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Natural Thymocytotoxic Autoantibody and Reactive Antigen in New Zealand Black and Other Mice

(sensitization phagocytosis/IgM/θ-alloantigen)

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ABSTRACT **Naturally occurring thymocytotoxic autoantibody (NTA) was detected by cytotoxic test in the sera of very young New Zealand Black mice (within 1 month after birth); the incidence was 100% at 3 months of age. Some mice from other strains also had NTA, but at an older age and with lower incidence and antibody titer. NTA had optimal activity at 4°C but was also strongly reactive at 37°C. It was cytotoxic for thymocytes of all strains of mice tested. Whereas only thymocytes were highly sensitive to NTA, the reactive antigen was demonstrated by absorption test in the thymus, lymph node, spleen, and brain of adult mice. It could be demonstrated only in the thymus of newborn mice.**

The distribution of NTA-reactive antigen suggests the presence of an antigen distinct from any so far described on the cell surface of mouse thymocytes. Gel filtration of NZB mouse serum suggests that NTA is an IgM. Mouse thymocytes sensitized with NTA *in vitro* became highly susceptible to phagocytosis by syngeneic macrophages.

The New Zealand Black (NZB) strain of mice is immunologically characterized by the spontaneous production of antierythrocyte autoantibodies beginning at about 4 months of age, followed by the development of autoimmune hemolytic anemia (1). Lupus erythematosus cell-inducing (2), antinuclear (3), anti-DNA (4), and anti-RNA (5) antibodies also occur in NZB mice but are more prevalent in (NZB × NZW) F_1 hybrid mice. NZB mice carry G (Gross)-type murine leukemia-like virus, apparently throughout life, but break tolerance to G (Gross) antigens and produce circulating free antibodies to G cell-surface (6) and viral-envelope (7) antigens in later life. In addition, thymic lesions (8, 9) and a depletion of recirculating, long-lived small lymphocytes (10) occur in NZB mice.

We describe here a naturally occurring thymocytotoxic autoantibody (NTA) that is detectable in the serum of NZB mice by cytotoxic test. This autoantibody appears early in the postnatal life of NZB mice and is present in virtually all NZB mice by 3 months of age. A somewhat similar NTA has been found by Schlesinger (11) in the sera of mice of the 129 strain.

MATERIALS AND METHODS

Cytotoxic test

Some procedural changes in the conventional cytotoxic test were necessary for the detection of NTA. No cytotoxic reaction was elicited when the cytotoxic test was carried out in the usual manner by incubating a suspension of test thymocytes, NTA-positive NZB mouse serum, and rabbit complement for 45 min at 37°C (12), but when complement was added to test thymocytes that had been washed to remove anticomplementary effect after incubation with NTA-positive NZB mouse serum, the cytotoxic reaction was clearly shown. The reactivity was stronger at 4°C and 22°C than at 37°C. In the procedure finally used, a mixture of 25 μl of test cell suspension (10^7 cells/ml) and 50 μl of serum at doubling dilutions was incubated for 30 min at 22°C and then for 30 min at 4°C, washed twice in cold Eagle's minimum essential medium (MEM) containing 3% fetal calf serum, and incubated for 30 min at 37°C with 50 μl of selected rabbit serum, 1:15 dilution, as a complement source (kindly provided by Dr. Lloyd J. Old, Sloan-Kettering Institute for Cancer Research, New York). The trypan blue dye-exclusion method was used to determine the percentage of living (unstained) and dead (stained) cells. Test serum alone and complement alone were always employed as negative controls; because these showed no more than 10% dead cells, the control data were excluded from the text. A cytotoxic test for NTA was graded positive if the undiluted serum killed more than 50% of test cells. C57BL/6J thymocytes were used as standard test cells for NTA. The cytotoxic titer of the serum was recorded as the serum dilution that produced 50% dead cells (cytotoxic endpoint).

Absorption test

Cells and tissue homogenates were prepared and tests were conducted as described by Takahashi *et al.* (13) with slight modification as follows: an equal volume of NTA-positive NZB serum pool, diluted two tubes below the cytotoxic endpoint, and washed packed cells or tissue homogenates to be tested were incubated for 30 min at 22°C and then for 30 min at 4°C. After centrifugation, the supernatant fluid was tested for residual cytotoxicity to standard test cells (C57BL/6J thymocytes).

Assay for *in vitro* phagocytosis by mouse peritoneal macrophages

Peritoneal exudate cells were obtained from C57BL/6J mice on the 4th day after an intraperitoneal injection of 1 ml of 1% glycogen. The cells were washed and cultured on coverslips in MEM with 10% fetal calf serum overnight at 37°C to obtain macrophages that attach themselves to glass (14). In

Abbreviations: NTA, natural thymocytotoxic autoantibody; NZB, New Zealand Black; NZW, New Zealand White.

the final step, the C57BL/6J thymocytes, previously treated with NTA-positive NZB mouse serum or with NTA-negative C57BL/6J mouse serum as in the cytotoxic test, were superimposed over the monolayer of macrophages. After incubation in MEM supplemented with 10% fetal calf serum for 60 min at 37°C, the cells were fixed with 10% neutral formalin and stained with hematoxylin and eosin. The percentage of macrophages showing phagocytosis of thymocytes was counted microscopically.

Mice

Strains NZB and NZW and (NZW × NZB) F_1 hybrid mice were from our colonies. Strains C57BL/6J, AKR/J, BALB/cJ, RF/J, SJL/J, DBA/J, SWR/J, A/J, C58/J, C3H/HeJ, C3HeB/FeJ,and 129/J were from Jackson Laboratories (Bar Harbor, Me). Some mice of the 129 strain were kindly provided by Dr. Lloyd J. Old (Sloan-Kettering Institute for Cancer Research, New York).

RESULTS

Distribution of NTA

Table 1 summarizes the incidence of NTA in NZB mice and several other strains of mice. The NTA was detected in five out of eight NZB mice at 2–3 weeks of age, but not in newborn NZB mice (pooled serum); 63% of NZB mice were positive for NTA at 1 month and 100% of mice were positive at 3 months. The age prevalence of positive tests for NTA was the same in male and female mice. Fig. 1 shows the NTA titration in representative sera from mice at 3 and 7 months of age. The NTA titer increased with age.

47% of (NZW × NZB) F_1 mice were positive for NTA at 5–7 months of age, and 100% were positive after 12 months of age. Several other mouse strains, NZW, C57BL/6J, AKR/J, BALB/cJ, and 129/J were positive for NTA after 5 months of age, but the prevalence and the titers of NTA were much lower than in NZB mice at corresponding ages.

Distribution of NTA-reactive antigen

Thymocytes of C57BL/6J mice were used as test cells in the preceding experiments. However, NTA was found to be cytotoxic also for thymocytes from all strains of mice tested: NZB, C57BL/6J, AKR/J, RF/J, SJL/J, DBA/2J, SWR/J, A/J, BALB/cJ, C58/J, C3H/HeJ, and C3HeB/FeJ. The thymocytes from these strains of mice displayed similar cytotoxic sensitivity to the NTA-positive NZB mouse serum pool. Absorption tests showed that the thymocytes from each of these strains of mice absorbed the cytotoxicity of the NTA-positive NZB mouse serum pool for C57BL/6J thymocytes.

Tests with cells of C57BL/6J mice indicated that the cytotoxicity of the NZB mouse serum was strong for thymocytes, very weak for lymph node cells, spleen cells, and blood leukocytes, and negative or questionable for bone marrow cells and peritoneal cells (Fig. 2). As shown by absorption tests with cells or tissue homogenates prepared from adult C57BL/6J mice, the thymus, lymph node, spleen, and brain absorbed the cytotoxic antibody (Table 2). Similar results were obtained with corresponding cells and homogenates prepared from adult NZB, BALB/cJ, and AKR/J mice, including NZB mice possessing NTA. In contrast, brain homogenates and spleen cells prepared from newborn NZB mice did not absorb NTA, whereas the thymocytes were strongly positive; lymph nodes were not available in sufficient amounts for testing. In addition to these normal tissues of mice, malignant lymphoid cells originating in the thymus also had NTA-reactive antigen: transplantable thymoma cells of NZB mice strongly absorbed NTA.

TABLE 1. *Occurrence of NTA in sera of NZB and other mice (cytotoxic test against thymocytes of C57BL/6J mice)*

Strain	No. positive/no. tested					
	Age of mice, months					
	1	2	3–4	5–7	8–11	>12
NZB	12/19	10/16	18/18	25/25	27/27	29/29
(NZW × NZB) F_1	0/6	0/2	0/5	8/17		7/7
NZW		0/5			2/18	1/4
C57BL/6J		0/20	0/5	10/40	4/24	
AKR/J		0/10	1/8	8/44	3/5	
BALB/cJ		0/4			2/10	
129/J	0/14			3/16		
129		0/5	10/1	0/3		0/1

Properties of NTA

Fractionation of NZB mouse serum pool on Sephadex G-200 showed that NTA occurred only in the first peak (excluded fractions), in contrast to the other natural antibodies which appeared in the second peak, e.g., antierythrocyte autoantibody, antinuclear antibody, and G (Gross) natural antibody. Thus NTA is probably an IgM immunoglobulin. NTA is stable for 30 min at 56°C, but rather labile when held for 15 or 30 min at 60°C. Cryoglobulin separated from an NTA-positive NZB serum pool did not react with mouse thymocytes.

In order to explore the immunobiological activity of NTA further, we studied the opsonic or immunophagocytic activity of NTA *in vitro*. As shown in Table 3 and Fig. 3, C57BL/6J thymocytes sensitized with NTA-positive serum were made highly susceptible to phagocytosis by syngeneic C57BL/6J macrophages in culture, in comparison with unsensitized thymocytes.

DISCUSSION

Natural thymocytotoxic autoantibody (NTA) in NZB mouse serum has the following properties. It is distinct from other

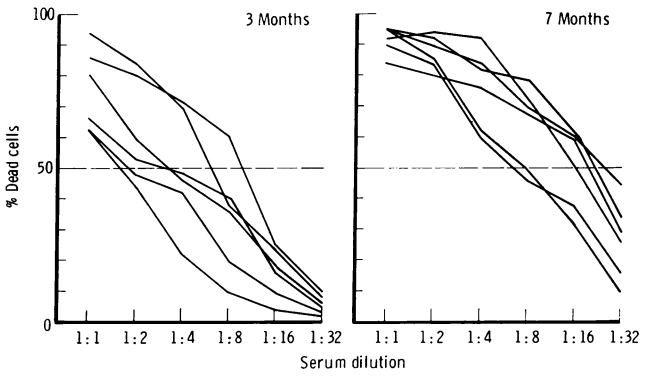

FIG. 1. NTA titrations in NZB mouse sera obtained at 3 and 7 months of age. The cytotoxicity was tested against standard test thymocytes of C57BL/6J mice. The cytotoxic end-point (50% dead cells) is indicated by dashed lines.

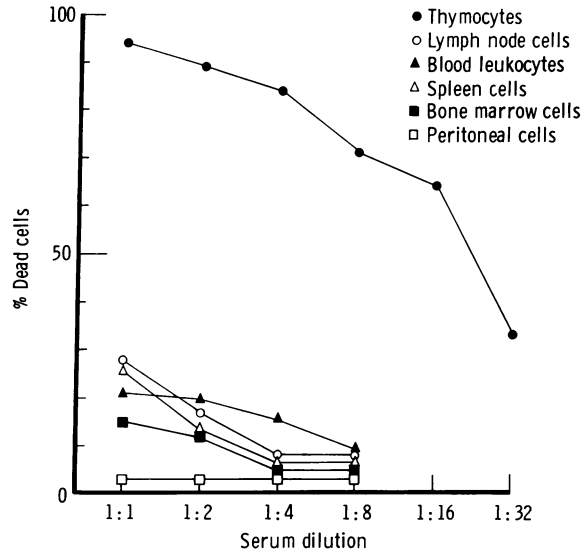

FIG. 2. Sensitivity of various cells from C57BL/6J mice to the cytotoxic effect of the NTA-positive NZB mouse serum pool. Only thymocytes are highly sensitive. The cells were prepared according to the method of Takahashi *et al.* (13).

natural antibodies in NZB mice and is apparently the earliest natural antibody produced in these mice. NTA is probably an IgM immunoglobulin and shows optimum reactivity with mouse thymocytes at 4°C, but is also reactive at 37°C. NTA of NZB mice is cytotoxic for syngeneic thymocytes as well as for the thymocytes of all other mouse strains tested. NTA is an autoantibody.

The occurrence of a somewhat similar NTA with the characteristics of IgM in the sera of mice of the 129 strain was described by Schlesinger (11). Our studies indicated that 3 of 16 mice of the 129 strain at 6 months of age had NTA as determined by the method used in the present work. The prevalence of NTA was almost the same as found in C57BL/6J and AKR/J mice at comparable ages. Additional preliminary studies (unpublished) indicated that 4 of 16 mice of the 129/J strain at 6 months of age and 2 of 10 mice of the 129 strain aged 2–14 months, obtained from Dr. Lloyd J. Old, were positive for NTA when the tests were carried out at 37°C, as

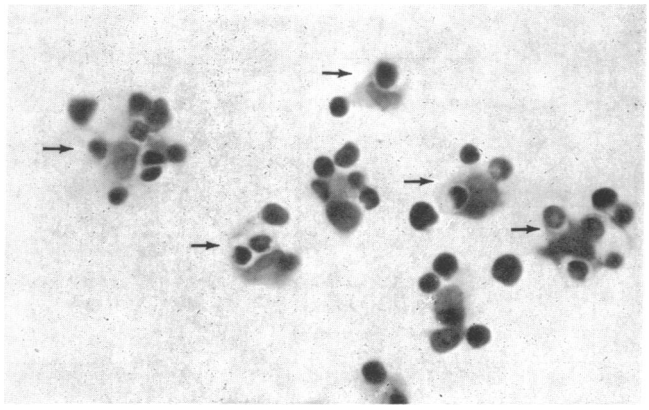

FIG. 3. Phagocytosis by syngeneic macrophages of C57BJ/6J thymocytes sensitized with NTA-positive NZB mouse serum. Arrows indicate thymocytes undergoing phagocytosis by macrophages.

TABLE 2. *Tissue distribution of NTA-reactive antigen in C57BL/6J and NZB mice as shown by absorption of cytotoxic effect of the NTA-positive NZB mouse serum pool against C57BL/6J thymocytes*

Serum absorbed with	% Dead cells (C57BL/6J thymocytes) Dilution of absorbed serum 1:1	1:2	Result of absorption
C57BL/6J (2 months)			
Thymocytes	<10	<10	+
Lymph node cells	13	<10	+
Spleen cells	11	<10	+
Bone marrow cells	59	50	−
Erythrocytes	82	70	−
Cells from testis	70	60	−
Brain homogenate	13	<10	+
Lung	77	60	−
Liver	71	48	−
Kidney	78	60	−
Salivary gland	71	66	−
Intestine	51	44	−
Heart muscle	73	65	−
Femur muscle	82	67	−
None	82	71	−
NZB (2 months)			
Thymocytes	<10	<10	+
Lymph node cells	14	<10	+
Spleen cells	26	11	+
Brain homogenate	26	<10	+
Liver	72	59	−
Kidney	69	60	−
NZB (newborn)			
Thymocytes	<10	...	+
Spleen cells	...	57	−
Brain homogenate	72	61	−
Liver	65	60	−
Kidney	...	60	−
None	81	70	−

in the work of Schlesinger (11). With but rare exceptions, mice of the 129 strain having NTA reactive at 37°C did not have NTA reactive at low temperatures. In addition, 37°C-reactive NTA of the 129 strain was rather labile when held for 30 min at 56°C. As noted previously, NTA in NZB mouse sera was most reactive at 4°C and rather stable when heated to 56°C. These findings suggest that two types of NTA,

TABLE 3. *Phagocytosis by syngeneic peritoneal macrophages of C57BL/6J thymocytes sensitized with NTA-positive serum*

C57BL/6J thymocytes treated with	Phagocytosis* (%)
NTA-positive NZB mouse serum	131/200 (65.5) $P < 0.001$
NTA-negative C57BL mouse serum	66/420 (15.7)

* (No. of macrophages showing phagocytosis)/(no. of macrophages observed).

cold- and warm-reactive, may be present in mice of a certain strain. NTA in NZB mouse sera is also reactive at 37°C: 5 of 15 mice at 2 months of age and 13 of 14 mice at 3–7 months of age were positive for NTA at 37°C (unpublished observation). Whether the individual sera of NZB mice contain both types of NTA is not yet clear.

Recently, Boyse *et al.* (15) also reported the presence in mouse alloantiserum of an autoantibody against mouse thymocytes detectable by a cytotoxic test conducted in the usual manner. This autoantibody is also an IgM immunoglobulin; its relation, if any, to NTA of NZB mice is at present unknown.

Analysis based on the absorption test indicated that NTA-reactive antigen was distributed in the thymus, spleen, lymph node, and brain of adult mice but only in the thymus of newborn mice (Table 2). However, only thymocytes were highly sensitive to the cytotoxic effect of NTA. Lymph node cells, spleen cells, and blood leukocytes were also sensitive but very weakly so. It is not known whether NTA reacts only with thymocytes (thymic lymphocytes) and thymus-derived lymphocytes in peripheral organs or with lymphocytes of other origin as well. If all the lymphocytes in peripheral organs or in blood had a low concentration of the reactive antigen on the cell surface, the lymphocytes could absorb NTA but without demonstrable sensitivity to its cytotoxic effect.

The observed characteristics of NTA-reactive antigen are very similar to the θ alloantigen in mice (16). The θ alloantigen system is also distributed in the thymus, spleen, lymph node, and brain of adult mice but only in the thymus of newborn mice and comprises at least two separate antigenic components: θ-AKR in AKR and RF mice and θ-C3H in C3H mice and in mouse strains other than AKR and RF. The NTA-reactive antigen described here is distributed in mice of all strains studied, including the AKR and C3H. The thymocytes from all mouse strains, whether possessing either θ-AKR or θ-C3H, displayed almost the same sensitivity to the cytotoxic action of the NTA-positive NZB mouse serum pool; this excludes the presence of θ alloantibody in the NZB mouse serum. The broad distribution of NTA-reactive antigen in various strains of mice suggests the existence on the cell surface of thymocytes of a newly discovered antigen, distinct from other known antigens on mouse thymocytes (17).

The biological and pathological significance of NTA is unknown at present but may prove to be of fundamental importance. Our experiments indicated that NTA promoted the opsonization, or the immune phagocytosis, of mouse thymocytes *in vitro*. If NTA were to have a regulatory role on thymocytes and thymus-derived lymphocytes *in vivo*, NTA could influence many of the cellular events involved in the induction of cellular and humoral immunity and the development of self-tolerance. Some normal mice of nonautoimmune strains had NTA, but in later life and in low titer. NZB mice, an autoimmune strain, produced NTA very early in life and in high titer. Young NZB mice are known to show hyperactivity of antibody production to some antigens and an impairment of the induction and maintenance of immunological tolerance (18); older NZB mice have an impairment of cellular immunity (19) and a depletion of recirculating, long-lived small lymphocytes (10). In the field of human medicine, lymphocytotoxic antibodies have been detected in high titer in the serum of patients with the autoimmune diseases systemic lupus erythematosus and rheumatoid arthritis, and in low titer in patients with the viral diseases infectious mononucleosis, rubella, and rubeola (20), and in the serum of patients with Hodgkin's disease (21). It is noteworthy that NZB mice carry murine leukemia-like virus particles, apparently throughout life (6); but the relation of this persisting viral infection to the production of NTA is unknown at present.

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