PRIMARY IN VITRO CELL-MEDIATED LYMPHOLYSIS REACTION OF NZB MICE AGAINST UNMODIFIED TARGETS SYNGENEIC AT THE MAJOR HISTOCOMPATIBILITY COMPLEX*

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NZB mice develop a progressive autoimmune disease resembling human systemic lupus erythematosus (1–3) in many respects. The natural history of this disease is characterized by the development of hemolytic anemia, splenomegaly, glomerulonephritis, and numerous autoantibodies (4–5). Humoral immune responses to a number of antigens have been found to be abnormal in NZB mice (4–8) and induction of tolerance to soluble protein antigens to be more difficult in NZB than in other strains (9). A variety of mechanisms have been proposed for these abnormalities (10–14). In older mice, T-cell functions like mitogen responsiveness (15), graft-versus-host reactivity (16), and cell-mediated cytotoxicity (17–20) have generally been decreased, although in a recent report (21) the level of some of these functions in the NZB strain has been observed to be within the range of normal.

In previous investigations (17–21) the phenomenon of cell-mediated lympholysis (CML)[1] in NZB mice has been examined by using targets differing at the major histocompatibility complex (MHC) from NZB or against virus-infected targets. In the experiments reported here, the primary in vitro CML activity of NZB mice was investigated against syngeneic targets and against targets carrying the NZB MHC type ($H\text{-}2^d$) on a background differing from NZB. In this situation primary in vitro CML activity is not generated by normal strains (22–26). In the present experiments a significant unidirectional CML was unexpectedly demonstrated against *H-2* identical allogeneic targets by NZB effector cells. These results represent the first demonstration of a CML reaction in a primary in vitro system directed against unmodified targets which do not differ from the cytotoxic effectors in the MHC. They provide evidence for a qualitative difference in T-cell cytotoxic function between NZB mice and control

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[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MIH, minor histocompatibility complex; PHA, phytohemagglutinin; UTHSCD, The University of Texas Health Science Center at Dallas.

strains. The capacity to respond in this system against H-2 identical targets may be related to the autoimmune disease of NZB mice.

Materials and Methods

Mice. Unless otherwise stated, 3-4 mo old male mice were used throughout the study. NZB mice were obtained from the breeding colony of the Rheumatic Disease Unit of the University of Texas Health Science Center at Dallas (UTHSCD), originally obtained from Dr. Marianne Bielschowsky, the University of Dunedin, New Zealand. BALB/c, C57BL/6, C57BL/10, B10.D2, and DBA/2 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. B6.C-H-2^d mice (HW19) were a generous gift of Doctors James Forman and Donald Bailey; all other strains used were bred and maintained in the colonies of J. K. at the UTHSCD.

Skin Grafting. Skin grafts were performed following the method described elsewhere (27).

Serological Testing. Serological tests for the presence of transplantation antigens were done according to Hauptfeld et al. (28).

Culture Media. Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.) was used for washings. RPMI-1640 (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 2 mM l-glutamine (Gibco), 100 U/ml penicillin, 0.25 μg/ml Fungizone, 100 mcg/ml streptomycin (Gibco), 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.), fetal calf serum (Gibco) 10% and 25 mM Hepes buffer (Gibco) was used for all cultures.

Cell Suspensions. Mice were sacrificed by cervical dislocation and their spleens aseptically removed. The spleens were gently teased apart in cold HBSS and the resulting suspension aspirated through a 24-gauge needle. Clumps were allowed to settle for 5 min and removed. The suspension was washed three times in cold HBSS, and after the third wash the cells were resuspended in RPMI-1640 and counted in a Coulter Counter, (Coulter Electronics, Inc., Hialeah, Fla.). All suspensions were adjusted to 4×10^6 cells/ml. Viability, as checked by trypan blue exclusion, was more than 95%.

Stimulating Cells. Stimulator cells were irradiated with 3,000 rads (cesium source, 100 rads/min).

Preparation of Effector Cells. 1/2 ml of responding cells (2×10^6 cells) and 0.5 ml of stimulator cells (2×10^6 cells) were placed in 35 × 10 mm polystyrene Petri dishes (Corning, no. 25,000, Corning Glass Works, Science Products Div., Corning, N.Y.). Each effector-stimulator combination was set up in triplicate. The dishes were incubated in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2 at 37°C on a rocking platform for 5 days (29). On the 5th day, target cells were added.

Preparation of Target Cells. 4 ml of the spleen cell suspensions containing 16×10^6 cells were incubated in Falcon 3013 flasks (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) at 37°C in an atmosphere of 5% CO_2 in air for 5 days. 48 h after the beginning of the culture, 5 μg/ml concanavalin A was added. On the 5th day, the resulting blast cell suspension was washed in HBSS and 200-400 μCi ^{51}Cr-sodium chromate in 0.2 ml saline and 50 μl FCS was added to the pellet. The cells were resuspended and incubated for 1 h at 37°C with occasional shaking. The cell suspension was then washed with HBSS, resuspended in 1 ml of medium, and layered on a Ficoll-Hypaque solution with a sp gr of 1.077 (Isolymph, Garrard-Schlesinger, Carle Place, N.Y.). The gradient was spun at 1,200 rpm for 10 min at 4°C. The cells at the interface were harvested with a Pasteur pipette and washed two times in HBSS, resuspended in medium, and counted. Viability of the cells was between 85 and 98% with most cells having a large blastoid-like appearance. The suspension was then adjusted to 1×10^5 cells/ml.

CML Assay. 10^4 target cells were added to each effector cell culture, and cultures were incubated for 4 h at 37°C in the gas mixture (83% N_2, 10% CO_2, 7% O_2). Thereafter, the content of the Petri dishes was transferred to 12 × 75-mm glass tubes and 2 ml of cold saline was added to stop the cytotoxic reaction. The tubes were shaken and spun down at 2,000 rpm for 5 min at 4°C. 1 ml of the supernate was transferred to another tube and the radioactivity in both corresponding tubes was counted in a gamma counter (Packard 5230, Packard Instrument Co., Inc., Downers Grove, Ill.). The counts were corrected for the counter background counts and ^{51}Cr release was calculated as percent of the total label incorporated according to the formula:

$$\% \text{ release} = \frac{(\text{cpm } 1/3 \text{ supernate}) \times 3}{(\text{cpm pellet} + \text{cpm } 2/3 \text{ supernate}) + (\text{cpm } 1/3 \text{ supernate})} \times 100.$$

Specific lysis was defined as the total lysis in a culture minus the background lysis of the target cells lysed. Background lysis was defined in every experiment as the release from target cells which were added to cultures of autologous effector and autologous stimulator cells. Additionally, control cultures of effector cells with allogenic stimulator cells tested against targets autologous with the effectors were set up.

Dose-Response Experiments. Effector cell cultures were pooled on the 5th day and the cell suspension washed two times in HBSS. The number of viable cells was determined by trypan blue exclusion. The desired number of potential effectors and 10^4 target cells in a total vol of 1 ml of fresh medium were placed into Falcon 2054 tubes, centrifuged at 300 rpm for 2 min, and then incubated for 4 h at 37°C in an atmosphere of 5% CO_2 in air. After 4 h, 2 ml of cold saline was added and ^{51}Cr release determined.

Anti-Thy 1 Treatment. Effector cell populations were pooled and washed two times in HBSS. The suspension was divided into two parts. One part was incubated with normal rabbit serum for 30 min in an ice bath and the other part with a rabbit anti-mouse brain serum. This serum was a gift of Dr. J. Cambier of the Department of Microbiology UTHSCD. Its production and characteristics have been described elsewhere (30). After 30 min, both the suspensions were washed in HBSS and incubated 30 min at 37°C in guinea pig serum diluted 1:4 with medium. Afterward, the suspensions were washed again two times with HBSS and viable cells were counted. The CML assay was performed in parallel with both suspensions in tubes as described above.

Mitogen Stimulation. 50,000 viable cells were incubated with PHA (Difco Laboratories, Detroit, Mich. 1:500), Con A (Pharmacia, Uppsala, Sweden, 5 μg/ml), or LPS (Difco, *E. coli* 0111:134 10 μg/ml for 2 days in round bottom microtiter plates. 1 μCi of [^{3}H]thymidine was then added to the cultures and after additional 6 h of incubation, the cultures were harvested with an automatic harvester (MASH II, Microbiological Associates) and radioactivity counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Statistical Calculations. Student's *t* tests were performed by using a program of the Medical Computing Resources Center, UTHSC at Dallas, on a DEC 10 computer.

Results

CML of NZB (H-2^d against BALB/c (H-2^d). Table I shows the results of an experiment, in which CML reactions of NZB mice of two different ages, 3 and 10 mo, against *H-2* identical BALB/c and *H-2* disparate C57BL/6 mice were examined. The NZB mice did not generate CML activity against each other, nor did BALB/c effectors react against NZB targets. In striking contrast, NZB mice, both young and old, generated a significant CML reaction against BALB/c targets. The fact that BALB/c exerted a strong CML reaction against C57BL/6 target cells indicated that the cytotoxic effector function of BALB/c was normal.

To verify the unexpected finding of primary in vitro CML against *H-2* identical targets by NZB effectors, five similar experiments were carried out. The results are summarized in Table II. In none of 12 CML tests of BALB/c effector cells against NZB targets, 6 of these against target cells of young animals and 6 against target cells of old animals, did BALB/c generate specific lysis of more than 10%, the mean specific release of all 12 reactions being 2.0 ± 2.1%. In contrast, 10 of 12 NZB versus BALB/c reactions yielded more than 10% specific lysis. The mean specific release was 26.5 ± 5.1%. The difference between the specific lysis obtained by NZB against BALB/c and that of BALB/c against NZB, is highly significant ($P < 0.001$, Student's *t* test). No significant CML reactivity was observed between old and young NZB.

Dose-Response Experiments. To establish that the NZB anti-*H-2^d* cytotoxic effect was caused by cells and not by a transferred soluble factor, two dose-response experiments were done. (Fig. 1) In the first experiment we investigated

TABLE I
Primary in Vitro CML of NZB Mice against BALB/c

Effector	Stimulator	Target	Release*	Specific release
			%	%
BALB/c	BALB/c	BALB/c	24.4 ± 2.2	–
C57BL/6			73.6 ± 1.2	49.2
NZB Old‡			53.0 ± 2.3	28.6
NZB Young§			55.8 ± 4.2	31.4
BALB/c	C57BL/6	C57BL/6	65.2 ± 1.1	44.8
C57BL/6			20.4 ± 0.6	–
NZB Old			56.0 ± 5.6	35.6
NZB Young			71.9 ± 1.2	51.5
BALB/6	NZB Old	NZB Old	34.5 ± 2.0	2.3
C57BL/6			66.8 ± 4.0	34.6
NZB Old			32.2 ± 1.3	–
NZB Young			42.5 ± 3.8	10.3
BALB/c	NZB Young	NZB Young	35.4 ± 3.8	7.1
C57BL/6			76.9 ± 1.2	48.6
NZB Old			27.2 ± 0.5	−1.1
NZB Young			28.3 ± 1.7	–

* Mean and standard error of the mean of triplicate determinations.
‡ 10 mo old.
§ 3 mo old.

TABLE II
Primary in Vitro CML of NZB Mice of Different Ages against BALB/c

Effector	Stimulator	Target	Specific release*	
			%	
C57BL/6	BALB/c	BALB/c	52.4 ± 4.9	
BALB/c	C57BL/6	C57BL/6	46.5 ± 1.6	
C57BL/6	NZB Old‡	NZB Old	40.0 ± 3.9	
C57BL/6	NZB Young§	NZB Young	42.2 ± 5.5	
NZB Old	BALB/c	BALB/c	22.8 ± 4.9	$P < 0.005$‖
BALB/c	NZB Old	NZB Old	4.5 ± 1.1	
NZB Young	BALB/c	BALB/c	30.3 ± 6.2	$P < 0.001$‖
BALB/c	NZB Young	NZB Young	0.3 ± 3.9	
NZB Old	NZB Young	NZB Young	−1.0 ± 2.2	
NZB Young	NZB Old	NZB Old	7.2 ± 2.4	

* Mean and standard error of the mean of the specific release in six experiments.
‡ 9–11 mo of age.
§ 2–4 mo of age.
‖ Student's *t* test.

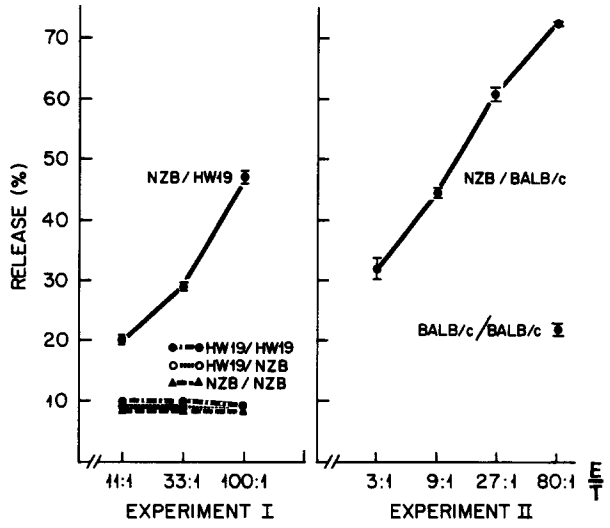

FIG. 1. Dependency of the NZB anti-*H-2*^{d} reaction on the effector/target cell ratio.

the reaction of NZB against HW19 (a congenic line, carrying *H-2*^{d}, derived from BALB/c on a C57BL/6 background); in the second experiment the NZB anti-BALB/c reaction was tested. The amount of label released from a constant number of targets (10^4) in both experiments was clearly dependent on the number of viable effector cells added, indicating that the cytotoxic effect was exerted by cells.

CML of NZB against Other H-2^d *Strains.* In subsequent experiments, the CML reactions of NZB against other *H-2*^{d} strains were tested. Table III gives a representative experiment of this series. As expected, no significant specific release was observed in primary in vitro cultures set up between the normal *H-2*^{d} carrying strains DBA/2, B10.D2, and BALB/c. Also, none of these strains reacted against NZB, although they did react with the *H-2* different target C57BL/10. In contrast, as observed above with the BALB/c target, NZB cells reacted against all tested *H-2*^{d} strains, indicating that the unidirectional CML activity of NZB against *H-2*^{d} targets was not restricted to the BALB/c *H-2*^{d}. These results were confirmed in two further experiments by using the strain HW19 in addition to the other strains.

Cross-Reactivity of NZB Effector Cells on Different H-2^d *Targets.* The specificity of the NZB effector cells obtained after BALB/c *H-2*^{d} primary in vitro sensitization was investigated in the experiment shown in Table IV. NZB effectors, sensitized with BALB/c cells, were tested against BALB/c, DBA/2, B10.D2, and C57BL/10 targets. Significant specific lysis of all three *H-2*^{d} carrying targets was observed, indicating cross-reactivity in this respect. A small reaction against C57BL/10 was observed, raising the question of cross-reactivity with a non-*H-2* identical target. This is examined below. Sensitization with C57BL/10 stimulators as a control resulted in lysis only of the appropriate target. In additional experiments, NZB effector cells sensitized with DBA/2 or B10.D2 showed the same *H-2*^{d} cross-reactions.

TABLE III
Primary in Vitro CML of NZB against Three Different H-2^d Strains

Effector	Stimulator	Target	Release*	Specific release
			%	%
C57BL/10	C57BL/10	C57BL/10	23.7 ± 2.3	–
B10.D2			66.7 ± 1.6	43.0
BALB/c			63.1 ± 1.2	39.4
DBA/2			63.5 ± 3.5	39.8
NZB			68.2 ± 3.0	44.4
B10.D2	NZB	NZB	24.4 ± 2.2	2.8
BALB/c			17.6 ± 1.6	−4.0
DBA/2			22.7 ± 1.9	1.1
NZB			21.6 ± 0.2	–
B10.D2	B10.D2	B10.D2	17.0 ± 1.9	–
BALB/c			18.6 ± 2.3	1.6
DBA/2			22.0 ± 1.0	5.0
NZB			52.1 ± 1.6	35.0
B10.D2	BALB/c	BALB/c	22.5 ± 2.6	3.0
BALB/c			19.5 ± 0.3	–
DBA/2			25.2 ± 0.5	5.7
NZB			75.2 ± 1.6	55.7
B10.D2	DBA/2	DBA/2	25.4 ± 2.3	2.7
BALB/c			22.5 ± 1.9	−0.2
DBA/2			22.7 ± 1.2	–
NZB			68.9 ± 8.2	46.2

* Mean and standard error of the mean of triplicate determinations.

TABLE IV
Cross-Reactivity of NZB CML Effectors Sensitized by BALB/c on Different H-2^d Targets

Effector	Stimulator	Target	Specific release
			%
NZB	BALB/c	BALB/c	29.1
NZB	C57BL/10	BALB/c	3.4
NZB	BALB/c	DBA/2	21.8
NZB	C57BL/10	DBA/2	6.3
NZB	BALB/c	B10.D2	19.5
NZB	C57BL/10	B10.D2	−1.0
NZB	BALB/c	C57BL/10	10.2
NZB	C57BL/10	C57BL/10	45.7
NZB	BALB/c	NZB	−1.9
NZB	C57BL/10	NZB	−2.5

TABLE V
Cross-Reactivity of NZB CML Effectors on the Congenic Lines B10 and B10D2

Effector	Stimulator	Target	Release*	Specific release
			%	%
C57BL/10	C57BL/10	C57BL/10	25.4 ± 0.8	–
C57BL/10	B10.D2		22.1 ± 0.5	−3.3
B10.D2	C57BL/10		76.2 ± 2.9	50.8
NZB	C57BL/10		79.5 ± 2.4	54.1
NZB	B10.D2		41.8 ± 11.2	16.4
C57BL/10	B10.D2	B10.D2	57.8 ± 3.4	37.0
C57BL/10	NZB		81.9 ± 1.9	60.1
B10.D2	C57BL/10		28.5 ± 2.2	7.7
B10.D2	B10.D2		20.8 ± 1.0	–
NZB	C57BL/10		77.4 ± 5.1	56.6
NZB	B10.D2		65.8 ± 6.3	45.0
B10.D2	C57BL/10	NZB	15.0 ± 0.8	−2.9
B10.D2	NZB		19.9 ± 1.8	2.0
NZB	C57BL/10		19.1 ± 1.3	1.2
NZB	B10.D2		21.7 ± 1.0	3.8
NZB	NZB		17.9 ± 1.5	–

* Mean and standard error of the mean of triplicate determinations.

Confirmation of the Presence of all H-2^d Transplantation Antigens in NZB. One possible explanation of the unidirectional cross-reactive CML response of NZB against *H-2^d* targets would be that other *H-2^d* strains possess *H-2^d* antigens which are lacking in NZB. NZB would then recognize such antigens as foreign and react against cells expressing the complete set of *H-2^d* antigens. To establish the presence of all transplantation antigens in the NZB *H-2^d* complex, an F_1 hybrid test was used. 10 (NZB × C57BL/10) F_1 hybrids were grafted with B10.D2 skin. No graft rejection occurred during the observation period of 100 days. Furthermore, serological tests for the presence of the *H-2^d* antigens *H-2* and *Ia* were carried out by the direct cytotoxic test (28) and by the absorption method (28). All tested *H-2^d* antigens were found to be expressed on NZB cells. So none of the antigens of the MHC, as defined either by the F_1 hybrid test or serologically is missing in NZB.

Cross-Reactivity of NZB Anti-H-2^d Effectors on H-2^b Targets. Another possible explanation of the NZB anti *H-2^d* CML is that the reaction is directed against minor histocompatibility (H) antigens recognized in the context of the *H-2^d* haplotype. If so, the reaction would be *H-2^d* restricted. Whether there is an *H-2* restriction of the cytotoxic effectors in NZB was investigated in experiments utilizing two pairs of congenic lines: C57BL/10 (*H-2^b*) B10.D2 (*H-2^d*), and C57BL/6 (*H-2^b*) and HW19 (*H-2^d*). The members of both pairs differ only in the *H-2* region. If NZB cells sensitized with one congenic partner were to lyse targets of both congenic partners, the CML would not be *H-2* restricted. If, on the other hand, only the *H-2* identical target were to be lysed, the *H-2* complex would be implicated in the reaction. Table V gives the result of a representative experiment and Table VI the summary of three experiments.

TABLE VI
Cross-Reactivity of NZB CML Effectors on the Congenic Lines C57BL/10 and B10.D2: Summary of Three Experiments

Effector	Stimulator	Target	Specific* release
			%
NZB	C57BL/10	C57BL/10	50.3 ± 6.4
NZB	C57BL/10	B10.D2	36.0 ± 12.5
NZB	B10.D2	B10.D2	34.3 ± 8.7
NZB	B10.D2	C57BL/10	15.3 ± 6.2
B10.D2	NZB	NZB	0.8 ± 1.3

* Mean and standard error of the mean of the specific lysis in three experiments.

TABLE VII
Effect of Anti-Thy 1 Serum Treatment on NZB Cytotoxic Reactions

Effector	Stimulator	Target	Incubated	Specific lysis
				%
NZB	C57BL/10	C57BL/10	NRS	55.6
NZB	C57BL/10	C57BL/10	Anti-Thy-1	−2.3
NZB	B10.D2	C57BL/10	NRS	23.1
NZB	B10.D2	C57BL/10	Anti-Thy-1	1.5
NZB	C57BL/10	B10.D2	NRS	60.4
NZB	C57BL/10	B10.D2	Anti-Thy-1	5.9
NZB	B10.D2	B10.D2	NRS	30.6
NZB	B10.D2	B10.D2	Anti-Thy-1	5.3

After sensitization of NZB effectors with C57BL/10, lysis is observed not only of the specific target, C57BL/10, but also of B10.D2 targets. After sensitization with B10.D2 stimulators, lysis not only of B10.D2 but also of C57BL/10 targets occurred. Similar findings were obtained in the reactions involving the congenic combination C57BL/6 and HW19. These results suggest that NZB effector cells in contrast to other strains may not be *H-2* restricted in their cytotoxic aggression. It should be pointed out, however, that the degree of lysis in the cross-reactive reactions was smaller than in the specific reactions.

T-Cell Character of the Cytotoxic Effector Cell. To determine whether cytotoxic T cells were responsible for the cytotoxic reactions demonstrated above, NZB effector sensitized either with C57BL/10 or B10.D2 were incubated with an anti-Thy 1 serum. After incubation in guinea pig serum as complement source, the effector populations were tested in an effector-to-target ratio of 35:1 on C57BL/10 and on B10.D2 targets. Additionally, the sensitized and the anti-Thy-1-treated sensitized populations were stimulated with the mitogens PHA, Con A, and LPS. Table VII gives the results of the CML tests. All CML reactions whether *H-2* allogeneic, NZB anti-H-2^d, or cross-reactive, were totally abolished

TABLE VIII
Selective T-Cell Elimination by Anti-Thy 1 Antiserum Treatment as Judged by Mitogen Stimulation

Mitogen	Treatment	cpm ± SE*
None	NRS	5,068 ± 548
None	Anti-Thy-1	1,810 ± 328
PHA	NRS	17,926 ± 1,782
PHA	Anti-Thy-1	2,169 ± 139
Con A	NRS	9,003 ± 278
Con A	Anti-Thy-1	2,139 ± 169
LPS	NRS	18,336 ± 2,121
LPS	Anti-Thy-1	22,710 ± 1,356

* Mean and standard error of the mean of triplicate determinations.

TABLE IX
*Cross-Reactivity of Cell-Mediated Lympholysis in NZB Mice**

Target		Effectors	
		BALB/c Sensitized with B10.D2	NZB Sensitized with B10.D2
C57BL/10	($H\text{-}2^b$)	−0.7	7.8
A.BY	($H\text{-}2^b$)	−0.9	9.1
B10.D2	($H\text{-}2^d$)	−2.0	42.0
DBA/2	($H\text{-}2^d$)	−5.9	27.9
B10.M	($H\text{-}2^f$)	−6.9	14.1
A.CA	($H\text{-}2^f$)	−0.4	3.4
B10.BR	($H\text{-}2^k$)	−6.3	9.2
CBA	($H\text{-}2^k$)	−1.3	12.1
B10.Q	($H\text{-}2^q$)	−0.5	10.4
DBA/1	($H\text{-}2^q$)	2.2	34.0
B10.S	($H\text{-}2^s$)	−2.6	19.1
A.SW	($H\text{-}2^s$)	−2.2	33.7

* Lysis of targets (*a*) identical to the stimulating cells, (*b*) only *H-2* identical, (*c*) only background identical, and (*d*) nonidentical. Numbers represent specific lysis in percent.

by the anti-Thy 1 treatment. The surviving cells in the antiserum treated population showed normal responses to LPS (Table VIII). These results clearly indicate that the effector cell in the CML reactions described is a T cell.

Cross-reactivity of NZB Effectors on Other B10 Lines and on Targets Allogenic in the H-2 Region and in the Background. The experiment shown in Table IX was done to determine whether NZB effector populations sensitized with $H\text{-}2^d$ cells on the B10 background would cross-react with other congenic

B10 lines expressing other *H-2* haplotypes than $H\text{-}2^d$ and $H\text{-}2^b$, like $H\text{-}2^f$ (B10.M), $H\text{-}2^k$ (B10.BR), $H\text{-}2^q$ (B10.Q), and $H\text{-}2^s$ (B10.S). Additionally, the question of whether NZB effector populations would recognize target cells which share neither the *H-2* haplotype nor the background with the stimulating cell population was investigated. In this experiment, therefore, the CML reactivity of the effector cell population against targets expressing the various *H-2* types on either B10 or non-B10 backgrounds was compared. Throughout the experiment the CML of BALB/c effector cells was assayed in parallel to the NZB effectors as a control. As expected, BALB/c, after sensitization with B10.D2, did not generate CML against any of the target cells used. In contrast, NZB sensitized with B10.D2 again reacted with the B10.D2 target, the sensitizing strain, and the DBA/2 target, carrying the $H\text{-}2^d$ haplotype of the sensitizing cell on a different background. However, there was also reaction with targets such as DBA/1 and A.SW carrying neither the *H-2* haplotype, nor the background of the sensitizing strain. These results indicate that NZB cytoxic effector cells sensitized with B10.D2 have the capacity to cross-react with background antigens of a number of mouse strains carrying different *H-2* haplotypes. They demonstrate that NZB effector cells, after $H\text{-}2^d$ sensitization, display a relatively broad cross-reactivity which is not found in the normal control strain BALB/c. These findings were confirmed in a similar experiment.

Discussion

The experiments reported here demonstrate that NZB mice after primary in vitro sensitization with *H-2* identical cells exert CML activity against target cells syngeneic at the MHC complex. These findings provide evidence for a qualitative T-cell abnormality in NZB mice compared to normal strains which do not generate cytotoxic cells under these conditions. The abnormal reactivity of NZB could be demonstrated as early as 2 mo of age, and was still present at 12 mo of age. These findings indicate that the observed defect is either inherited or acquired early in life and continues after the emergence of the autoimmune disease.

NZB effector cells, sensitized with one $H\text{-}2^d$ strain, exerted CML not only on targets identical to the strain used for sensitization, but also against all other $H\text{-}2^d$ strains tested. However, they did not lyse NZB target cells. In some experiments NZB effectors sensitized with $H\text{-}2^d$ cells cross-reacted with non-$H\text{-}2^d$ targets independently of whether these targets shared the genetic background with the stimulating cell or not. Additionally, NZB anti-$H\text{-}2^b$ effector cells were found to cross-react with $H\text{-}2^d$ carrying target cells bearing the same genetic background. Thus, for example, NZB anti-C57BL/10 effector cells were able to lyse C57BL/10 and B10.D2 target cells.

Several explanations can be considered for the unexpected observation of CML activity of NZB against MHC identical strains and for the cross-reactions described. The first possibility is that the reactions were a nonspecific effect of the in vitro system used. This explanation is ruled out by control experiments involving responding cells derived from strains other than NZB. In all cases, these cells responded with the specificity of the CML reactions as commonly recorded (22).

The second possiblity is that the NZB anti-*H-2*^{d} reactivity noted is an autoimmune response to *H-2*^{d} antigens. Such an explanation is unlikely since the same effector cells which lysed BALB/c targets did not lyse NZB targets. Were the NZB cytotoxic cells reacting with *H-2*^{d} antigens, the NZB targets would have been killed to the same extent as the BALB/c and other *H-2*^{d} targets.

The third possibility is that NZB mice differ from BALB/c and other *H-2*^{d} strains (B10.D2, HW19, DBA/2) in the *H-2* complex and that, for example, the NZB anti-BALB/c reaction is an *H-2* allogeneic reaction. Since the reaction is unidirectional, i.e. BALB/c responders do not kill NZB targets, one would have to presume that BALB/c and other *H-2*^{d} strains carry *H-2* antigens that are absent in NZB. Such an assumption is contradicted by the finding that B10.D2 skin grafts are permanently accepted by (B10 × NZB) F_1 hybrids (31). This observation was reconfirmed by grafting such F_1 hybrids derived by mating mice maintained in our animal colonies. The identity of the *H-2* haplotypes of NZB and B10.D2 is further supported by serological analysis which failed to reveal any difference between the two strains, and by the fact that grafts exchanged between B10.D2 and B10.NZB, a congenic line carrying the *H-2* haplotype of NZB on B10 background, survived indefinitely (P. Ivanyi, personal communication).

The fourth and, in our view, the most likely explanation of the NZB anti-BALB/c reactivity is that the reaction is directed against minor H antigens and as such is *H-2* restricted. As originally demonstrated by Bevan (23) and Gordon and co-workers (24), minor H antigens can be recognized by T cells, but only in the context of the *H-2* carried by the stimulating cells. The effector cells thus produced can then react with target cells sharing with the stimulators not only minor H antigens, but also *H-2* antigens. Since the NZB strain was derived independently of other *H-2*^{d} strains, such as BALB/c, B10.D2, DBA/2, HW19 (1), it very likely differs from them at a number of H loci. Furthermore, it is likely that BALB/c, B10.D2, and DBA/2 share alleles at some of these loci and differ at these same loci from NZB (22). One may, therefore, postulate that during culture of NZB cells with BALB/c stimulators, effector cells are generated against minor H antigens. However, since both strains carry the *H-2*^{d} haplotype, the recognition of minor H antigens would occur in the context of the *H-2*^{d} molecules. Such effectors would then be capable of lysing not only BALB/c target cells but also all targets that share with BALB/c the *H-2*^{d} haplotype and at least some of the minor H antigens.

If the above explanation of the NZB anti-BALB/c reactivity is correct, then the observed reactivity is the first documented instance in which CML against minor H antigens has been obtained in primary in vitro culture. In the experiments of Bevan (23) and of Gordon et al. (24), CML to minor antigens was observed only after extensive in vivo preimmunization of the prospective donors of potential effector cells in CML. Similar requirement for in vivo pre-sensitization has also been observed in all other studies of CML against MIH antigens (25, 26). What could be the reason for the unusual behavior of the NZB cells? The one striking difference between the NZB mice and other mouse strains is the development of autoimmune disease in the NZB strain, characterized by a

number of abnormalities in both humoral and cellular immunity. Prominent among these is an increased resistance to tolerance induction (9). Expanded T-cell clones specific for minor H antigens may exist in the NZB already in vivo, so that the observed reaction of NZB against *H-2* identical target cells would constitute a secondary immune reaction against these antigens. Alternatively, the NZB strain might contain factors which nonspecifically enhance the CML reaction through activation of cytotoxic T cells. Experiments to solve the question of the role of the NZB-*H-2* complex as compared to background antigens in the observed CML responses utilizing the previously mentioned B10.NZB line could not be carried out because this line has been discontinued (P. Ivanyi, personal communication, M. Micková, personal communication).

The observation that in some experiments the NZB anti-B10.D2 effectors also reacted with targets carrying haplotypes other than $H\text{-}2^d$ can be explained in one of two ways. First, it is possible that CML to some minor H antigens is not *H-2* restricted in the NZB and that the observed cross-reactivity of the NZB anti-B10.D2 cells is caused by such antigens. Second, one could argue that the cross-reactivity reflects similarity between certain seemingly unrelated *H-2* haplotypes so that T-cell effectors generated against a minor H antigen in the context of $H\text{-}2^d$ could, in the NZB, recognize this antigen in the context of $H\text{-}2^q$, for example. The reason why such cross-reactivity would be more apparent with NZB than other effectors could, again, be related to the autoimmune status of the NZB mouse. An indication that NZB effectors might be more cross-reactive than effectors derived from other mouse strains was also obtained in some allogeneic CML reactions carried out in this study. In most strain combinations CML cross-reactivity among unrelated *H-2* haplotypes is relatively difficult to demonstrate (32). In our experiments, however, cross-reactivity of NZB anti-C57BL/10 effectors on B10.D2 target cells and of NZB anti-C57BL/10 effectors on B10.D2 target cells and of NZB effectors sensitized with C57BL/6 on HW19 target cells was observed. The question of what causes the unusual CML reactivity of NZB effector cells remains open. However, by investigating this question, one may gain important information about the cellular mechanisms involved in autoimmunity.

Summary

T-cell cytotoxicity of NZB mice was tested after in vitro sensitization against a group of *H-2* identical strains (BALB/c, B10.D2, DBA/2, HW19). A highly significant and unexpected unidirectional cell-mediated lympholysis (CML) reaction by the sensitized NZB effector cells on these targets was found. After sensitization in vitro with stimulator cells of one $H\text{-}2^d$ strain, NZB effector cells ($H\text{-}2^d$) lysed all other $H\text{-}2^d$ targets and to a lesser degree, some non-$H\text{-}2^d$ targets (C57BL/10, DBA/1, B10.Q, CBA, B10.S, A.SW). NZB targets were not lysed. Differences in the major histocompatibility region between NZB and other $H\text{-}2^d$ strains could be excluded as a possible explanation for the observed reaction of NZB ($H\text{-}2^d$) against other $H\text{-}2^d$ strains. These results consequently represent the first description of a primary in vitro CML directed against determinants not coded for in the major histocompatibility complex. The responsible effector cells are demonstrated to be T cells. The CML of NZB against *H-2* identical targets appears best explained by a reaction against minor histocompatibility anti-

gens. This, and the observed cross-reactions, would indicate that the cytotoxic T-cell system in NZB mice is not subjected to restrictions found in all normal mouse strains tested until now under similar conditions. It is suggested that this hyperreactivity is related to the autoimmune responsiveness of the NZB strain.

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