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Independent Appearance of Anti-thymocyte and Anti-RNA Antibodies in NZB/NZW F1 Mice

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Summary. NZB and NZB/NZW (B/W) mice were studied for natural thymocytotoxic autoantibody (NTA) and antibody to reovirus RNA by cytotoxicity and radioimmunoassay respectively. NZB mice developed NTA by 2–3 months of age. RNA antibodies appeared later if at all. NTA and anti-RNA were present in B/W mice starting at 4–5 months and increased in frequency with age. Either activity could appear first and could be present without the other. Thus, all four possible patterns of autoantibody occurrence were found. NTA and anti-RNA in 12-month B/W sera were separable by sucrose density gradient ultracentrifugation; NTA is a 19S IgM antibody whereas anti-RNA is 7S.

INTRODUCTION

New Zealand Black (NZB) and NZB/NZW F1 hybrid (B/W) mice spontaneously develop various manifestations of autoimmunity and lymphoid malignancy. These include the formation of antinuclear antibodies, lupus erythematosus (LE) cells (Helyer and Howie, 1963), Coombs' positive haemolytic anaemia (Bielschowsky, Helyer and Howie, 1959), an immune complex glomerulonephritis (Lambert and Dixon, 1968; Dixon, Oldstone and Tonietti, 1971) B-cell malignancies (Mellors, 1966; Greenspan, Gutman, Talal, Weissman and Sugai, 1974) and monoclonal macroglobulinaemia (Sugai, Pillarisetty and Talal, 1973). Antibodies to DNA (Steinberg, Pincus and Talal, 1969) and RNA (Talal, Steinberg and Daley, 1971a; Talal, Steinberg, Jacobs, Chused and Gazdar, 1971b) develop in both NZB and B/W mice, although with greater frequency in the latter. A naturally occurring thymocytotoxic autoantibody (NTA) is found very early in life in NZB mice and in high titre in old NZB mice. It also occurs in B/W mice, although later in life, and in lower titre (Shirai and Mellors, 1971; 1972).

Certain abnormalities of thymus function are present in 1–2-month-old NZB and B/W mice prior to the onset of autoimmunity and the more gross defects in cellular immunity. These early thymus abnormalities include: (1) a relative resistance to the induction of immunological tolerance in thymocytes (Staples and Talal, 1969; Jacobs, Gordon and Talal, 1971; Playfair, 1971); (2) an abnormal proliferative response of NZB thymocytes to alloantigens (Dauphinee and Talal, 1973); (3) a loss of suppressor T cells (Barthold, Kysela and Steinberg, 1974; Talal, Dauphinee, Pillarisetty and Goldblum,

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1974); (4) a marked decline in the serum level of thymic hormone present in the blood of normal mice (Bach, Dardenne and Salomon, 1973).

A loss of suppressor T cells within the first weeks of life could explain the loss of tolerance, the abnormal thymocyte proliferation and the emergence of autoimmunity in NZB and B/W mice. Shirai, Yoshiki and Mellors (1972 a,c, 1973) have proposed that NTA might be responsible for the suppressor cell deficiency and development of autoimmunity. This important hypothesis has not yet been tested.

This present investigation was designed to study the relationship between NTA and anti-RNA antibodies. In particular, we were interested in whether NTA was always present prior to anti-RNA, or if anti-RNA appeared without prior NTA. The latter situation was found, suggesting that these autoantibodies may be separate and independent expressions of autoimmunity.

MATERIALS AND METHODS

Mice

NZB, NZB/NZW and BD F1 mice were from our colonies maintained at the Vivarium of the University of California, San Francisco and Fort Miley Veterans Administration Hospital. C57Bl/6 mice were from Jackson Laboratory, Bar Harbor, Maine.

Cytotoxic assay for NTA

Cytotoxicity assays were performed according to the method of Shirai and Mellors (1971). A suspension of thymocytes from C57Bl/6 mice aged 6–10 weeks was washed three times in Eagle's minimum essential medium (MEM) supplemented with 3 per cent foetal calf serum (heat-inactivated) and adjusted to a concentration of 1×10^7 cells/cc. Twenty-five microlitres of the cell suspension was then incubated at 4° for 60 minutes with 50 μl of undiluted serum to be tested. The cells were then washed twice and incubated for 30 minutes at 37° with 50 μl of rabbit complement at a 1:20 dilution (Hyland Laboratories, Costa Mesa, California). The complement was selected for low toxicity towards thymocytes and high complement activity. Cytotoxicity was determined by trypan blue dye exclusion.

Sera which killed more than 50 per cent of thymocytes were considered to be positive for NTA. All doubtful reactions were repeated once or twice. A control consisting of only thymocytes and complement (no serum) was performed with every set of determinations. Known positive and negative controls were included with each set of tests. Initially, additional controls consisting of sera and thymocytes (no complement) were included. After the initial hundred of these latter controls demonstrated essentially no cytotoxicity, they were abandoned in order to conserve serum.

Anti-RNA assays

Antibodies to RNA were measured by a cellulose ester filter radioimmunoassay method (Attias, Sylvester and Talal, 1973). Ten microlitres of serum was diluted with 85 μl of borate sodium chloride buffer (0·15 M, pH 8·0) and incubated at 56° for 30 minutes to destroy complement components which bind some nucleic acids non-specifically. Five microlitres of reovirus [^{3}H]RNA (specific activity 900 counts per minute (ct/min) in 25 mμg) was then added to give a total volume of 100 μl. Incubation was carried out for 30 minutes at 37° followed by 60 minutes at 4°. The antigen–antibody mixture was then

diluted with buffer and passed over a cellulose ester filter under suction. The filter was washed with buffer, dried, placed in counting vials and covered with 10 cc of Liquifluor–toluene scintillation medium. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3380, Packard Instrument Company, Downers Grove, Illinois). The results are expressed as ct/min retained on the filter.

Ten BD F1 mice, a strain which does not normally develop autoantibodies, were assayed for anti-RNA antibodies. Their mean ct/min was 25 and the range 14–38 ct/min. Values in excess of two standard deviations above the normal mean (40 ct/min) were considered positive for anti-RNA antibodies.

Sucrose density gradient ultracentrifugation

Two B/W mouse sera containing both NTA and anti-RNA antibodies were pooled and subjected to ultracentrifugation in a 1–35 per cent sucrose density gradient (0·15 M NaCl, pH 8·0) using an SW 40 rotor (Beckman Instruments, Incorporated, Spinco Division, Palo Alto, California). The centrifugation was at 39,000 rev/min for 20 hours. The 13·5 ml gradient was collected dropwise into fifty fractions and analysed for NTA and antibodies to RNA. Two proteins of known sedimentation constants were run in a companion gradient and served as reference markers.

Results of thymocytoxicity testing of sucrose density gradient fractions were expressed as a cytotoxicity index (CI), according to the following formula (Shirai, Yoshiki and Mellors, 1972b):

$$CI = [(\text{percentage dead cells with serum} - \text{percentage dead cells with complement})/(100 - \text{percentage dead cells with complement})] \times 100.$$

RESULTS

INCIDENCE OF NTA AND ANTI-RNA

NZB and B/W mice of varying ages were tested for NTA. Every NZB mouse older than 2 months had NTA (Table 1). By contrast, only one of twenty B/W mice between 2 and 3 months of age had NTA. The incidence of NTA increased progressively in B/W mice, so that 90 per cent were positive by 12 months of age. There was no sex difference in occurrence of NTA in either strain.

NZB and B/W sera were also tested for antibody to double-stranded reovirus RNA. In contrast to the results with NTA, NZB mice were less often positive for antibodies to RNA (Table 2). By 12 months of age, only 44 per cent of NZB mice were positive with a mean

TABLE 1

INCIDENCE OF THYMOTOTOXIC ANTIBODY IN NZB AND NZB/NZW MICE

Age (months)	NZB (Number positive)/(number tested)	NZB Percentage	NZB/NZW (Number positive)/(number tested)	NZB/NZW Percentage
1	7/17	41	0/14	0
2–3	10/10	100	1/20	5
4–5	–	–	6/26	23
6–11	–	–	16/28	57
>12	25/25	100	26/29	90

binding of 74 ct/min. Thus, NZB mice develop NTA early in life and RNA antibodies later, if at all. In B/W mice, however, NTA and anti-RNA occur with similar frequencies at all ages. We therefore selected B/W mice for further studies designed to compare these two activities in individual mice.

TABLE 2
INCIDENCE OF ANTI-REOVIRUS RNA IN NZB AND NZB/NZW MICE

Age (months)	NZB (Number positive)/ (number tested)	NZB Percentage	NZB/NZW (Number positive)/ (number tested)	NZB/NZW Percentage
1	1/19	5	0/5	0
2–3	1/8	13	0/11	0
4–5	–	–	4/24	17
6–11	–	–	13/28	46
>12	7/16	44	13/18	72

IMMUNOCHEMICAL CHARACTERIZATION OF NTA AND SEPARATION FROM ANTI-RNA

The results of subjecting pooled sera positive for both NTA and RNA antibodies to sucrose density gradient ultracentrifugation and subsequent testing of individual reactions is depicted in Fig. 1. Thymocytotoxic activity was found as a peak in the 19S region whereas [^{3}H]RNA binding activity was recovered as a peak in the 7S region of the gradient.

Gradient tubes from the 19S region (fractions 10–19) and 7S region (fractions 20–39) were pooled separately, dialyzed against 0·15 M phosphate-buffered saline (pH 7·2), lyophilized, reconstituted to a constant volume and tested for thymocytotoxicity. As expected, the 19S peak had a greater CI (62) than the 7S pool (CI=31). The 19S pool was then incubated with various reagents in an attempt to neutralize its cytotoxicity (Table 3). Anti-IgM at a 1:10 dilution neutralized the cytotoxicity completely and was

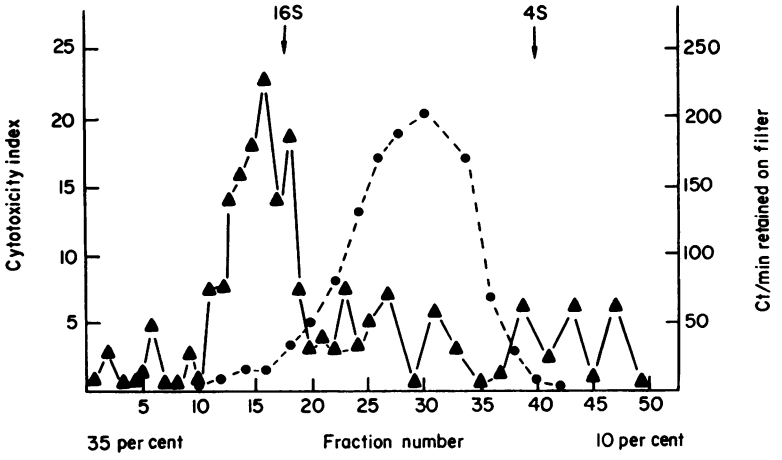

FIG. 1. Separation of natural thymocytotoxic antibody (▲) from anti-^{3}H-labelled reovirus RNA antibody (●) by sucrose gradient ultracentrifugation of NZB/NZW serum containing both antibodies. (- - -) Ct/min retained on filter. (——) CI.

TABLE 3

NEUTRALIZATION OF CYTOTOXICITY OF POOLED 19S FRACTION*

Treatment	Cytotoxicity index
Medium (MEM)	62
0·2 M 2-mercaptoethanol	26
Anti-IgM 1/10†	1
Anti-IgM 1/20	21
Anti-IgM 1/40	35
Anti-IgG 1/10	47

* In each experiment, 25 μl of the 19S pool was incubated with 25 μl of the appropriate reagent and the mixture was then tested for thymocytotoxicity in the usual manner.

† Antisera kindly supplied by Dr Richard Asofsky, National Institutes of Health.

still effective at 1:20 and 1:40 dilutions. Anti-IgG (γ_2) had little effect. Treatment with 0·2 M 2-mercaptoethanol reduced the cytotoxicity index of the pooled 19S fractions from 62 to 26, despite the demonstration that 0·2 M 2-mercaptoethanol alone was toxic to thymocytes (CI of 38). None of the other reagents by themselves were toxic to thymocytes (CI < 1). The thymocytotoxicity of pooled 7S fraction was probably due to contaminating IgM, since anti-IgM inhibited its cytotoxicity completely.

RELATIONSHIP OF NTA AND ANTI-RNA ANTIBODIES

Sera from seventy-four B/W mice were tested simultaneously for both NTA and anti-RNA antibodies. All four possible patterns of autoantibody occurrence were found (Table 4). Thirty-two mice (with a mean age of 4·3 months) had neither antibody activity. Fifteen mice (mean age 8·8 months) had NTA but no anti-RNA. However, nine mice (mean age 8·8 months) had anti-RNA in the absence of NTA. Anti-RNA binding activity in these mice did not differ significantly from that found in mice containing both NTA and anti-RNA. Eighteen mice (mean age 11 months) had both activities.

These results suggest that NTA and anti-RNA appear at approximately the same age in B/W mice. Either may appear first; older mice generally have both antibodies.

TABLE 4

RELATIONSHIPS OF AUTOANTIBODIES IN NZB/NZW MICE

Number of Mice	NTA*	Anti-RNA*	Mean ct/min	Mean age (months)
32	0	0	23	4·3
15	+	0	30	8·8
9	0	+	119	8·8
18	+	+	135	11·0

* Symbol 0 indicates the absence of antibody; + indicates the presence of antibody.

DISCUSSION

NTA is an IgM autoantibody easily separable from 7S anti-RNA antibody by sucrose density gradient ultracentrifugation. NTA was found predominantly in the 19S peak; anti-IgM antiserum and 2-mercaptoethanol considerably reduced its thymocytoxic activity. NTA is excluded from Sephadex G-200 (Shirai and Mellors, 1972). Indirect immunofluorescent studies also confirm the IgM nature of NTA (Parker, Chused and Steinberg, 1974).

NZB mice develop NTA earlier and in higher titre than other mouse strains; B/W mice develop NTA later and in lower titre (Shirai and Mellors, 1972). NTA is species-specific and reacts against thymocytes bearing either θ marker. Absorption with adult mouse thymus, lymph node, spleen and brain removes NTA from serum (Shirai and Mellors, 1972).

Humans with systemic lupus erythematosus (SLE) frequently develop anti-lymphocytic antibody (Terasaki, Mottironi and Barnett, 1970; Mittal, Rossen, Sharp, Lidsky and Butler, 1970). This antibody, like NTA, has specificity for T cells and thymocytes (Lies, Messner and Williams, 1973). Thus, information about the role of NTA in New Zealand mouse disease may have important implications for understanding SLE.

Our results suggest that NTA and anti-RNA are probably independent criteria of autoimmunity since either may be present without the other. B/W mice were initially negative for both autoantibodies and later expressed their autoimmunity by the production of either NTA or anti-RNA. Subsequently, many mice produced both autoantibodies.

NTA and anti-RNA are generally not present intermittently in B/W mice; once present, titres frequently increase as the animals grow older. NTA could be absorbed onto thymocytes *in vivo* and be absent from the serum. However, we were unable to find direct cytotoxicity of B/W mouse thymocytes upon addition of rabbit complement. Such cells might have been lysed *in vivo* and eliminated. The specificity of NTA is that of a natural anti-thymocyte serum (Shirai and Mellors, 1972), making it unlikely to have selective specificity for a particular T-cell subpopulation.

Our results do not support the hypothesis (Shirai *et al.*, 1972a, b, 1973) that NTA is responsible for a deficiency of suppressor T cells and the subsequent emergence of autoimmunity. Cellular immunity directed against thymocytes remains a possibility.

While the role for NTA in the genesis of other autoantibodies is doubtful, it may be responsible for the progressive decrease in recirculating T cells (Denman and Denman, 1970; Zatz, Mellors and Lance, 1971) and loss of cellular immunity (Cantor, Asofsky and Talal, 1970; Leventhal and Talal, 1970; Gazdar, Beitzel and Talal, 1971) that occurs with age in New Zealand mice. NTA promotes the *in vitro* immune phagocytosis of mouse thymocytes by peritoneal macrophages (Shirai and Mellors, 1971). In addition, NTA alters the migration pattern of ^{51}Cr-labelled C57Bl/6 lymph node cells injected intravenously in syngeneic mice. Cells exposed to NTA were located relatively more in liver and less in lymph nodes and spleen, thus suggesting a process of autosensitization phagocytosis (Shirai, 1973). Furthermore, NTA-sensitive cells decrease progressively with age in mesenteric lymph nodes, spleen, and blood of NZB mice in comparison with normal mouse strains (Shirai *et al.*, 1972a).

Antibodies to cell surface antigens are often seen in malignancy and may be related to viral infection. New Zealand mice harbour one or more mouse leukaemia viruses which are readily apparent as C-type particles budding off cell membranes in thymus and other

organs (Mellors and Huang, 1966). Modification of a T-cell surface antigen or formation of a new surface antigen as a consequence of viral infection might alter the immunogenicity of thymocytes and lead to the formation of thymocytotoxic antibody. Antibodies induced in this way could be cross-reactive with theta and other antigens on normal mouse thymocytes.

The occurrence of both antibodies to viral double-stranded RNA (Talal *et al.*, 1971; Schur, Stollar, Steinberg, and Talal, 1971) and antibodies to thymocyte surface antigens in SLE patients and New Zealand mice is a further example of the remarkable similarity between these conditions and a further suggestion of a common immunological, viral pathogenetic mechanism. Concurrent testing for anti-lymphocyte antibody and antinuclear antibody (ANA) in SLE revealed that many patients have ANA in the absence of anti-lymphocyte antibody (Butler, Sharp, Rossen, Lidsky, Mittal and Gard, 1972), a finding analogous to our results in mice.

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