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NATURAL CYTOTOXIC AUTOANTIBODY AGAINST THYMOCYTES IN NZB MICE

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SUMMARY

NZB mice were found to produce natural thymocytotoxic autoantibody in high prevalence and antibody titre. This autoantibody in NZB mice was detectable by the cytotoxicity test at both 4°C and 37°C; the prevalence and antibody titre were generally higher at 4°C. Mice of other strains also produced natural thymocytotoxic autoantibody although in lower prevalence and antibody titre and in some instances the activity was greater at 37°C than at 4°C. Natural thymocytotoxic autoantibody in NZB mice reacted equally with the thymocytes of virtually all strains of mice tested but to a lesser degree with the thymocytes of SJL/J mice. A serum pool obtained from old NZB mice had an extremely high titre of natural thymocytotoxic autoantibody (1:1024 at 4°C). Nevertheless, the cells in lymph nodes, spleen and blood leucocytes were only partially sensitive to this serum pool, and bone marrow cells were for the most part negative. By absorption, the antigen reacting with natural thymocytotoxic autoantibody was found in thymus, lymph node, spleen and brain of adult mice, thymus of newborn mice and some leukaemias. Natural thymocytotoxic autoantibody in NZB mice was an IgM-globulin as determined by sensitivity to 2-mercaptoethanol treatment and by Sephadex G-200 column chromatography in contrast to other natural antibodies (antinuclear, antierythrocyte and G antibodies) of IgG-globulin class. NZB mice also produced natural antibodies against thymocytes of the rat and the hamster; these antibodies were species-specific and did not react with the thymocytes of any but the homologous species.

INTRODUCTION

Accumulating evidence has indicated that a progressive decline of cellular immune function occurs in NZB mice with ageing (Stutman, Yunis & Good, 1968; Cantor, Asofsky & Talal, 1970; Leventhal & Talal, 1970; Rodey, Good & Yunis, 1971). NZB mice are known to develop autoimmune haemolytic anaemia (Bielschowsky, Helyer & Howie, 1959) and

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immune-complex type glomerulonephritis (Lambert & Dixon, 1968; Mellors *et al.*, 1971). The relation between the deficient cellular immune functions and the development of the autoimmune disease of NZB mice is not yet clear; the possible significance is suggested by the finding that neonatal thymectomy accelerates the onset of autoimmune disease in NZB and (NZB × NZW)F_1 hybrid mice (Howie & Helyer, 1966) and induces autoimmune manifestations in mice of some other strains (Teague *et al.*, 1970; Yunis *et al.*, 1967).

Recently we discovered natural thymocytotoxic autoantibody (NTA) in the sera of NZB mice (Shirai & Mellors, 1971). Although NTA is not unique for NZB mice, the prevalence and antibody titre are much higher even in early life than found in other strains of mice. The purpose of this paper is to present detailed information on this point.

MATERIALS AND METHODS

Animals

NZB, NZW and (NZB × NZW)F_1 and (NZB × C57BL/6)F_1 mice were obtained from our colonies. The general methods of maintenance and care of these mice have been described elsewhere (Mellors, 1965). Other strains of mice were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. Wistar/Fu rats were obtained from Microbiological Associates (Bethesda, Maryland); Syrian hamsters from Lakeview Hamsters (Newfield, N.J.); English short hair guinea-pigs from Camm Research Institute (Wayne, N.J.); and New Zealand white rabbits from Summit View Farm (Mountain Lakes, N.J.).

Tumours

All the tumours in NZB mice were induced in our colonies. They were maintained in the syngeneic young mice (2 months of age) of the same sex. The origins and natures of the tumours were identified in the text. EL_4, ERLD, E♂G_2, K36 (Old & Boyse, 1965) and MOPC70A (Takahashi, Old & Boyse, 1970) were donated by Dr L. J. Old, Sloan-Kettering Institute for Cancer Research, New York. Primary leukaemias of AKR/J mice were also used.

Sera

Blood for serological studies was taken from the peri-orbital sinus. All the sera were kept at −70°C before use. G (Gross) typing serum was produced according to the method described by Old, Boyse & Stockert (1965). NZB sera used for standard serological tests were pooled from mice at 8–10 and 17–19 months of age. The sera were kept at 4°C for 48 hr to exclude the cryoprecipitates before storage at −70°C. Blood from newborn mice was obtained by cardiac puncture using capillary glass pipettes.

Cell suspensions

Cells from thymus, lymph node, spleen and testis were obtained by gently twisting the tissues with glass homogenizers (Tissue grinder No. 1977, Bellco Glass Inc., Vineland, N.J.) in medium 199 supplemented with 3% foetal calf serum (Microbiological Associates, Bethesda, Maryland) previously heated to 56°C for 30 min. Free cells were collected from the supernatant after large fragments of tissue settled, and were washed twice at 1000 rev/min for 5 min prior to use. Blood leucocytes were collected by mixing one volume of heparinized blood and two volumes of 6% W/V Dextran in 0·9% NaCl (Grade H, Pharma-

chem Corp., Bethlehem, Pennsylvania). The mixture was incubated at 37°C for 2 hr and the supernatant rich in leucocytes was collected. After centrifugation at 1000 rev/min for 15 min, the cells were washed twice in MEM supplemented with 3% foetal calf serum and treated once in the solution with nine volumes of 0·83% aqueous ammonium chloride and one volume of Tris-buffer (pH 7·6) of which pH was finally adjusted to 7·2 (Boyle, 1968). The final cell suspension obtained after two quick washings was free from erythrocytes and the viability of leucocytes was always more than 95% as determined by trypan blue dye exclusion. Bone marrow cells were obtained by dispersing them into cold medium 199 with 3% foetal calf serum and then washed twice in cold.

Tissue homogenates

Tissues were minced with scissors and gently homogenized with a glass homogenizer in cold Earle's balanced salt solution. The homogenates were kept in an ice bath until the large fragments of tissue settled. The homogenates in the supernatant fluid were washed at least four times followed by centrifugation for 15 min at 2000 rev/min in each instance. For the absorption test these homogenates were transferred into small glass tubes (6 × 50 mm) and were centrifuged again in the same manner. These homogenates were mainly composed of the mixture of free intact cells and cell-membrane ghosts.

Cytotoxicity test

In order to test the reactivity of NTA at 4°C and at 37°C, a mixture of 0·025 ml of test cell suspension (10^7 cells/ml) in medium 199 supplemented with 3% foetal calf serum and 0·05 ml serum at doubling dilutions was incubated in parallel at 4°C and 37°C respectively for 1 hr, then washed twice and incubated again with 0·05 ml selected rabbit serum at concentrations of 1:20 as a complement source. This rabbit serum was previously selected individually for low cytotoxicity for mouse thymocytes and bone marrow cells combined with a high complement level. A selected guinea-pig serum was used at a concentration of 1:4 as a source of complement for some tests on thymocytes of rats, hamsters and guinea-pigs, because we were unable to find any rabbit serum suitable for all tests. The trypan blue dye exclusion method was employed to determine the dead (stained) cells and live (unstained) cells. The serum which killed more than 50% of the cells in the test was graded positive for NTA. Thymocytes of male C57BL/6J at 2 months of age were used as standard test cells for NTA. The cytotoxic titre of the serum tested was tabulated as the reciprocal of the serum dilution which produced more than 50% dead cells (cytotoxic end-point). Test serum without complement and complement without test serum were always employed as negative controls. Positive control serum was NZB mouse serum pool collected from the mice at 8–10 months of age. Background level of cell death in control groups was less than 10%. When the control data exceeded 10%, all the test data were discarded and the experiments were repeated.

Absorption test

Portions of standard serum pool diluted two tubes below the cytotoxic end-point were absorbed with an equal volume of washed packed cells or tissue homogenates to be tested for 60 min at 4°C or at 37°C. The residual cytotoxicity of the supernatant fluid was tested on C57BL/6J thymocytes by cytotoxicity test.

Test for antinuclear antibody (ANA)

The indirect method was used on cryostat sections of normal guinea-pig liver which had been air dried, fixed in 95% ethyl alcohol and washed with 0·01 M phosphate buffered isotonic saline solution (PBS pH 7·6). The sections were incubated at room temperature for 30 min with 0·1 ml of each serum to be tested, followed by washing with PBS solution and treatment with fluorescein-labelled goat antibody to mouse globulins. For every test, positive control serum (NZB mouse serum) and negative control serum (CFW mouse serum) were employed.

Test for anti-erythrocyte antibody (AEA)

The mixture of 0·05 ml serum to be tested and 0·025 ml of 6% suspension of ficin-treated mouse erythrocytes were incubated for three min at room temperature, centrifuged for 30 sec in a Sero-fuge (Clay-Adams Corp., New York) and then observed for macroscopic agglutination. Tubes showing no or doubtful agglutination at this time were re-examined after further incubation for 30 min at 37°C. Positive control serum was obtained from NZB mice, negative control serum from CFW mice.

Test for G natural antibody

The indirect immunofluorescence method with suspension of viable G positive E♂G_2 indicator cells was used. A complete description of this method has been given elsewhere (Aoki, Boyse & Old, 1966).

Gel filtration of mouse serum

Chromatography on Sephadex G-200 was performed with 0·01 M phosphate buffer in 0·2 M NaCl, pH 7·6, in SR25/45 columns. Fractions obtained were adjusted to isotonic and concentrated by vacuum dialysis (Schleicher and Schuell Co., Keene, New Hampshire).

2-Mercaptoethanol (2-ME) treatment of mouse serum

The method described by Hall *et al.* (1969) was employed. 0·2 M 2-ME (Eastman Organic Chemicals, Rochester, New York) was mixed with an equal volume of mouse serum pool and incubated for 15 min at 37°C followed by 4 hr at room temperature, and then dialysed in running tap water for 2 hr followed by phosphate buffered saline solution, pH 7·6, overnight.

RESULTS

Incidence of cold-reactive and warm-reactive NTA in mice

In the previous communication (Shirai & Mellors, 1971), the incidence of NTA in several strains of mice was tested by incubating the sera to be tested and C57BL/6J thymocytes for 30 min at 22°C and then for 30 min at 4°C. In this report, however, all the serum samples were tested simultaneously both at 4°C and 37°C, because it was found that some sera from certain strains of mice were reactive only at 37°C. As shown in Table 1, the cold-reactive NTA (CR-NTA) appeared much earlier and in higher titre (Table 2) in the NZB mouse strain. A 50% incidence of CR-NTA is reached within one month after birth and almost 100% at 3 months of age. Several other strains, C3HeB/FeJ, 129/J, BALB/cJ, A/J and DBA/2J showed considerably high prevalence of CR-NTA after 7 months of age. Table 2 shows average titres of these NTA-positive sera selected from several strains of mice. NTA titres

TABLE 1. Incidence of natural cytotoxic antibody against C57BL/6J thymocytes in mice of several strains

Strains	Ages (months)	No. positive/No. tested by the test at:		Positive reaction:		
		4°C	37°C	Only at 4°C	Both at 4°C and 37°C	Only at 37°C
NZB						
	1	7/13	3/13	4	3	0
	2	27/36	14/36	14	13	1
	3–6	38/39	35/39	3	35	0
	7–11	22/22	20/22	2	20	0
	12–24	31/31	31/31	0	31	0
C57BL/6J						
	2	0/42	0/42	0	0	0
	3–6	3/28	1/28	2	1	0
	7–11	7/20	1/20	6	1	0
	12–24	5/20	4/20	3	2	2
AKR/J						
	2	9/33	3/33	8	1	2
	3–6	6/31	3/31	4	2	1
	7–11	9/56	8/56	4	5	3
129/J						
	1	0/14	0/14	0	0	0
	2	2/22	9/22	0	2	7
	3–6	9/45	8/45	5	4	4
	7–11	43/53	29/53	16	27	2
	12	10/10	10/10	0	10	0
C58J						
	2	2/29	13/29	1	1	12
	3–6	1/11	4/11	1	0	4
BALB/cJ						
	1	0/8	0/8	0	0	0
	2	2/11	0/11	2	0	0
	7–11	6/10	1/10	5	1	0
C3HeB/FeJ						
	1	2/10	0/10	2	0	0
	2	4/10	0/10	4	0	0
	7–11	10/10	3/10	7	3	0
DBA/2J						
	2	0/9	0/9	0	0	0
	3–6	2/4	0/4	2	0	0
	7–11	4/10	0/10	4	0	0
A/J						
	2	1/10	0/10	1	0	0
	7–11	4/10	0/10	4	0	0
SJL/J						
	2	2/11	0/11	2	0	0
	3–6	1/10	1/10	0	1	0
NZW						
	2	0/5	0/5	0	0	0
	3–6	0/7	1/7	0	0	1
	7–11	4/13	5/13	1	3	2
	12–24	4/20	8/20	1	3	5

Table 1. (*cont.*)

Strains	Ages (months)	No. positive/No. tested by the test at:		Positive reaction:		
		4°C	37°C	Only at 4°C	Both at 4°C and 37°C	Only at 37°C
(NZB × NZW)F_1						
	2	5/27	0/27	5	0	0
	3–6	15/28	6/28	9	6	0
(NZB × C57BL/6)F_1						
	3–6	9/16	1/16	9	0	1
	7–11	6/22	2/22	4	2	0
	12–24	18/27	17/27	2	16	1

of NZB mice were tested on randomly selected sera at varying ages. The average CR-NTA titres of NZB mouse sera at comparable ages (8–11 months) were 7·3–16-fold higher than those of CR-NTA-positive sera of any other strains. The maximum CR-NTA titre of any sera from mice other than NZB was 1:4.

Table 1 also shows the incidence of warm-reactive NTA (WR-NTA) in the same samples of serum. Almost 40% of NZB mice were positive for WR-NTA at 2 months of age and more than 90% at 5 months of age. As compared with CR-NTA, WR-NTA is less prevalent in other mouse strains tested. A rather high incidence of WR-NTA was observed in C58/J and 129/J mouse strains at 2 months of age. The tests which so far have been done showed that the titres of WR-NTA in C58/J, 129/J, AKR/J, NZW and C3HeB/FeJ did not exceed 1:2 and most of the positive were detectable only by undiluted serum (Table 2). The titres (Table 2) of WR-NTA in NZB mice were 4·2–5·8-fold higher than found in other mouse strains at comparable ages (8–11 months).

The results shown above (Table 1) indicate that certain strains of mice, particularly C58/J and 129/J at younger age, produce NTA which reacts more strongly at 37°C than at 4°C with corresponding antigen. Most sera of NZB mice were cytotoxic for C57BL/6J thymocytes both at 4°C and 37°C. This finding raises the question as to whether CR-NTA and WR-NTA in the sera of NZB mice are the same antibody molecule. If the cold and warm reactivities of NZB mouse sera for mouse thymocytes result from the activity of the same antibody molecules, one would expect that the ratio of cytotoxic titres determined at 4°C and at 37°C would be relatively constant in each individual serum. Table 3 shows the comparison of CR-NTA and WR-NTA titrations in the sera of several individual NZB mice. Generally, the titre of CR-NTA was greater than that of WR-NTA although there was considerable variation in the ratio. Four of twenty-seven sera showed higher titres in the warm than in the cold, and another three had the same titre in both tests. The incidence of CR- and WR-NTA in (NZB × NZW) F_1 and (NZB × C57BL/6) F_1 hybrids was lower than in the parental NZB strain. No significant sex differences in NTA prevalence and titre in NZB mice was observed.

Cytotoxicity of NZB mouse serum against thymocytes of mouse, rat, hamster and guinea-pig

Although the preceding tests for NTA in mice were conducted on C57BL/6J thymocytes, the NTA was cytotoxic also for thymocytes of all strains of mice tested (C57BL/6J,

TABLE 2. Cytotoxic titres of NTA in several strains of mice by the tests at 4°C and 37°C

Strains	Ages (months)	Test at 4°C		Test at 37°C	
		No. Tested	Mean arithmetic titres	No. Tested	Mean arithmetic titres
NZB					
	1–2	9	1·9	3	1·7
	3–4	17	6·2	8	2·6
	5–7	17	13·5	9	4·6
	8–11	24	16·0	15	5·8
	12–15	28	23·6	16	8·8
C57BL/6J					
	5–7	6	1·7		
	8–11	4	1·0		
AKR/J					
	2	9	1·1	2	1·0
	5–7	6	2·0		
	8–11	7	1·4	3	1·0
129/J					
	2–3	4	1·0	10	1·0
	5–7	8	1·0	6	1·2
	8–11	11	1·7	5	1·4
	12–15	6	3·3		
C58/J					
	2–3	1	1·0	14	1·1
BALB/cJ					
	8–11	6	2·2	1	1·0
C3HeB/FeJ					
	8–11	7	1·6	3	1·0
DBA/2J					
	8–11	4	1·3		
A/J					
	8–11	5	1·2		
NZW					
	5–7	1	1·0	1	1·0
	8–11	1	2 0	2	1·0
	12–15	1	2·0	1	2·0
	16–24	2	2·5	5	1·0

C58/J, BALB/cJ, SJL/J, SWR/J, DBA/2J, C3H/HeJ, C3HeB/FeJ, RF/J, AKR/J, 129/J, CFW, NZB). All tests were carried out on the thymocytes of mice of both sexes at 2 months of age. Table 4 shows the sensitivity of thymocytes of several strains of mice for the cytotoxicity of CR-NTA in the serum pool of NZB mice at 8–10 months of age. Repeated experiments indicated that the thymocytes of most strains of mice displayed similar sensitivities to the cytotoxicity of CR-NTA in the NZB mouse serum pool. In some tests however, thymocytes of the SJL/J mice displayed rather low sensitivity. The same was true for the tests at 37°C (Table 5). In this case, however, the low sensitivity of SJL/J thymocytes was

TABLE 3. Differences of cytotoxic titres of NZB mouse sera against C57BL/6J thymocytes by the simultaneous tests at 4°C and 37°C

Identification No. of NZB mouse serum (age, months)	Titres of cytotoxicity by the tests at:		Ratio of cold-reactive titre/ warm-reactive titre
	4°C	37°C	
10481♀ (4)	16	4	4
10482♀ (4)	8	2	4
10484♀ (4)	16	4	4
10494♀ (4)	4	2	2
10501♀ (4)	16	2	8
10502♀ (4)	1	2	0·5
10520♂ (4)	16	1	16
10521♂ (4)	1	4	0·25
10526♀ (4)	8	0	>8
10444♀ (5)	4	1	4
10464♀ (5)	2	2	1
10465♀ (5)	8	4	2
10367♀ (6)	32	8	4
10380♀ (6)	2	8	0·25
10391♀ (6)	16	4	4
10402♀ (6)	1	2	0·5
10403♀ (6)	32	8	4
10410♀ (6)	16	4	4
10530♂ (9)	16	16	1
10236♀ (10)	32	8	4
10229♀ (10)	32	4	8
10215♀ (10)	4	4	1
10451♀ (11)	8	4	2
10453♀ (11)	64	16	4
10472♀ (11)	16	8	2
10024♂ (15)	64	32	2
10054♀ (15)	64	32	2

more manifest. Thymocytes of all the strains of mice including SJL/J clearly absorbed the cytotoxicity of NZB mouse serum for C57BL/6J thymocytes.

In addition to NTA against mouse thymocytes, NZB mouse serum was found to contain antibodies against rat and hamster thymocytes. Fig. 1 shows the cytotoxicity against thymocytes of Wistar/Fu rats, Syrian hamsters and English short hair guinea-pigs of NZB mouse serum pool collected from the mice at 8–10 months of age. NZB mouse serum showed high cytotoxicity for hamster thymocytes, medium cytotoxicity for rat thymocytes, and low or questionable cytotoxicity for guinea-pig thymocytes.

Species specificity of natural antibodies in NZB mouse serum to the thymocytes of mouse, rat and hamster

The specificity of the cytotoxicities of NZB mouse serum against thymocytes of mouse, rat and hamster was tested by absorption. The results are shown in Table 6 indicating that the antibody against mouse thymocytes was only absorbed with mouse thymocytes (C57BL/

TABLE 4. Sensitivity of thymocytes of various strains of mice for the cytotoxicity of NZB mouse serum pool at 4°C

Thymocytes of	No. tested	Average percentage of dead cells Serum dilution 1:2	1:4	1:8	1:16	1:32	1:63
C57BL/6J	6	86	82	74	60	38	15
C58J	4	82	82	75	61	41	28
BALB/cJ	3	85	81	72	61	46	21
129/J	3	78	74	68	59	44	23
NZB	2	85	78	69	60	39	21
C3H/HeJ	1	82	80	76	62	40	26
C3HeB/FeJ	1	84	82	70	62	53	22
A/J	1	80	86	68	60	42	26
DBA/2J	1	90	84	72	63	43	26
AKR/J	1	82	72	67	57	33	16
RF/J	1	82	76	68	60	42	15
SJL	4	76	70	59	49	30	17

6J) but not with thymocytes of rats or hamsters. The same was true for the antibodies against the thymocytes of rats and hamsters. The antibodies against rats and hamsters were absorbed only with homologous thymocytes. The results shown above indicated that cytotoxic antibodies against thymocytes of mouse, rat and hamster found in NZB mouse serum are species specific and do not cross-react.

NTA-reactive antigen distribution in mouse tissues

Table 7 shows the absorption tests of NZB mouse serum pool, collected at 8–10 months of age, with several cells and tissues of mice of C57BL/6J, AKR/J and BALB/cJ strains at 2

TABLE 5. Sensitivity of thymocytes of various strains of mice for the cytotoxicity of NZB mouse serum pool at 37°C

Thymocytes of	No. tested	Average percentage of dead cells Serum dilution 1:1	1:2	1:4	1:8	1:16
C57BL/6J	3	87	76	63	31	19
C58/J	3	79	70	54	26	11
BALB/cJ	1	74	64	57	34	20
129/J	3	86	80	67	39	19
C3H/HeJ	2	78	81	70	47	21
C3HeB/FeJ	1	88	80	65	43	20
A/J	1	84	76	60	39	24
DBA/2J	1	86	80	70	45	28
SJL/J	3	53	54	40	26	17

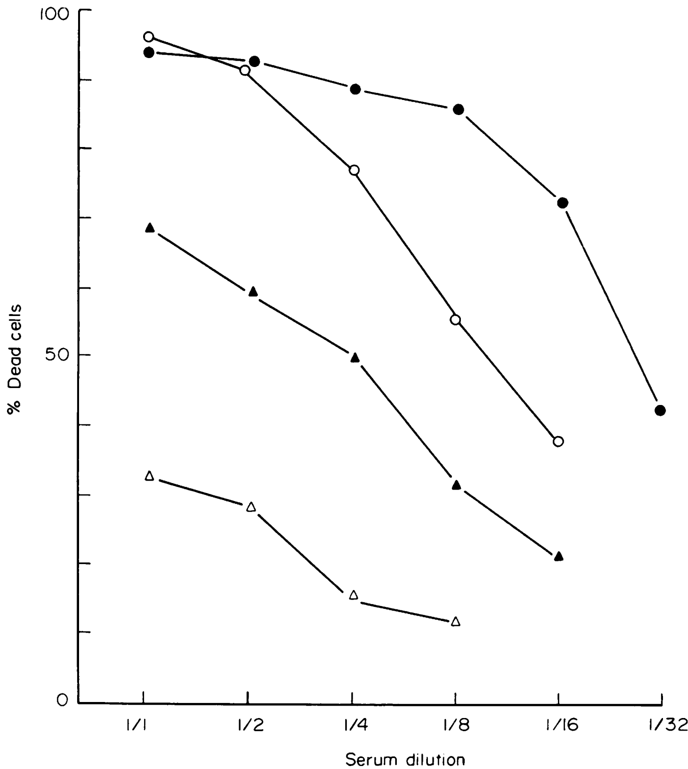

FIG. 1. Natural cytotoxic antibodies in NZB mouse serum against thymocytes of mouse, rat, hamster and guinea-pig. Average percentage of dead cells of 4 experiments. ●, C57BL/6 mouse thymocytes; ○, Syrian hamster thymocytes; ▲, Wistar/Fu rat thymocytes; ▲, English short hair guinea-pig thymoctyes.

months of age. The absorptions and the final cytotoxicity tests on C57BL/6J thymocytes were conducted at 4°C. As shown in Table 7, the thymus, lymph node, spleen and brain absorbed the cytotoxic antibody. Comparable absorption tests were carried out with NZB mouse cells and tissues collected from mice at birth and at 2 weeks and 2 months of age (Table 8). The thymus, lymph node, spleen and brain of NZB mice at 2 months of age clearly absorbed the cytotoxicity. Only the thymus of newborn mice was strongly positive for absorption, whereas brain homogenate and spleen cells of newborn NZB mice did not absorb NTA. (Lymph nodes were not available for testing.) At 2 weeks of age, the spleen and brain homogenates of NZB mice showed intermediate absorbing activity.

Similar absorption tests were carried out with C57BL/6J cells and tissues at 37°C. The test for residual cytotoxicity for C57BL/6J thymocytes was also conducted at 37°C. As shown in Table 7, the thymus, lymph node, spleen and brain absorbed WR-NTA. The absorption test with the NTA-positive 129/J or C58/J mouse serum has not been successful yet because of the low titres.

The absorption of NZB mouse serum with C57BL/6J thymocytes at 4°C and 37°C respectively abolished the residual cytotoxicity for mouse thymocytes at 37°C and 4°C respectively.

TABLE 6. Species specificity of natural antibodies in NZB mouse serum against thymocytes of mouse, rat and hamster tested by cross-absorption

Residual cytotoxicity to	NZB mouse serum pool* absorbed with											
	C57BL/6J mouse thymocytes			Wistar/Fu rat thymocytes			Syrian hamster thymocytes			None		
	Dilution of absorbed serum			Dilution of absorbed serum			Dilution of absorbed serum			Dilution of unabsorbed serum		
	1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4
C57BL/6J mouse thymocytes	<10	<10	<10	94	90	85	95	94	90	94	94	93
Wistar/Fu rat thymocytes	60	45	29	<10	<10	<10	72	64	39	65	56	48
Syrian hamster thymocytes	92	61	25	80	64	38	<10	<10	<10	94	90	70

* NZB mouse serum collected from the mice at ages between 8–10 months at a dilution of 1:1.

Cytotoxicity of NZB mouse serum for cells from thymus, lymph node, spleen and blood leucocytes in mice.

The cytotoxic activities of NTA in NZB mouse serum were compared against thymocytes, lymph node, spleen, bone marrow cells and blood leucocytes of C57BL/6J mice. As presented in the previous communication (Shirai & Mellors, 1971), the cytotoxicity of NZB mouse serum pool collected at ages between 8–10 months was strong only for thymocytes, very weak for lymph node cells, spleen cells and blood leucocytes, and negative or questionable for bone marrow cells.

Recently, we had a serum pool which was randomly collected from NZB mice at 17–19 months of age and shown to have extremely high titre of NTA: 1:1024 for CR-NTA and 1:256 for WR-NTA. In the cytotoxicity tests conducted at 4°C using this NZB mouse serum pool the cytotoxicity of NTA for lymph node cells, spleen cells and blood leucocytes was clearly detected (Fig. 2). Bone marrow cells were not affected by NTA. However, repeated experiments showed that only 50–70% of the lymph node cells, 30–50% of spleen cells and 50–75% of blood leucocytes were killed even by high concentration of the NZB mouse serum pool.

Specificity of the cytotoxicity of NTA against lymph node and spleen cells

The results shown above suggest that the cytotoxicity of NTA in NZB mouse serum is specific for thymocytes and thymus-derived lymphocytes (T-cells) in peripheral lymphoid tissues. In order to test this possibility, a preliminary study was carried out by absorption. An NZB mouse serum pool collected at 17–19 months of age was first absorbed with either C57BL/6J thymocytes or bone marrow cells and then tests were conducted for the residual

TABLE 7. Tissue distribution of NTA-reactive antigen in mice tested by absorption

NZB mouse serum pool* absorbed with	Absorption and final cytotoxicity test at 4°C						At 37°C		Result of absorption
	C57BL/6J tissues		AKR/J tissues		BALB/cJ tissues		C57BL/6J tissues		
	Dilution of absorbed serum		Dilution of absorbed serum		Dilution of absorbed serum		Dilution of absorbed serum		
	1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2	
	Residual cytotoxicity on C57BL/6J thymocytes after absorption (%)								
Cells									
Thymocytes	<10	<10	12	<10	<10	<10	<10	<10	+
Lymph node cells	18	<10	38	12	<10	<10	40	18	+
Spleen cells	20	<10	48	20	<10	<10	45	16	+
Bone marrow cells	80	55	90	78	—	—	90	68	−
Red blood cells	83	76	90	84	84	76	90	72	—
Homogenates									
Brain	<10	<10	45	20	22	<10	54	18	+
Liver	86	66	93	80	76	67	87	55	−
Kidney	83	70	94	83	75	66	84	56	−
Testis	86	75	94	83	74	71	86	75	−
None	85	81	95	84	86	72	86	73	−

* NZB mouse serum collected from the mice at ages between 8–10 months at a dilution of 1:4.

TABLE 8. Tissue distribution of NTA-reactive antigen in mice at different ages

NZB mouse serum pool* absorbed with	Tissues of NZB mice at newborn — Dilution of absorbed serum 1:1	1:2	Tissues of NZB mice at 14-days old — Dilution of absorbed serum 1:1	1:2	Tissues of NZB mice at 60-days old — Dilution of absorbed serum 1:1	1:2
	% Residual cytotoxicity on C57BL/6J thymocytes after absorption					
Cells						
Thymocytes	<10	—	<10	<10	<10	<10
Lymph node cells	—	—	—	—	14	<10
Spleen cells	—	57	44	18	26	11
Homogenates						
Brain	72	61	44	19	26	<10
Liver	65	60	70	60	72	59
Kidney	—	60	71	61	69	60
None	81	70	—	—	—	—

* NZB mouse serum collected from the mice at ages between 8–10 months at a dilution of 1:4.

cytotoxicity for C57BL/6J thymocytes, lymph node cells and spleen cells. The results clearly indicated that the cytotoxicity of the NZB mouse serum pool for lymph node cells and spleen cells was completely absorbed with thymocytes but not with bone marrow cells (Table 9).

NTA-reactive antigen distribution on tumour cells

Several tumour cell lines of C57BL/6, AKR, NZB and BALB/c mice were tested for the presence or absence of NTA-reactive antigen by absorption. Tests for residual cytotoxicity were conducted on C57BL/6J thymocytes. Transplantable thymomas 7007 and 7062 which arose in NZB mice after the administration of Imuran were strongly positive for NTA-reactive antigen. Leukaemia EL_4 induced by dimethylbenzanthracene and ERLD induced by X-irradiation of C57BL/ 6 mice also strongly absorbed NTA. However other leukaemias tested, such as E♂G_2 (Gross virus induced, C57BL/6), K36 (spontaneous, AKR), some primary leukaemias of AKR/J strain of mice and a spontaneous lymphoma 7747 of NZB mice, were negative or questionable as also a spontaneous osteogenic sarcoma 2061 of NZB mice and a myeloma MOPC70A of BALB/c strain.

Some properties of NTA

Gel-filtration on Sephadex G-200 showed that NZB-NTA occurred only in the first peak (excluded fraction) containing IgM-globulins. All the other natural antibodies in NZB mouse serum tested (antinuclear, antierythrocyte and G [Gross] natural antibody) came out in the second peak containing IgG-globulins.

2-ME treatment of a NZB mouse serum pool destroyed the cytotoxic effect of CR- and WR-NTA in contrast to the other natural antibodies shown above which appeared to be

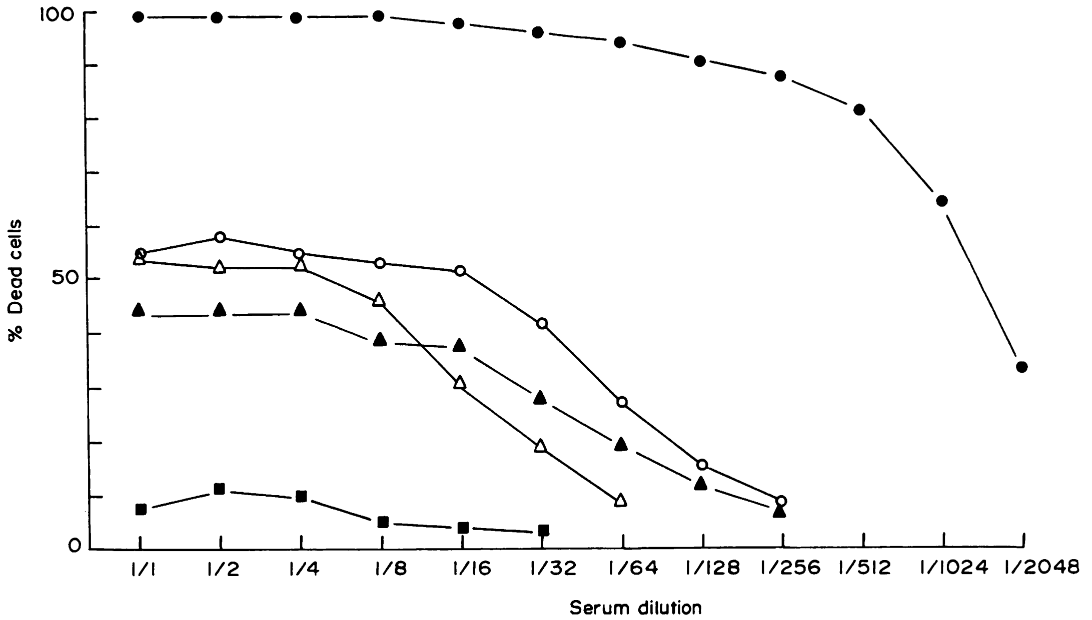

FIG. 2. Cytotoxicity of NZB mouse serum pool collected from the mice at ages between 17 to 19 months against several sources of C57BL/6J cells. Average percentage of dead cells of two experiments. ●, Thymocytes; ○, lymph node cells; ▲, spleen cells; △, blood leucocytes; ■, bone marrow cells.

intact after the treatment (Table 10). The cytotoxic antibodies in NZB mouse serum against the thymocytes of Wistar/Fu rats and Syrian hamsters were also destroyed completely by 2-ME treatment. These results indicated that natural cytotoxic antibodies in NZB mouse serum against thymocytes of mice, rats and hamsters have the properties of IgM immuno-

TABLE 9. Absorption of the cytotoxicity of NZB mouse serum pool on C57BL/6J lymph node and spleen cells by C57BL/6J thymocytes

Residual cytotoxicity to	NZB mouse serum pool* absorbed with: C57BL/6J mouse thymocytes — Dilution of absorbed serum 1:1	1:2	1:4	C57BL/6J mouse bone marrow cells — Dilution of absorbed serum 1:1	1:2	1:4	None — Dilution of serum 1:1	1:2	1:4
	Residual cytotoxicity (%)								
C57BL/6J mouse									
Thymocytes	<10	<10	<10	98	97	97	100	99	99
Lymph node cells	<10	<10	<10	44	45	28	48	47	50
Spleen cells	<10	<10	<10	40	38	27	40	41	37

* NZB mouse serum collected from the mice at ages between 17–19 months at a dilution of 1:4.

TABLE 10. Effect of 2-mercaptoethanol treatment of natural antibodies in NZB mouse serum pool

Tests for natural antibodies	Titres of natural antibodies	
	Treated	Untreated
CR-NTA	Negative	1024
WR-NTA	Negative	256
ANA	16	32
AEA	32	32
G natural antibody	8	8

globulins; the other natural antibodies, antinuclear antibody (Norins & Holmes, 1964a), erythrocyte autoantibody (Norins & Holmes, 1964b) and G natural antibody are mainly IgG immunoglobulins. The NTA in the other mouse strains was not available for these tests because of its low titre.

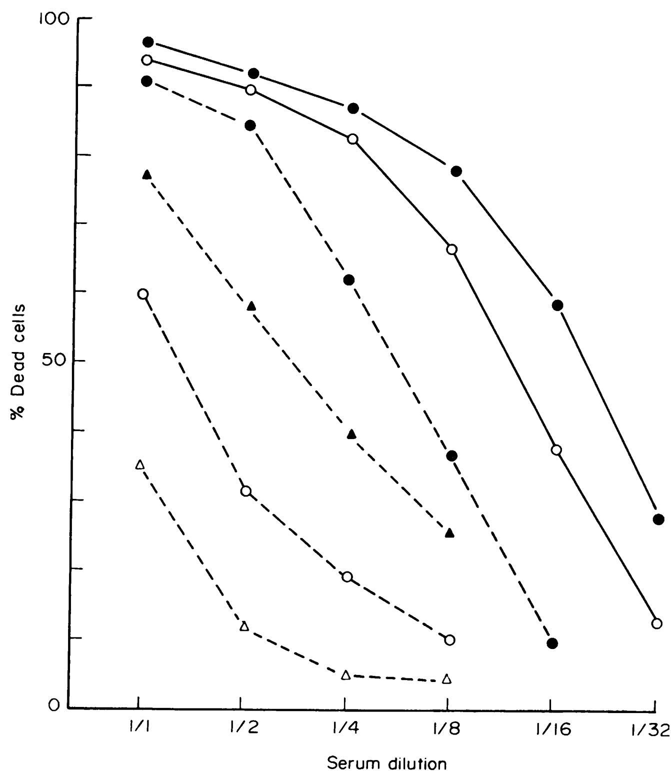

FIG. 3. Heat stability (56°C for 30 min) of cold-reactive and warm-reactive NTA in mice of NZB and 129 strains. Average percentage of dead cells of four experiments with NZB mouse serum pool and one with 129 mouse serum. ●, Untreated NZB mouse serum; ○ heat-treated NZB mouse serum; ▲, untreated 129 mouse serum; △, heat-treated 129 mouse serum; (—) cytotoxicity test at 4°C; (- - -) cytotoxicity test at 37°C.

The heat-stability of CR-NTA and WR-NTA in NZB mouse serum and of WR-NTA in 129 mouse serum was examined by incubating the sera for 30 min at 56°C. Fig. 3 summarizes the four repeated experiments, indicating that CR-NTA in NZB mouse serum is rather stable for 30 min at 56°C and WR-NTA rather labile, although it was not completely destroyed. WR-NTA in the serum of 129 strain showed a similar nature.

DISCUSSION

NZB mice, an autoimmune strain, start producing NTA in early post-natal life. Most sera of NZB mice showed cytotoxicity for mouse thymocytes both at 4°C and at 37°C. Generally, the titre of NTA was greater in the cold, but some sera of NZB mice at younger ages showed greater warm reactivity, and individual sera showed variations in the ratio of cytotoxic titre at 4°C and 37°C. This raised the question as to whether NTA comprises two types of antibodies or but one with wide thermal amplitude. The fact that 48 of 842 mouse serum samples tested were positive for NTA only when tested at 37°C strongly suggested the existence of two types of NTA in mice, namely cold-reactive NTA and warm-reactive NTA. A definitive conclusion is still difficult at present because both cold-reactivity and warm-reactivity of NZB mouse sera were absorbed with mouse thymocytes at either 4°C or 37°C.

In addition to this type of NTA in NZB mice, somewhat similar types of mouse autoantibodies against mouse thymocytes have been described (Schlesinger, 1965; Boyse *et al.*, 1970; Raff, 1971a). All these antibodies were detected by cytotoxicity test carried out at 37°C, although the assay systems were different in each instance. Schlesinger (1965) has reported the early presence of IgM natural autoantibody against mouse thymocytes in mice of the 129 strain. The cytotoxicity was very strong for thymocytes of the 129 strain, medium for those of C57BL/6, A and BALB/c and low for C3H, C58 and SJL strains of mice. While the cytotoxicity tests indicated a specific effect of the serum on thymocytes of mouse and rat and some leukaemic cells of mouse, the cytotoxicity was absorbed by all nucleated cells and tissues of mouse and rat, including kidney. The antibody was destroyed completely by heating at 56°C for 20 min. Recently Raff (1971a) described a rather similar IgM autoantibody in CBA, AKR, C3H, BALB/c, C57BL/6 and A strains of mice. There were no strain variations of thymocyte sensitivity for the cytotoxicity of a single serum tested. The reactive antigen was distributed in thymus, lymph node, spleen, brain, liver and kidney of the mouse. This autoantibody was also heat-labile. Another IgM autoantibody against mouse thymocytes was described by Boyse *et al.* (1970) which appeared in some alloantisera of mice. These authors observed strain variations in the sensitivity of thymocytes for the cytotoxicity of the autoantibody. In contrast to the natural antibodies described by Schlesinger (1965) and Raff (1971a), heat-treatment did not destroy this autoantibody. Boyse *et al.* (1970) suggested that the autoantibody in alloantiserum reacted with a complex antigenic system. In the case of NTA in NZB mouse serum, repeated experiments indicated that the cytotoxic sensitivities to NTA were similar for the thymocytes of most of the strains tested. Only SJL thymocytes were less sensitive in some tests, particularly in the warm reaction. By absorption, the thymocytes of all strains including SJL completely absorbed the cytotoxicity of NTA in NZB mice for C57BL/6 thymocytes. Cold-reactive NTA was heat stable (56°C for 30 min) and warm-reactive NTA was rather heat-labile, although it was not completely destroyed. The antigen reacting with NTA in NZB mouse serum is distributed

in the thymus, lymph node, spleen and brain of adult mice and only in the thymus of newborn mice but is not present in the liver, kidney, testis, bone marrow, and red blood cells of mice. Thus the distribution of antigen reacting with NTA in NZB mouse serum differs from that of the antigens reacting with the natural autoantibodies described by Schlesinger (1965) and Raff (1971a). NZB mouse serum also contained natural antibodies against the thymocytes of rat and hamster, but the specificities of these antibodies differed from that of NTA. Some of the differences in the nature and specificity of NTA in NZB mice as compared with other mouse strains could not be fully investigated because the titre of NTA in these strains was low as measured by the method employed. Further clarification is necessary. These efforts may contribute to a better understanding of the nature and specificity of lymphocytotoxins found in human patients with systemic lupus erythematosus, rheumatoid arthritis, infectious mononucleosis, rubella, rubeola (Terasaki, Mottironi & Barnett, 1970; Mottironi & Terasaki, 1970) and Hodgkins' disease (Grifoni *et al.*, 1970).

It is of particular interest that the tissue distribution of NZB-NTA reactive antigen is fairly similar to that of θ-alloantigen in mice (Reif & Allen, 1964). θ-alloantigen is determined by a single locus with two alleles: θ-AKR in AKR and RF mice and θ-C3H in most other strains of mice. Because NTA in NZB mice reacts with and is absorbed by thymocytes of strains of mice carrying either θ-AKR or θ-C3H allele, NTA-reactive antigen differs from these antigenic determinants of the θ system. Another comparable antigen on mouse thymocytes is mouse specific lymphocyte antigen (MSLA) recognized by the antiserum produced in rabbit (Shigeno *et al.*, 1968). However, this antigen is not present in brain tissue, unlike the antigens of the NTA reactive system and the θ-alloantigen system. Thus, the NTA-reactive antigen appeared to be distinct from other heretofore described antigens on the mouse thymocyte (Boyse & Old, 1969).

The T-cell specificity of θ-alloantigen system has recently been well defined (Raff, 1969; Schlesinger & Yron, 1969). Analogously, in the present study the cytotoxic effect of NTA in NZB mouse serum against mouse lymph node and spleen cells was absorbed with thymocytes and not with bone marrow cells. In addition, when a cytotoxic test was carried out with a serum pool obtained from NZB mice at 17–19 months of age, which pool proved to have CR-NTA titre of 1:1024 for C57BL/6 thymocytes, only a proportion of peripheral lymphocytes were susceptible to the cytotoxicity, a result comparable to that obtained with θ-alloantiserum (Raff, 1971b). T-cells in peripheral lymphoid tissues and in the circulation are known to have a reduced concentration of θ-antigen on the cell-surface (Aoki *et al.*, 1969) and to show much lower sensitivity to the cytotoxic effect of θ-antibody (Takahaski, Old & Boyse, 1970). The similar finding was made for NTA-sensitive cells in lymph node, spleen and blood leucocytes (Fig. 2). Recently, further evidence of T-cell specificity of NTA was obtained indicating that neonatal thymectomy decreased the NTA-sensitive cells in peripheral lymphoid tissues (Shirai, Yoshiki & Mellors, unpublished observation).

The immunological significance of NTA in NZB mice is presently unknown. However, older NZB mice are known to have a depletion of circulating long-lived lymphocytes (Denman & Denman, 1970) and of the lymph node-seeking population of cells (Zatz, Mellors & Lance, 1971), both of which are believed to be thymus-dependent. An impairment of cellular immune capacity to elicit graft-versus-host reaction, which is also thymus-dependent, is another feature in older NZB mice (Stutman *et al.*, 1968; Cantor *et al.*, 1970). Further, the spleen cells of NZB mice are deficient in response to phytohaemagglutinin or to allogeneic cells (Leventhal & Talal, 1970; Rodey *et al.*, 1971). The important fact is that the

cells involved in the preceding findings are thought to be T-cells. NTA may contribute to the T-cell deficiency in aged NZB mice.

It has been reported that neonatal thymectomy induces the development of glomerulonephritis (Teague *et al.*, 1970) and haemolytic anaemia associated with positive Coombs' test conversion (Yunis *et al.*, 1967) in some strains of mice and accelerates positive Coombs' test conversion in NZB mice and glomerulonephritis and positive Coombs' test conversion in (NZB × NZW) F_1 mice (Howie & Helyer, 1966). Further clarification of the possible relation between NTA production and the autoimmune manifestations in NZB mice is necessary.

On the other hand, it was noted that most strains of mice tested had NTA. Although cold-reactive NTA was the most common in these mice, it may have sufficiently wide thermal amplitude to react with the autologous thymocytes or T-cells *in vivo*. A decrease of both cellular and humoral immune capacity occurs in aged mice of non-autoimmune strains (Teller *et al.*, 1964; Makinodan & Peterson, 1964). Further, the contributions of NTA to the regulation of the magnitude and duration of cellular and humoral antibody responses in normal mice against certain exogenous antigens, as a function of immune homeostatic control, may be one of the possible hypotheses. Observations reported by Boyse *et al.* (1970) are of interest in this respect.

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ABBREVIATIONS

CR	cold reactive
G	Gross
MSLA	mouse specific lymphocyte antigen
NTA	natural thymocytotoxic antibody
T-cells	thymus-dependent lymphocytes
WR	warm reactive