Dietary Influence on Breeding Behavior, Hemolytic Anemia, and Longevity in NZB Mice[1] (36327)

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Mice of the NZB strain become Coombs positive after 3–4 months (1–3). In old age renal failure with histologic lesions resembling those of lupus erythematosus occur in both sexes (4, 5). This disease is genetically determined and its severity differs in males and females (6). NZB mice therefore serve throughout the world as a model system for study of autoimmune diseases. These mice seem to grow and develop normally up to a certain age when autoantibodies directed against red blood cells appear in their circulation (7). This development is succeeded by occurrence of a Coombs positive hemolytic anemia and loss of vigor of cell-mediated immune capacity (9, 10). Somewhat later, renal lesions characteristic of those observed with the indirect type of immune assault are encountered which lead to progressive renal damage, renal failure, and death (11, 12). The appearance of the renal lesions correlates with the appearance of antinuclear and anti-DNA antibodies (13–15). Evidence implicating a virus in the pathogenesis of the disease of NZB mice has been presented (16–18). Further, the full disease picture appears to have been adoptively transferred by NZB thymus grafts or by transfer of spleen cells or lymphoid cells (19–21) and contributions of these transfers of virus agent(s) and immunologic potentialities of the NZB donors have been analyzed (22). Infection with a virus which the host could not eliminate, *e.g.*, MuL or LCM virus, markedly accelerated development of the anti-DNA and anti-DNP antibodies and typical renal lesions, but did not influence development of the hemolytic anemia (23–25).

In the present study, which was initially designed to investigate the influence of diet on breeding capacity of NZB and other mice of inbred strains in our laboratory (26), we found that two different diets used were associated with differences in sex and earlier onset of hemolytic disease and with even greater differences in longevity of NZB mice. A diet relatively low in fat and higher in protein was associated with poor breeding behavior, later onset of hemolytic anemia, and greater longevity in male NZB mice than was the diet that was lower in protein and higher in fat.

Materials and Methods. Source of our NZB strain, inbreeding of our mice are described elsewhere (27). Weanling NZB mice were selected at 4 weeks from parents that had been fed either diet I or II (Table I) throughout their lives. Experimental animals were, in every instance, derived from second litters of these parents which belonged to generation 68 of NZB mice. Thirty-three offspring from each parent stock were relegated to this study. For study of breeding capacity, 10 males and 10 females of each stock were caged as pairs. An additional 13 mice (6 females and 7 males) from each group were also housed in groups of 3–4 males or females. Thus 33 mice from parents fed either diet I or II were in turn fed diet I or II and studied with respect to time of onset of Coombs positive hemolytic anemia, breeding capacity, and longevity. Since it was difficult to obtain a larger group of mice of the same age, our present study is based on a relatively small number of animals.

Two standard diets were obtained from

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TABLE I. Approximate Chemical Composition of Diets.

Diet I: Mouse chow (high fat–low protein)	(%)	Diet II: Lab chow (low fat–high protein) (%)
Protein	17	23
Fat	11	4.5
Fiber	2.15	5.2
TDN[a]	80	75
NFE[b] (by difference)	51.7	50.8
Gross energy (kCal/g)	4.31	4.25
Ash	6.38	7.3
Calcium	0.89	1.20
Vit. A (IU/g)	30	12.00

[a] TDN = total digestible nutrients.
[b] NFE = nitrogen-free extract.

Purina Company once every month and were fed *ad libitum*. These diets were composed of approximately the following estrogen free ingredients (Table I, Source: Purina Co., St. Louis).

All animals were bled from the tails when 2, 4, 6, 9, and 12 months of age. Hematocrit and Coombs tests were carried out with standard methods used in our laboratories (49). Breeding records were kept for individual females and the number of young born and number weaned was carefully observed and recorded, as were the time of death of the experimental mice. These mice were maintained, along with our other inbred strains, in one breeding room. The same standard of housing and environment as previously reported was strictly followed. The data were analyzed on an Olivetti desk computer for the mean values ± standard error for all parameters and the p values given from t (Student's) test; p values $> .5$ were considered not significant (ns).

Results. 1. Breeding behavior. Females on high fat, low protein diet delivered their first litter at a mean age of 74.8 days (SE ± 2.02); whereas those on the low fat, high protein diet presented a first litter slightly later, mean 84.9 days (SE ± 5.6). This difference is not significant.

The average first litter size of the females on the higher fat diet was 7.4 and for low fat diet was 7.2. The second litters were smaller than the first litters; the mean being 5.6 and 5.8 for diets I and II, respectively. Again, these differences were not significant. However, a few females on diet I had as many as 5–6 litters; whereas, those on diet II stopped breeding after 3–4 litters. The total number of young born from females on diet I was 241 (41 litters); whereas, the number from females on diet II was only 177 (29 litters). Overall weaning percentages were 58 and 54 for females on diets I and II, respectively.

2. Body weights. No significant difference in body weights between the females and males on either diet was observed up to 4 months (Table II). However, at 6 and 9 months of age, females on diet I had a significantly greater body weight than did those on diet II. Males on diet I were much larger at 9 months of age than were males on diet II.

3. Coombs tests. Table III compares the times of appearance of direct Coombs positivity in mice on the two dietary regimens.

TABLE II. Average Body Weights of Female and Male NZB Mice on Diets of Differing Fat and Protein Composition.

Diet	Sex	Wt (g); age (months): 2	4	6	9
I	Females	24.9 ± 0.3	30.4 ± 0.8	34.4 ± 0.6	45.8 ± 1.8
II	Females	25.2 ± 0.4	29.3 ± 0.7	30.7 ± 0.5	38.5 ± 1.2
p		ns	ns	$<.01$	$<.05$
I	Males	29.1 ± 0.5	36.1 ± 0.5	38.3 ± 1.2	53.9 ± 3.2
II	Males	28.5 ± 0.4	35.4 ± 0.8	36.6 ± 0.8	43.8 ± 2.1
p		ns	ns	ns	$<.001$

TABLE III. Dietary Influence on Direct Coombs Tests and Strength of Agglutination in NZB Mice.

Age (months)		Females				Comparison of Coombs test (p)	Males				Comparison of Coombs test (p)
		Diet I		Diet II			Diet I		Diet II		
		(%)	No.	(%)	No.		(%)	No.	(%)	No.	
2	Coombs test[a]	6.2	(1/16)[c]	0	(0/17)	ns	5.8	(1/17)	0	(0/16)	ns
	Aggl. str.[b]	0.06 ± 0.05		—			0.05 ± 0.05		—		
4	Coombs test	31.2	(5/16)	17.6	(3/17)	ns	76.4	(13/17)	31.2	(5/16)	<.01
	Aggl. str.	0.8 ± 0.3		0.3 ± 0.2			2.1 ± 0.4		0.7 ± 0.3		
6	Coombs test	62.5	(10/16)	68.7	(11/16)	ns	93.7	(15/16)	62.5	(10/16)	<.01
	Aggl. str.	1.3 ± 0.4		1.4 ± 0.3			1.9 ± 0.2		0.9 ± 0.2		
9	Coombs test	76.9	(10/13)	62.5	(10/16)	ns	86.6	(13/15)	93.7	(15/16)	ns
	Aggl. str.	1.8 ± 0.4		1.5 ± 0.4			2.4 ± 0.3		3.1 ± 0.3		
12	Coombs test	83.3	(5/6)	78.5	(11/14)	ns	100	(8/8)	93.3	(14/15)	ns
	Aggl. str.	1.2 ± 0.3		1.6 ± 0.3			2.4 ± 0.5		2.5 ± 0.3		

[a] Antimouse serum was obtained from Hyland Laboratories and absorbed 2–3 times in mouse red blood cells (49).

[b] Agglutination strength was graded from + to ++++ according to our serological methods (49).

[c] Positive/total test animals.

TABLE IV. Influence of Diet on Mean Hematocrit Levels and Incidence of Anemia of NZB Mice.

Age (months)	Females[a]			Males[a]		
	Diet I	Diet II	p	Diet I	Diet II	p
2	49.2 ± 0.6 (0/16)[b]	49.2 ± 0.5 (0/17)	ns	48.2 ± 0.5 (0/17)	50.5 ± 0.8 (0/16)	<.05
4	46.1 ± 0.7 (3/16)	46.8 ± 0.8 (4/17)	ns	44.1 ± 1.2 (4/17)	47.7 ± 0.5 (0/16)	<.02
6	40.6 ± 1.2 (13/16)	44.2 ± 1.1 (11/16)	<.05	39.2 ± 0.8 (16/16)	41.0 ± 0.5 (12/16)	ns
9	37.3 ± 1.6 (11/13)	40.6 ± 1.5 (12/16)	<.05	35.2 ± 1.1 (14/15)	38.5 ± 1.4 (13/15)	<.05
12	35.5 ± 2.9 (5/6)	34.7 ± 1.1 (14/14)	ns	31.2 ± 2.3 (8/8)	37.0 ± 1.1 (13/15)	<.02

[a] Mean ± 1 SE.

[b] The number in parentheses indicates the number of mice with anemia of the surviving mice at that age. Anemia was scored when the hematocrit was below the value obtained by subtracting 2 ± SD from the mean hematocrit at 2 months of age in each group.

Table III shows that the Coombs test tended to become positive at an earlier age in mice on diet I than in those on diet II. Further, it was noted, as shown in Table III, that the degree of Coombs positivity in the 4- and 6-month-old male mice on diet I was significantly greater than that for mice on diet II. Females on diets I and II showed no significant influences. By 9 months of age, however, the influence of diet on incidence of Coombs positivity on the males was no longer detectable. At 4 months, males showed a higher frequency of Coombs positivity than did females on either diet.

4. Hematocrit levels. Males on diet I showed at 2, 4, 9, and 12 months of age significantly lower hematocrit values than did males fed diet II. Females on diet I showed significantly lower hematocrits at 6 and 9 months. Table IV analyzes these data in another way. Table IV shows that more males on diet I developed anemia at 4 and 6 months than is the case with the male mice on diet II. The incidence of anemia in females on the two diets was nearly equal.

5. Reticulocyte counts. Table V summarizes studies on the reticulocyte counts of these animals. Table V shows that, by this parameter as well, animals on diet I develop abnormalities earlier and have more severe disease than those on diet II. Both males and females on diet I develop higher reticulocyte counts than do the animals fed diet II. Statistical significance varied with the sexes as indicated.

6. Leukocyte count. Animals on the two diets were also compared with respect to total leukocyte counts. These data are summarized in Table VI. Table VI shows that no differences were observed in total leukocyte

TABLE V. Influence of Diet on Reticulocyte Count in NZB Mice.[a]

Age (months)	Females			Males		
	Diet I	Diet II	p	Diet I	Diet II	p
6	14.2 ± 4.8	2.1 ± 0.6	<.05	2.8 ± 0.6	1.8 ± 0.4	ns
9	15.1 ± 3.2	6.2 ± 3.6	<.05	26.6 ± 6.5	9.9 ± 3.7	<.01
12	45.6 ± 13.1	32.2 ± 7.4	ns	67.2 ± 8.6	22.8 ± 5.4	<.001

[a] Total (%) out of 500 red blood cell counts.

TABLE VI. Mean White Blood Cell Count at Various Ages in NZB Mice Fed Diets I and II.

Diet	Age (months)					
		Differentials				
	6	Lymph.	Neutro.	Mono.	9	12
I	(13)[a]				(17)	(14)
	5761 ± 1030	56.5	40.2	2.9	5935 ± 486	13021 ± 2869
II	(13)				(19)	(29)
	3857 ± 324	63.4	32.2	4.1	5673 ± 425	6950 ± 622
p	ns	<.02	<.02	<.02	ns	<.02

[a] Number of animals indicated in parentheses.

counts between the two groups at 6–9 months of age; but the surviving mice on diet I showed a significantly higher leukocyte count at 12 months than did those on diet II. Further, a significant difference in the differential counts was observed at 6 months of age. Differential counts were not done at 9 and 12 months of age.

7. Mortality rate. Finally, Table VII and Fig. 1 show that the mean longevity of both males and females was significantly shorter in NZB mice on diets I and II. Figure 1 summarizes in graphic form the comulative mortalities. Once again the influence of the dietary regimen was more pronounced in males than in females. Animals of the two sexes died at approximately the same time on diet I. However, males on diet II lived much longer than did females and the difference between the males on diets I and II is highly significant.

Discussion. NZB mice represent a well-established form of autoimmunity in which genetic factors, endocrine factors, and viral agents play interacting roles (28–30). In this disease, autoimmune phenomena are regularly expressed, breeding efficiency decreased, and life span is short (31). In this study, we present consistent evidence from analysis of several parameters in NZB mice that differences in diet alter significantly the time of onset of hemolytic anemia, breeding efficiency, and short life span of these animals. Animals fed complex diets which differ from one another in protein and fat distribution ex-

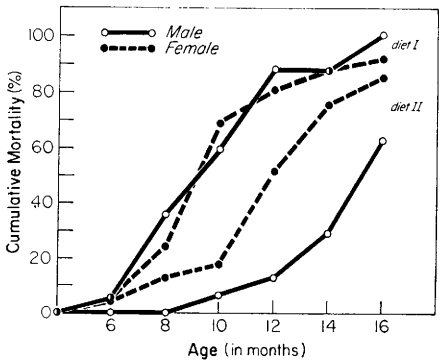

FIG. 1. Mortality curves for NZB female and male mice on diets I and II.

press differently the autoimmune hemolytic disease. Differences in breeding efficiency of several strains of mice with slight changes in protein and fat levels of the diet has been previously recognized (32, 33). Atrial throm-

TABLE VII. Longevity of NZB Mice on Two Different Diets.[a]

Diet	Sex	Age (days)	Sex	Age (days)	*p*
I	Females	305 ± 21.0	Males	295 ± 20.5	ns
II	Females	367 ± 18.3	Males	456 ± 15.0	<.001
p		<.05		<.001	

[a] Mean ± SE for all mice either died or survivors killed at 16 months of age (see Fig. 1).

bosis in mice and anemia in guinea pigs has been associated with fat and cholesterol in the diet (34–36).

For many years, an influence of nutrition on immune responses has been studied; and these influences have been recently reviewed (37). They range from the classical association of starvation and pestilence to inhibition of tumor and cancer development by dietary variations (38, 39). An essential relationship between the lymphoid system, immunity, and malignancy (40), as well as evidence of immunosuppression by chronic protein and chronic protein plus calorie deprivation have been presented recently from our laboratories (41). These studies show that, with moderate chronic protein restriction, depression of humoral immunity occurs in both man and animals, while cellular immunity is left intact or may even be increased (42–45). More severe chronic protein deprivation on the other hand, can interfere with development of both humoral and cellular immunity (46). Influences of both acute and chronic vitamin restriction on immune reactions has been described (47, 48). From our present studies, since we used commercially obtained standard diets, it is impossible to determine which, if any, of the several nutritional variables that were manipulated account for the influence on the immunologically based disease of NZB mice. Although it seems likely that the influence of diet on this disease is attributable to an influence on immunologic mechanism, this is by no means certain. Alteration of exposure to exogenous antigens by dietary manipulation of bacterial, viral, or fungal flora, for example, could play a part. Also, the composition of the dietary ingredients, such as protein and fat levels, might be crucial since these components of the diet could vary in antigens that cross react with those of host.

Future studies must dissect specifically the several possibilities. It will be of real interest, for example, to determine whether expression of autoimmune diseases and survival in other autoimmune susceptible strains of mice (49, 50) are influenced by these diets as is the autoimmune disease of NZB mice. More important perhaps will be careful dissection of the nature of the dietary basis or bases of the influences observed in these studies. This analysis can be achieved by varying independently protein, lipid, calories, and vitamins in the diets; and studying the influence of such manipulations on the several autoimmune processes and expressions of virus infection in NZB mice. Also, since autoimmune processes in both man and animals relate so intimately to aging (51), particularly of the lymphoid apparatus (52, 53), consideration of relation of dietary influences on the aging of immunologic functions will require extensive study (54, 55). Indeed, one of the most provocative current theories of aging relates the aging process to oxidants and retardation of aging to antioxidants in the diet (56, 57). Thus a definitive study of dietary influence on aging of inbred mice could profitably stand additional attention and the results might be relevant to the observation recorded here.

Summary. NZB mice are extensively used throughout the world as a model system for studying autoimmune disease. Apparently associated with a propensity to early development of autoimmune hemolytic anemia, the breeding capacity of NZB mice is defective; and the life span of these animals is short. As part of a general effort to improve breeding in our colonies, comparison of the influence of two commercial diets on NZB mice was made. One diet provided low protein and relatively high fat; the other, somewhat higher protein and lower fat content. Improved breeding performance was observed in the animals fed higher fat, lower protein intake. But, this diet influenced the animals adversely by fostering development of hemolytic anemia and shortening the life span both in males and females; whereas higher protein and lower fat extended particularly the longevity of male mice. These observations are taken as preliminary evidence that dietary intake can profoundly influence the development of autoimmune disease and to suggest that further systematic studies must be carried out to dissect more specifically the nature of these dietary influences.

1. Bielschowsky, M., Helyer, B. J., and Howie, J. B., Proc. Univ. Otago Med. Sch. **37**, 9 (1959).

2. Helyer, B. J., and Howie, J. B., Brit. J. Haematol. **9**, 119 (1963).

3. Miyasato, F., Manaligod, J. R., and Pollak, V. E., Arch. Pathol. **83**, 20 (1967).

4. Holmes, M. C., and Burnet, F. M., Ann. Intern. Med. **59**, 265 (1963).

5. Mellors, R. C., J. Exp. Med. **122**, 25 (1965).

6. Burnet, F. M., and Holmes, M. C., Nature (London) **207**, 368 (1965).

7. Long, G., Holmes, M. C., and Burnet, F. M., Aust. J. Exp. Biol. Med. Sci. **41**, 315 (1963).

8. Helyer, J. B., and Howie, J. B., Nature (London) **197**, 97 (1963b).

9. Burnet, F. M., and Holmes, M. C., J. Pathol. Bacteriol. **88**, 229 (1964).

10. Stutman, O., Yunis, E. J., and Good, R. A., Proc. Soc. Exp. Biol. Med. **127**, 1204 (1968).

11. Mellors, R. C., Int. Rev. Exp. Pathol. **5**, 127 (1966).

12. East, J., Sousa, M. A B. De, and Parrot, D. M. V., Transplantation **3**, 711 (1965).

13. Hicks, J. D., J. Pathol. Bacteriol. **91**, 479 (1966).

14. Norins, L., and Holmes, M. C., J. Immunol. **93**, 148 (1964).

15. Aaron, I., Nature (London) **203**, 1080 (1964).

16. Lambert, P. H., and Dixon, F. J., J. Exp. Med. **127**, 507 (1968).

17. Mellors, R. C., and Huang, C. Y., J. Exp. Med. **124**, 1031 (1966).

18. Prosser, P. R., Clin. Exp. Immunol. **3**, 213 (1968).

19. East, J., Prosser, P. R., Holborow, E. J., and Jacquet, H., Lancet **1**, 755 (1967).

20. Holmes, M. C., Aust. J. Exp. Biol. Med. Sci. **43**, 399 (1965).

21. Masters, J. M., and Spurling, C. L., Blood **30**, 569 (1967).

22. Denman, A. M., Russell, A. S., and Denman, E. J., Clin. Exp. Immunol. **5**, 567 (1969).

23. Oldstone, M. B. A., and Dixon, F. J., J. Exp. Med. **129**, 483 (1969).

24. Aoki, T., Boyse, E. A., and Old, L. J., J. Nat. Cancer Inst. **41**, 89 (1968).

25. Mellors, R. C., Aoki, T., and Huebner, R. J., J. Exp. Med. **129**, 1045 (1969).

26. Smith, J., Fernandes, G., and Hanson, A., (Abstr.), Amer. Ass. Lab. Anim. Sci. Meet., 20th, Dallas, 1969.

27. Inbred Strains of Mice, No. 6, July 1969, Jackson Laboratory.

28. Braverman, J. M., Arthritis Rheum. **11**, 95 (1968).

29. East, J., and DeSousa, M. A. B., Nat. Cancer Inst. Monogr. **22**, 605 (1966).

30. East, J., Vox Sang. **16**, 318 (1969).

31. Bileschowsky, M., and Bielschowsky, F., Aust. J. Exp. Biol. Med. Sci. **42**, 561 (1964).

32. Hoag, W. G., and Dickie, M. M., Proc. Anim. Care Panel **10**, 109 (1960).

33. Hoag, W. G., and Dickie, M. M., Proc. Anim. Care Panel **12**, 7 (1962).

34. Yamanaka, W., Ostwald, R., and French, S. W., Proc. Soc. Exp. Biol. Med. **125**, 303 (1967).

35. Ostwald, R., Yamaka, W., Irwin, D., Hansma, H., Light, M., and Tom, K., J. Nutr. **101**, 699 (1971).

36. Clover, R. B., J. Atheroscler. Res. **8**, 885 (1968).

37. Scrimshaw, N. S., Taylor, C. E., and Gordon, J. E., W. H. O. Monogr. Ser. No. 57, Geneva, 1968.

38. Cannon, P. R., J. Immunol. **44**, 107 (1942).

39. Dubos, R., "Man Adapting." Yale University Press (1965).

40. Good, R. A., and Finstad, J., Nat. Cancer Inst. Monogr. **31**, 41 (1969).

41. Jose, D. G., and Good, R. A., Nature (London) **231**, 323 (1971).

42. Jose, D. G., Welch, J. S., and Doherty, D. A., Aust. Paediat. J. **6**, 192 (1970).

43. Jose, D. G., Cooper, W. C., and Good, R. A. (editorial), J. Amer. Med. Ass. **218**, 1428 (1972).

44. Cooper, W. C., Mariani, T., and Good, R. A., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 364 (1970).

45. Cooper, W. C., and Good, R. A., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **30**, 351 (1971).

46. Jose, D. G., and Good, R. A., Proc. Leukocyte Culture Congr., 6th, Academy Press Inc., New York (1971).

47. Axelrod, A. E., *in* "Modern Nutrition in Health and Disease" (M. G. Wohl, and R. S. Goodhart, eds.). Lea and Febiger, Philadelphia (1968).

48. Panda, B., and Combs, G. F., Proc. Soc. Exp. Biol. Med. **113**, 530 (1963).

49. Yunis, E. J., Hong, R., Grewe, M. A., Martinez, C., Cornelius, E., and Good, R. A., J. Exp. Med. **125**, 947 (1967).

50. Yunis, E. J., Teague, P. O., Stutman, O., and Good, R. A., Lab. Invest. **20**, 46 (1969).

51. Yunis, E. J., Stutman, O., and Good, R. A., Nat. Acad. Sci. **183**, 205 (1971).

52. Yunis, E. J., Fernandes, G., and Stutman, O., Amer. J. Clin. Pathol. **56**, 280 (1971).

53. Yunis, E. J., Stutman, O., Fernandes, G., Teague, P. O., and Good, R. A., *in* "Tolerance, Im-

munity, and Molecular Aging," (Siegel and Good, eds.), Chap. 8. Univ. of Miami Press, Coral Gables, FL (1971).

54. Tannenbaum, A., and Silverstone, H., *in* "Cancer" (R. W. Raven, ed.), Vol. 1, p. 306. Butterworth, London (1959).

55. Walford, R. L., "The Immunologic Theory of Aging." Munksgaard, Copenhagen (1969).

56. Tappel, A. L., Geriatrics **23**, 97 (1968).

57. Comfort, A., Youthotsky-Gok, I., and Pathmanathan, K., Nature (London) **229**, 254 (1971).

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