Effects of Calorie Restriction on Immunologic Functions and Development of Autoimmune Disease in NZB Mice (43498)

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Abstract. **Chronic energy (calorie) intake restriction (CEIR) prolonged life, inhibited autoimmune disease, and influenced immunologic and hematologic parameters in NZB mice. Abnormalities in numbers and proportions of T and B cell populations were corrected. Deficient responses to phytomitogens, mixed lymphocyte reactions, formation of plaque-forming cells to sheep red blood cells *in vitro*, production of cytotoxic T lymphocytes after *in vitro* stimulation, and interleukin 2 production were also corrected. CEIR prevented the extreme splenomegaly that normally occurs with age in NZB mice. This influence was associated with reduction of a greatly expanded non-T, non-B lymphoid cell population. Calorie restriction also prevented in NZB mice the rapid decrease in total numbers of colony-forming B cells in bone marrow that is also characteristic of mice of this strain. The influences of CEIR on immune parameters and hematopoiesis were generally less marked in non-autoimmune-prone DBA/2 mice than in autoimmune-prone NZB mice. CEIR has been shown to produce profound influences on several strains of autoimmune-prone mice (NZB × NZW)F1, MRL/*lpr*, BXSB, and NZB herein). In each of these strains, the pathogenesis and manifestations of autoimmune disease are dissimilar. Therefore, it seems likely that calorie restriction acts on an as yet elusive mechanism that operates to foster development of the diseases associated with aging common to each of these autoimmune strains as well as autoimmune-resistant mice and rats. Further investigation of the molecular and cellular bases of the benefits of CEIR seems urgent.** [P.S.E.B.M. 1992, Vol 201]

The NZB mouse strain is an experimental model of spontaneous autoimmune disease characterized by autoantibodies to erythrocytes, T lymphocytes, and nucleic acids. Such mice die with autoimmune hemolytic anemia, lymphoid malignancy or immune complex nephritis at a mean age of 15–18 months (1, 2). We have shown that feeding a standard nonpurified diet relatively low in protein and high in fat improved breeding performance in NZB mice, but fostered development of autoimmune hemolytic anemia and shortened life-span, whereas a diet higher in protein and lower in fat yielded a longer median life-span (3, 4). These studies were not ideal, however, because the complete composition of the diets was not defined.

We also showed that selective protein restriction altered some immune functions in NZB and (NZB × NZW)F1 (B/W) mice, but did not prolong life-span (5, 6). On the other hand, chronic energy intake restriction (CEIR), consisting of 40% calorie restriction and a diet relatively high in carbohydrate and relatively low in fat, doubled or even tripled the life-span of B/W or other autoimmune strains (7–10) and prevented the decline of certain immunologic functions in these mice.

To examine further the influences of CEIR on life-span and the development of autoimmune disease, we analyzed the effects of CEIR on immune functions in the spleen, thymus, and bone marrow of NZB mice. CEIR profoundly affected life-span and development of autoimmune disease in NZB mice. Furthermore, CEIR was associated with maintenance of immune

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functions that had become progressively deficient in full-fed NZB mice.

Materials and Methods

Mice. Inbred, 6-week-old, female NZB/Bin and DBA/2 mice (Jackson Laboratory, Bar Harbor, ME) were housed individually and fed as specified in animal rooms operated on a 12:12-hr light:dark cycle. CBA/H mice (Jackson Laboratory) were housed in groups and fed a commercial diet *ad libitum*. Each group comprised 20 mice.

Diets. Diets used in this study were prepared weekly, as described (7–10), and stored at 4°C. Full-fed mice were given a fixed amount of food equal to 18 kcal/day/mouse every other day. Half that amount of food (9 kcal/day/mouse) was given to the CEIR mice. With the full-fed mice, some of the diet was left over so that the restriction was actually approximately 40%. However, mice fed the CEIR diets were given vitamin and salt mixtures adjusted to provide intakes equivalent to those of the full-fed mice. The diets were begun at weaning (age 6 weeks). Representative mice were sacrificed at 9 and 13 months of age for evaluation.

Cell Preparation for Immunologic Assay. Mice were bled by cutting the femoral arteries and sacrificed by cervical dislocation. Spleens and bone marrow were collected aseptically. Single cell suspensions were made by gently squeezing spleen tissue between two glass slides in Hanks' balanced salt solution (Gibco, Grand Island, NY), with gauze filtration to remove residual large fragments. Cells were washed three times with Hanks' balanced salt solution before use. Bone marrow cells were obtained by flushing the femurs with cold RPMI 1640 medium (Gibco) supplemented with 5% fetal calf serum (FCS). Single cell suspensions were prepared by vigorous pipetting through a 13-gauge needle. Large debris and bone fractions were removed by layering the suspensions over FCS, letting fragments settle, and decanting the suspended cells.

Culture Medium. RPMI 1640 medium was made to 1 μM sodium pyruvate, 5 mM HEPES. Penicillin (100 units/ml), 100 μg/ml of streptomycin, 5×10^{-5} moles of 2-mercaptoethanol, and 10% FCS were added to the culture medium. Normal CBA/H mouse serum (1%) was used instead of FCS to assay responses to mitogen stimulation or mixed lymphocyte reaction.

Agar Culture. B lymphocytes were plated to form colonies in semisolid agar cultures containing 2-mercaptoethanol, 15% FCS, amino acids, and vitamins in McCoy medium (11). Lipopolysaccharide (25 μg/plate) was used to potentiate colony formation. After 6–7 days of incubation at 7% CO_2, colonies were counted using a dissecting microscope. Granulocyte-macrophage progenitors (colony-forming unit culture [CFU-c]) were cultured in semisolid agar medium containing an appropriate source of colony-stimulating activities (CSA). Conditioned medium from L cells predominantly elicits macrophage colonies from full-fed mice of several strains, and cells from NZB mice respond normally to this stimulus. In contrast, proliferation of marrow progenitor cells from NZB mice is suboptimal when conditioned medium from WEHI-3 cells is used as a source of CSA.

Immunofluorescence. Cells bearing sIg or Thy 1.2 antigen were detected using fluorescein isothiocyanate-labeled goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) or fluorescein-labeled anti-Thy 1.2 monoclonal antibodies (Becton-Dickinson, Mountain View, CA). A macromolecular antigen characteristic of cells of the B lineage was detected using monoclonal rat antibody 14.8 and purified, fluorescein-labeled mouse anti-rat Ig antibodies (12).

Mitogen Stimulation. Mitogen-induced blastogenesis (9) was measured using: phytohemagglutinin P ([PHA-P] Difco, Detroit, MI), 0.1% v/v; concanavalin A ([Con A] Calbiochem, La Jolla, CA), 2 μg/ml; *Salmonella typhosa* lipopolysaccharide (Difco), 50 μg/ml; or pokeweed mitogen ([PWM] Gibco), 1% v/v in RPMI 1640.

Cytotoxic Assay. Cytolytic activity was tested by microcytotoxic assay as described (9).

Mixed Lymphocyte Reaction. Cultures containing 4×10^5 responding cells and the same number of allogeneic stimulator cells were subjected to 2000 rad gamma ray irradiation in a total of 200 μl of RPMI 1640 medium. Cultures were set up in flat-bottomed microtiter plates and incubated for 64 hr at 37°C with 5% CO_2, then 0.4 μCi of [^{3}H]thymidine was added for 8 hr more. Samples were collected on glass fiber filters using a mini-MASH harvester (Microbiological Associates, Walkersville, MD), placed in vials containing Scintilene (Fisher, Fair Lawn, NJ) and counted using a liquid scintillation spectrometer.

Induction of Cytotoxic Cells. *In vitro* allogeneic immunization was performed in 24 flat-bottom well plates (Linbro Scientific, McLean, VA). Duplicate cultures were set up with 500 μl containing 1×10^7/ml spleen cells and 250 μl of 2000-rad ^{137}Cs-irradiated C57BL/6 spleen cells containing 1×10^7 cells/ml in RPMI 1640 and incubated for 4 days at 37°C with 5% CO_2. EL-4 tumor cells, a benzo[α]pyrene-induced lymphoma line of C57BL/6 origin, were used as target cells.

Induction of Plaque-Forming Cells. Triplicate cultures of 500 μl of 1×10^7/ml spleen cells and the same volume and number of sheep red blood cells (SRBC) in 24 flat-bottom well plates were incubated for 5 days at 37°C with 5% CO_2. Cultures were pooled and plaque-forming cell numbers determined (13).

Interleukin 2 Production. Spleen cells (5×10^6) suspended in 1 ml of RPMI 1640 supplemented with 2 μg/ml of Con A were cultured in 24-well tissue culture plates (Linbro) for 24 hr at 37°C with 5% CO_2. After

centrifugation at 1500*g* for 10 min, cell-free supernatants were collected and stored at −20°C. Interleukin (IL) 2 activity of supernatants was determined by quantifying the influence of supernatants on growth of an IL-2-dependent T cell line (14).

Hematology. Blood samples were obtained from the retro-orbital sinus using heparinized microcapillary tubes. Hematocrit and white blood cell counts were determined with standard methodology.

Assay of Circulating Immune Complexes. The Raji cell radioimmunoassay adapted for mice was used to measure circulating immune complexes (CIC) in sera (15). Results are expressed as μg equivalents of aggregated murine IgG per ml of serum.

Statistics. Statistical analyses were performed using Student's *t* test; *P*-values <0.05 were considered significant.

Results

Growth of Mice. Full-fed, *ad libitum*-fed, NZB mice that had calorie intake compared with CEIR-fed mice of approximately 100/60 gained weight steadily, reaching a mean weight of approximately 42 g at 10 months of age. Full-fed DBA/2 mice gained less weight (mean weight, 33 g at 10 months). Mice subjected to CEIR from time of weaning showed only little change in weight throughout the study, but did grow over the course of the investigation. Although NZB and DBA/2 mice in the CEIR groups consumed the same diets, NZB mice on the CEIR diets were slightly heavier than DBA/2 mice, but these differences were not significant. Growth curves for mean body weights are shown in Figure 1.

Organ Weights. Spleen weight (mean weight, 237 ± 79 g), particularly spleen weight relative to total body weight (0.57% ± 0.19%), was greatly increased in full-fed NZB mice at 9 months of age. By contrast, mean actual spleen weight (50 ± 3 g) and relative spleen weight (0.20% ± 0.02%) were significantly reduced in NZB mice fed the CEIR diet ($P < 0.01$). In DBA/2 mice, however, spleen weight was not decreased relative to body weight as a function of CEIR. The mean weights of liver and kidney were somewhat reduced by CEIR. Thymus weights were well maintained in the CEIR mice, a finding taken as evidence that such dietary intervention prevented thymic wasting and involution.

Survival. Full-fed NZB female mice had short, median, and absolute life-spans: 90% died by 18 months of age. Only 10% of NZB female mice fed the CEIR diet, and none of the DBA/2 female mice, were dead at 18 months of age (Fig. 2).

Hematology. As shown in Table I, leukocyte counts were low at 9 and 13 months of age in CEIR mice. By contrast, full-fed NZB mice had elevated leukocyte counts at age 13 months ($P < 0.01$). Full-fed NZB mice ($P < 0.01$) had lower hematocrit values than did NZB mice fed their CEIR diet ($P < 0.01$). All DBA/2 mice had normal hematocrit levels. CIC were dramatically reduced by CEIR in the NZB mice. DBA/2 mice had low levels of CIC regardless of the amount of calories consumed.

Total Cells, sIg$^+$ Cell and Thy 1.2$^+$ Cells in Spleen. Absolute and relative numbers of cells of several subpopulations among the spleen cells were influenced by CEIR (Table II). NZB mice fed *ad libitum* had enlarged spleens and showed the largest number of total spleen cells. In NZB mice, the absolute number of spleen cells from full-fed mice was nearly 4 times that of the CEIR mice at 9 months of age, and 20 times greater at 13 months. That the NZB mice on the CEIR diet did not develop splenomegaly could be attributed to a great decrease in the cells that did not bear surface Ig or Thy 1 surface markers. Full-fed DBA/2 mice showed no splenomegaly and had normal cell numbers that were significantly higher than those of DBA mice fed the CEIR diet.

In NZB mice, the relative proportions of Ig$^+$ cells and Thy 1.2$^+$ cells present in the spleen of full-fed mice were lower than in CEIR mice at 13 months of age ($P < 0.05$). DBA/2 mice fed either diet had equivalent

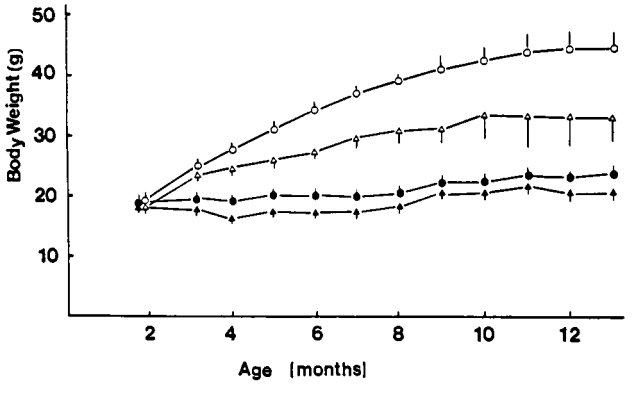

Figure 1. Body weights (mean ± SE) of full-fed or CEIR NZB and DBA/2 mice: ○, full-fed NZB; △, full-fed DBA/2; ●, CEIR NZB; and ▲, CEIR DBA/2.

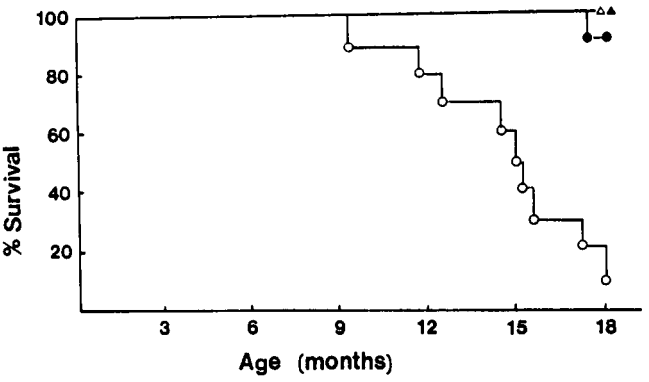

Figure 2. Survival of full-fed NZB mice (○), CEIR NZB mice (●), full-fed DBA/2 mice (△), and CEIR DBA/2 mice (▲).

Table I. Dietary Influence on Peripheral White Blood Cells, Hematocrit, and Serum CIC[a]

Strain	Calorie	WBC (/mm³)		Ht (%)	CIC (μg/ml)[b]	
		9 mo	13 mo	13 mo	9 mo	13 mo
NZB	High	3,060 ± 300	27,349 ± 10,875[c]	36.0 ± 1.6[c]	989 ± 176[c]	2,472 ± 864[c]
	Low	2,300 ± 156	3,009 ± 258	42.0 ± 0.7	141 ± 6	447 ± 59
DBA/2	High	3,420 ± 366	4,300 ± 373	47.0 ± 2.0	29 ± 9	<20
	Low	2,340 ± 261	2,920 ± 277	49.8 ± 0.6	27 ± 17	<20
CBA/H	Lab chow	6,780 ± 840	ND	50.2 ± 0.8	143 ± 7	390 ± 161

[a] Results are shown as mean ± SE for three mice of each group.
[b] Results are expressed as μg equivalents of aggregated murine IgG per ml of murine serum.
[c] $P < 0.01$ compared with low calorie group.

Table II. Effects of Calorie Restriction on Spleen Cells in NZB and DBA/2 Mice[a]

Strain	Calorie	Cell number (×10⁻⁷)		IgG⁺ cell (%)	Thy 1.2⁺ cell (%)	CFU-B at 9 mo	
		9 mo	13 mo	13 mo	13 mo	Colonies/10⁵	Colonies/spleen
NZB	High	34.5 ± 15.5[b]	116.0 ± 23.1[b]	9.8 ± 5.3[c]	7.3 ± 2.5[c]	675 ± 65[c]	2194 ± 1028
	Low	8.0 ± 1.6	5.5 ± 0.2	29.7 ± 1.8	30.1 ± 1.8	2133 ± 80	1658 ± 308
DBA/2	High	15.1 ± 1.1[c]	10.0 ± 2.0	31.4 ± 3.6	10.9 ± 0.9	1466 ± 186	2246 ± 411
	Low	7.8 ± 1.2	8.7 ± 0.8	37.7 ± 2.5	13.1 ± 1.6	2003 ± 160	1591 ± 329
CBA/H	Lab chow	7.6 ± 0.6	ND	31.3 ± 1.5	23.6 ± 2.7	ND	ND

[a] Results are shown as mean ± SE for three mice of each group.
[b] $P < 0.01$ compared with low calorie group.
[c] $P < 0.05$ compared with low calorie group.

proportions of Ig^+ cells and Thy 1.2^+ cells. The relative number of Thy 1.2^+ cells in spleens from DBA/2 mice was lower than that in spleens from NZB mice on CEIR. The most dramatic changes were noted in total numbers of non-T and non-B (Thy-1^-, sIg^-) cells. The number of such cells was increased 44-fold in full-fed NZB mice compared with CEIR mice (Table II).

Clonable B cells (CFU-B) in spleens were also investigated (Table II). No significant differences in total CFU-B per spleen were observed at 9 months. However, the proportion of DFU-B per 10^5 cells was much lower in the full-fed NZB mice than in the NZB mice on CEIR ($P < 0.05$). Again, this dramatic change was attributed to expansion of "null" cells in the spleen in full-fed mice and the absence of such a change in the CEIR mice.

Spleen Cell Response to Mitogens. At 9 months of age, full-fed NZB mice showed decreased responses of the total spleen cell population to stimulation with PHA, Con A, and PWM compared with CEIR mice of the same strain (Table III). Full-fed DBA/2 mice also showed decreased responses to PHA and PWM compared with their counterparts on CEIR. This difference was more pronounced at 13 months of age. Total spleen cells of full-fed NZB mice responded poorly to all mitogens studied. By contrast, NZB mice consuming the CEIR diet had vigorous proliferative responses to PHA, Con A, lipopolysaccharide, and PWM. However, the change in these parameters again seems to most dramatically reflect the greatly increased numbers of non-T and non-B cells in the full-fed mice, an expansion that is characteristic of the NZB strain. Responses were almost the same order of magnitude when considered as response per number of cultured T cells to PHA or Con A, or per number of cultured B cells to lipopolysaccharide.

Mixed Lymphocyte Reaction. Table IV summarizes *in vitro* proliferative responses of total spleen cells against irradiated allogeneic C57BL/6 or CBA/H spleen cells. In NZB mice, spleen cells of CEIR mice responded more vigorously than did those of full-fed mice at 9 months of age ($P < 0.05$); this difference was even more dramatic at 13 months ($P < 0.01$). This finding may also reflect the reduced proportion of T cells relative to total cells in the spleens of full-fed mice. In the DBA/2 mouse strain, responses were comparatively more vigorous in CEIR mice when CBA/H stimulator cells were used ($P < 0.05$).

Cytotoxic Responses to *In Vitro* Immunization with Alloantigen. Cytotoxic responses of total spleen cells from NZB or DBA/2 mice to C57BL/6 spleen cells was studied. At 9 months, no significant differences were observed in NZB mice as a function of diet (data not shown). At 13 months, however, responses of full-fed NZB mice were markedly decreased, but those of the CEIR group were maintained. Full-fed DBA/2

Table III. Effects of Calorie Restriction on Mitogen Responses of Spleen Cells in Mice[a]

Age (mo)	Strain	Calorie	Mitogen				
			(−)	PHA-P	Con A	Lipopolysaccharide	PWM
9	NZB	High	7,107 ± 2,074	18,165 ± 8,355[b]	56,201 ± 16,734[b]	143,713 ± 13,755	84,705 ± 31,427[b]
		Low	10,564 ± 1,071	58,642 ± 13,843	105,102 ± 7,764	156,026 ± 1,785	179,252 ± 10,850
	DBA/2	High	5,875 ± 803	13,166 ± 1,699[b]	148,952 ± 4,160	94,806 ± 5,107	76,053 ± 5,753[b]
		Low	8,420 ± 1,691	51,399 ± 10,582	114,693 ± 4,836	90,693 ± 11,248	134,819 ± 6,164
13	NZB	High	781 ± 262	3,051 ± 352[c]	41,521 ± 27,216[b]	18,458 ± 12,052[c]	2,163 ± 1,000[c]
		Low	4,905 ± 1,243	23,125 ± 10,746	104,664 ± 26,842	100,282 ± 18,990	139,217 ± 5,686
	DBA/2	High	8,583 ± 2,662	20,221 ± 1,798	146,582 ± 15,796	80,661 ± 7,718	62,242 ± 13,867
		Low	8,385 ± 1,008	24,165 ± 1,571	120,657 ± 7,172	99,303 ± 4,846	92,984 ± 4,221
	CBA/H	Lab chow	5,732 ± 28	75,620 ± 365	169,219 ± 3,885	67,093 ± 2,477	111,002 ± 8,018

[a] Spleen cells were cultured for 64 hr at 37°C and then [^{3}H]thymidine was added for an additional 8 hr of incubation.
[b] $P < 0.05$ compared with low calorie group.
[c] $P < 0.01$ compared with low calorie group.

Table IV. Effects of Calorie Restriction on the Mixed Lymphocyte Reaction of Spleen Cells from NZB or DBA/2 Mice[a]

Age (mo)	Strain	Calorie	Stimulator cells		
			(−)	C57BL/6	CBA/H
9	NZB	High	5,082 ± 2,240	21,900 ± 9,880[b]	ND
		Low	7,549 ± 1,286	47,154 ± 1,972	ND
	DBA/2	High	3,695 ± 658	25,151 ± 5,469	ND
		Low	6,693 ± 1,489	38,369 ± 8,272	ND
13	NZB	High	604 ± 174	1,135 ± 351[c]	931 ± 256[c]
		Low	3,585 ± 948	28,797 ± 1,875	23,811 ± 455
	DBA/2	High	6,333 ± 1,460	26,128 ± 1,844	28,461 ± 2,346[b]
		Low	7,676 ± 929	36,612 ± 7,678	33,882 ± 224
	CBA/H	Lab chow	6,247 ± 1,335	42,202 ± 3,690	5,977 ± 336

[a] Three individual spleens were tested in each group using triplicate samples. Cell suspensions were incubated for 64 hr at 37°C and then [^{3}H]thymidine was added for an additional 8 hr of incubation. Results are shown as mean ± SE.
[b] $P < 0.05$ compared with low calorie group.
[c] $P < 0.01$ compared with low calorie group.

mice showed decreased responses compared with DBA/2 mice on the CEIR diet at 9 months of age, but differences were not significant between the two groups at 13 months.

IL-2 Production by Spleen Cells. As was expected, total spleen cells taken from autoimmune-prone NZB mice produced less IL-2 than did autoimmune-resistant DBA/2 mice (data not shown). IL-2 production by total spleen cells from full-fed NZB mice decreased markedly with age, as was expected given the decreased proportions of T cells in the enlarged NZB spleens. This deficiency, like the splenomegaly, was significantly correlated by CEIR (data not shown). In the DBA/2 groups, IL-2 production by spleen cells from the CEIR group was not significantly affected by diet at 9 or 13 months of age.

Antibody Formation after *In Vitro* Immunization with SRBC. Full-fed NZB mice showed strikingly deficient responses of the total spleen cell population to SRBC at 13 months of age (not shown). Plaque-forming cell responses of total spleen cells from NZB mice on CEIR were similar to levels observed with total spleen cells of CEIR DBA/2 mice, which in turn were slightly lower than those in full-fed DBA/2 mice (not shown).

B Lymphocyte Lineage Cells in Bone Marrow. Since calorie restriction dramatically affected relative B cell numbers but not total numbers of functional B cells (CFU-B) in peripheral lymphoid tissues, we investigated the influence of CEIR on bone marrow function (Table V). Mice of the two strains and dietary groups did not differ significantly in total numbers of nucleated cells in bone marrow taken from the femur.

Cells bearing the 14.8 marker include $c\mu^+$ B cell precursors (pre-B cells) as well as sIg^+ B cells (16, 17), and also a particular subset of peripheral T lymphocytes (12, 18, 19). The 14.8^+ cells were shown to decrease progressively with age in NZB mice. Indeed, this decline of 14.8^+ cells regularly preceded evidence of development of autoimmunity and autoimmune disease (20). In the present study, the total number of 14.8^+ cells

Table V. Influence of Calorie Restriction on Bone Marrow Cells[a]

Mice	Calorie	Cell number/femur ($\times 10^{-7}$)		14.8^+ (%)	CRU-B			
					9 mo		13 mo	
		9 mo	13 mo	9 mo	/10^5	/femur	/10^5	/femur
NZB	High	1.51 ± 0.71	1.11 ± 0.10	7.2 ± 0.5[b]	113 ± 10	13,009 ± 495[b]	42 ± 18	4,578 ± 1,805[b]
	Low	1.54 ± 0.12	1.07 ± 0.11	11.9 ± 1.5	362 ± 169	43,233 ± 18,787	428 ± 71	44,532 ± 4,013
DBA/2	High	1.34 ± 0.07	1.04 ± 0.11	17.3 ± 1.4	66 ± 40	9,751 ± 5,929	76 ± 23	7,460 ± 1,398
	Low	1.20 ± 0.16	0.96 ± 0.19	19.7 ± 1.1	81 ± 55	7,442 ± 5,063	52 ± 10	4,251 ± 109

[a] Results are shown as mean ± SE. Each group consisted of three mice.
[b] $P < 0.05$ compared with low calorie group.

was found to be lower in bone marrow of NZB mice than in DBA/2 mice. This parameter was significantly, but not dramatically, influenced by diet. The numbers of 14.8^+ cells in bone marrow from full-fed NZB mice were significantly lower than those in NZB mice on the restricted diet ($P < 0.05$).

The numbers of clonable B cells in bone marrow were substantially higher in NZB than in DBA/2 mice at 9 months of age. The numbers of these cells in full-fed NZB mice became lower than in DBA/2 mice by 13 months of age. It has been reported that clonable B cells decreased with aging in NZB mice fed a lab chow diet (20). In the present study, total number NZB mice on the CEIR diet showed a marked increase of clonable B cells at 9 months of age and maintained this increase up to 13 months of age, when the experiments were terminated.

Myeloid Progenitor Cells. Cells capable of giving rise to colonies of granulocytes and macrophages were also studied (Table VI). NZB mice are atypical in terms of responsiveness of their bone marrow cells to a particular type of stimulus (21). The incidence of colony-forming myeloid progenitor cells (CFU-c) from marrow of DBA/2 mice was much higher than that of the marrow of NZB mice when WEHI-3 supernatant (CSA) was used as a stimulus. No difference was seen between the strains when L cell-conditioned medium was the source of CSA. CFU-c capacity was not dramatically influenced by diet.

Discussion

In the 1930s, McCay *et al.* (22, 23) showed that the life-span of rats was significantly prolonged by dietary restriction. These findings were confirmed and extended by Weindruch *et al.* (24) and Weindruch and Walford (25) for mice and rats of long-lived strains in which undernutrition without malnutrition prolonged life-span even when such restriction was delayed until midlife. Extension of median and maximal life-span in these animals is associated with maintenance of immunologic functions that otherwise declines with age (6, 9, 24).

We have demonstrated that CEIR can extend life-span and prevent or delay autoimmune disease in mice of short-lived, autoimmune-prone strains. The prolongation of life achieved by CEIR in B/W and MRL/*l* mice was associated with dramatic effects on certain immune responses that generally decline with aging in these strains (6–9). Moreover, CEIR dramatically inhibited disease development, prevented development of the pathology of glomerulonephritis, and reduced circulating immune complexes, especially in B/W mice (6, 9, 10, 26–28). CEIR doubled and sometimes tripled the life-span of autoimmune-prone mice, with the greatest extension of life-span achieved by a diet low in calories, low in fat, and relatively high in carbohydrates (10).

In the present study, we show that CEIR can also extend survival times, prevent autoimmunities, and reduce CIC in another autoimmune-prone strain, the

Table VI. Effect of Calorie Restriction on Myeloid Progenitor Cells of Bone Marrow at 9 Months of Age[a]

Mice	Calorie	WEHI-3 CSA[b]		LCCM[c]	
		Colonies/10^5	Colonies/femur	Colonies/10^5	Colonies/femur
NZB	High	35 ± 2	5,565 ± 318	142 ± 10	22,578 ± 1,590
	Low	44 ± 3	6,996 ± 477	165 ± 4	26,235 ± 636
DBA/2	High	93 ± 12	14,229 ± 1,836	147 ± 9	22,491 ± 1,377
	Low	120 ± 15	17,640 ± 2,205	165 ± 8	24,108 ± 1,176

[a] Results are shown as mean ± SE. Each group consisted of three mice.
[b] Colony-stimulating activity from supernatants of WEHI-3 cells.
[c] LCCM, L cell conditioned medium.

NZB strain. Female NZB mice develop a very different pattern of autoimmune-based disease that do B/W or MRL/*l* mice. CEIR also restored toward or to normal deficiencies in proliferative responses to mitogens, deficient antibody production, decreased mixed lymphocyte reaction, and even deficient IL-2 production in the autoimmune-prone mice.

We did not use limiting-dilution assays for most of these evaluations, and it is obvious that many of the apparent deficiencies of the NZB strain are related to the development of splenomegaly that is accompanied by a significant expansion of a non-T, non-B lymphoid cell population. Thus, equivalent numbers of nucleated cells were present in cultures from both the full-fed and CEIR groups. However, in the older, full-fed NZB mice, many, if not most, of the cells present were non-T non-B lymphoid cells. For example, when mitogen-dependent proliferative responses and IL-2 production were expressed on a per T cell or per B cell basis, the full-fed NZB mice were not markedly deficient. In contrast, antibody responses of spleen cells from full-fed female NZB mice were diminished even when this cell dilution effect was accounted for. It may be that the null cells interfere with T cell-macrophage-B cell interactions required for effective responses to SRBC. Splenomegaly, one of the most striking changes to occur in full-fed NZB mice, is markedly decreased in the CEIR animals, and T and B cell proportions are maintained closer to normal by CEIR. Thus, the finding that calorie restriction decreased the size of the spleen makes studies using spleen cells subject to alternative interpretations. The apparent increase in vigor of numerous proliferative responses and increased antibody production with spleen cells in the CEIR mice should also be done with defined numbers of T cells and/or B cells to determine whether the responses of these cells themselves are altered by CEIR. Future studies must surely involve investigations with enriched populations of cells or other ways to correct for the striking decrease of null cells that occurred with calorie restriction. Thus, the responses to mitogens, mixed lymphocyte reactions, cytokine responses, and even *in vitro* antibody production versus SRBC currently could be explained, at least in part, by the decrease in relative numbers of T cells or altered proportions of T cells and B cells in the mice fed a normal diet. Thus, the enhanced immune responses attributed to the energy-restricted diet can only be taken as suggestive. Dietary restriction had less of an effect on lymphoid cells of DBA/2 mice, but certain immunoparameters that decline with age were also corrected toward normal in these long-lived, autoimmune-resistant mice.

Of particular interest was the effect of calorie restriction on hematopoietic parameters in NZB mice. Hematopoiesis, as well as the number of clonable B cells, is abnormal in NZB mice from an early age (20). Colony-forming cells (CFU-c) that respond to WEHI-3 CSA were especially low in NZB mice. This hematopoietic defect was not corrected by calorie restriction. Total numbers of B cell precursors and B cells bearing the molecular antigen recognized by 14.8 monoclonal antibodies are transiently high early in life in NZB mice and then decline rapidly thereafter. This decline precedes evidence of development of autoimmune disease (20). NZB mice fed a CEIR diet had slightly higher numbers of cells bearing this antigen than did full-fed NZB mice, but overall, these numbers remained lower than in DBA/2 mice fed either diet. This fall in the number of clonable B lymphocytes from the high numbers present early in life in NZB mice was prevented by a lower calorie intake.

Our findings indicate that CEIR does not completely reverse certain genetically determined deficiencies in NZB mice, such as the response to a particular type of colony-stimulating activity. However, certain central lymphoid tissue changes were modulated by diet; for example, the decline in numbers of functional B cells and their precursors in bone marrow could be forestalled by CEIR. However, these effects are not nearly as striking as the diet-related changes in spleen size that we observed.

Thus, we have shown that in NZB mice, CEIR can curb or correct anemia, inhibit formation of CIC, reduce spleen size, and prolong median life-span, life-span, and span of health. The fundamental mechanism(s) underlying these beneficial effects of CEIR is only now beginning to be examined (29, 30). Given the global influence of CEIR in preventing immunologic disease and extending life-span in the NZB strain, continued investigation of the cellular and molecular means by which CEIR controls so many critical biological processes seems paramount.

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1. Howie JB, Helyer BJ. The immunology and pathology of NZB mice. Adv Immunol **9**:215–266, 1968.
2. Talal N, Steinberg AD. The pathogenesis of autoimmunity in NZB mice. Curr Top Microbiol Immunol **64**:79–103, 1974.
3. Fernandes G, Yunis EJ, Smith J, Good RA. Dietary influence on breeding behavior, hemolytic anemia, and longevity in NZB mice. Proc Soc Exp Biol Med **139**:1189–1196, 1972.
4. Fernandes G, Yunis EJ, Jose DG, Good RA. Dietary influence on antinuclear antibodies and cell-mediated immunity in NZB mice. Int Arch Allergy Appl Immunol **44**:770–782, 1973.
5. Fernandes G, Yunis EJ, Good RA. Influence of protein restriction on immune functions in NZB mice. J Immunol **116**:782–790, 1976.
6. Fernandes G, Friend P, Yunis EJ, Good RA. Influence of dietary

restriction on immunologic function and renal disease in (NZB × NZW)F1 mice. Proc Natl Acad Sci USA **75**:1500–1504, 1978.
7. Fernandes G, Yunis EJ, Good RA. Influence of diet on survival of mice. Proc Natl Acad Sci USA **73**:1279–1283, 1976.
8. Kubo C, Day NK, Good RA. Influence of early or late dietary restriction on life span and immunological parameters in MRL/Mp-lpr/lpr mice. Proc Natl Acad Sci USA **81**:5831–5835, 1984.
9. Kubo C, Johnson BC, Day NK, Good RA. Calorie source, calorie restriction, immunity and aging of (NZB/NZW)F1 mice. J Nutr **114**:1884–1899, 1984.
10. Kubo C, Johnson BC, Gajjar A, Good RA. Crucial dietary factors on maximizing life span and longevity in autoimmune-prone mice. J Nutr **117**:1129–1135, 1987.
11. Kincade PW, Paige CJ, Parkhouse RME, Lee G. Characterization of murine colony-forming B cells. I. Distribution, resistance to anti-immunoglobulin antibodies, and expression of Ia antigens. J Immunol **120**:1289–1296, 1978.
12. Scheid MP, Landreth KS, Tung JS, Kincade PW. Preferential but nonexclusive expression of macro-molecular antigens on B-lineage cells. Immunol Rev **69**:141–159, 1982.
13. Cunningham AJ, Szenberg A. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology **14**:599–600, 1968.
14. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor: Parameters of production and a quantitative microassay for activity. J Immunol **120**:2027–2032, 1978.
15. Safai-Kutti S, Fernandes G, Wang Y, Safai B, Good RA, Day NK. Reduction of circulating immune complexes by calorie restriction in (NZB × NZW)F1 mice. Clin Immunol Immunopathol **15**:293–300, 1980.
16. Landreth KS, Rosse C, Clagett J. Myelogenous production and maturation of B lymphocytes in the mouse. J Immunol **127**:2027–2034, 1981.
17. Jyonouchi H, Kincade PW. Changes in B lineage cell populations in liver and spleen of normal neonatal mice. J Immunol **130**:1616–1621, 1983.
18. Kincade PW, Lee G, Watanabe T, Sun L, Scheid MP. Antigens displayed on murine B lymphocyte precursors. J Immunol **127**:2262–2268, 1981.
19. Kincade PW. Formation of B lymphocytes in fetal and adult life. Adv Immunol **31**:177–245, 1981.
20. Jyonouchi H, Kincade PW, Landreth KS, Lee G, Good RA, Gershwin ME. Age-dependent deficiency of B-lymphocyte lineage precursors in NZB mice. J Exp Med **155**:1665–1678, 1982.
21. Kincade PW, Lee G, Fernandes G, Moore MAS, William N, Good RA. Abnormalities in clonable B lymphocytes and myeloid progenitors in autoimmune NZB mice. Proc Natl Acad Sci USA **76**:3464–3468, 1979.
22. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. J Nutr **10**:63–79, 1935.
23. McCay CM, Maynard LA, Sperling G, Barnes LL. Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. J Nutr **18**:1–13, 1939.
24. Weindruch R, Gottesman SRS, Walford RL. Modifications of age-related immune decline in mice dietarily restricted from or after mid-adulthood. Proc Natl Acad Sci USA **79**:898–902, 1939.
25. Weindruch R, Walford RL. Dietary restriction in mice beginning at 1 year of age: Effect of life span and spontaneous cancer incidence. Science **215**:1415–1418, 1982.
26. Gajjar A, Kubo C, Johnson BC, Good RA. Influence of extremes of protein and energy intake on survival of B/W mice. J Nutr **117**:1136–1140, 1987.
27. Izui S, Fernandes G, Hara I, McConahey PJ, Jensen FC, Good RA. Low-calorie diet selectively reduces expression of retroviral envelope glycoprotein gp70 in sera of NZB × NZW F1 hybrid mice. J Exp Med **154**:1116–1124, 1981.
28. Johnson BC, Good RA. Chronic dietary restriction and longevity. Proc Soc Exp Biol Med **193**:4–5, 1990.
29. Walford RL, Crew M. How dietary restriction retards aging: An integrative hypothesis [Editorial]. Growth Development & Aging **53**:139–140, 1989.
30. Masoro EJ, Katz MS, McMahan CA. Evidence for the glycation hypothesis of aging from the food-restricted rodent model. J Gerontol.: Biological Sciences **44**:B20–22, 1989.