress the production of anti-DNA antibodies by extra-splenic lymphocytes. This effect is seen when both thymus and spleen are removed. Such animals achieve the highest 7S DNA binding seen in any group studied. Whereas 19S antibodies remain decreased in splenectomized animals, they are increased in thymectomized mice. This suggests that the thymic suppressor mechanism regulates both 19S and 7S responses, while the splenic suppressor mechanism may influence only the 7S response. Further evidence for this suggestion was obtained in older splenectomized mice (Roubinian, Papoian & Talal, 1977).

Antibodies to Poly A were exclusively 19S at the ages studied. Thymectomy caused an increase in Poly A binding, which may reflect the removal of suppressor cells. Splenectomy resulted in decreased binding, suggesting that synthesis of 19S antibodies to Poly A occurs predominantly in the spleen. Synthesis remained low in mice subjected to combined splenectomy and thymectomy.

The secondary lymphoid organs of one-month-old female B/W mice are already producing large amounts of 7S antibodies to DNA. This antibody is relatively resistant to the effects of neonatal thymec-

tomy and may reflect a premature peripheralization of helper T cells. Neonatal thymectomy did produce an increase in 19S antibodies to DNA, suggesting the removal of suppressor cells capable of regulating the 19S response. This observation represents a difference between female and male mice, since thymic suppressor cells in male mice control both 19S and 7S antibodies to DNA. Splenectomy in female mice has the same effect as in males, resulting in a persistent depression of 19S antibodies and a delayed recovery of 7S antibodies to DNA. However, combined thymectomized and splenectomized female mice have a decrease in 7S antibodies to DNA at 2 months compared to sex-matched controls, whereas male mice subjected to the combined procedures have the highest levels of 7S anti-DNA antibodies seen in any group. Thus, the predominant effect of thymic and splenic ablation in the male is the elimination of suppressor cells, while in female it may be the removal of a helper mechanism.

This study suggests that spontaneous antibodies to nucleic acids in B/W mice appear in a sequential and ordered fashion. This order may reflect the presence of regulatory mechanism(s) that are strongly dependent upon factors and/or cells present in the thymus and spleen. Antibodies to DNA and Poly A appear to be under separate regulatory controls.

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