Effect of low dietary lipid on the development of Sjögren's syndrome and haematological abnormalities in (NZB×NZW)_{F1} mice

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SUMMARY A diet low in fat was found to retard the development of autoimmune disease in (NZB×NZW)_{F1} mice, whereas diets high in fat content were associated with more severe disease. The ability of a reduced lipid intake to ameliorate the progression of autoimmune disease was indicated by preserved lacrimal gland secretion (measured by a modified Schirmer test), decreased infiltration of inflammatory cells into the exocrine tissue, and decreased severity of immunohaemolytic anaemia as indicated by near-normal packed cell volume and reticulocyte values. These results suggest that nutritional intervention may be of some help in reducing the severity of pathological abnormalities associated with human systemic lupus erythematosus and Sjögren's syndrome.

Numerous studies investigating the aetiology and pathogenesis of murine autoimmune disease have shown the importance of genetic, viral, and environmental factors in determining both the onset and severity of disease.¹ The (NZB×NZW)_{F1} (NZB/W) hybrid mouse spontaneously develops immunopathological abnormalities similar to those of human systemic lupus erythematosus and Sjögren's syndrome,^{2–4} and is a well established model for these disorders.

Immunologically, systemic lupus erythematosus is characterised by many cellular and humoral defects, including the production of numerous autoantibodies to DNA, RNA, and cellular antigens. Sjögren's syndrome is a chronic inflammatory autoimmune disease, in which lymphocytic and plasma cell infiltration of the salivary glands and lacrimal glands is associated with tissue destruction of these organs and concomitant decreased production of saliva (xerostomia) and tears (xerophthalmia).⁵

The NZB/W mouse has been used for numerous studies on autoimmunity and experimental therapeutics. Virtually all these investigations, however,

have focused on the systemic lupus erythematosus components—namely, the production of autoantibodies and the occurrence of immune complex mediated inflammation, particularly in the kidneys. The NZB/W mouse also develops autoimmune related exocrine gland disease, which resembles the abnormalities seen in human Sjögren's syndrome, as well as other less specific autoimmune related immunopathological phenomena.

Our laboratory and others have shown that autoimmune disease in NZB/W mice can be affected by nutritional variables such as lipids, energy value, proteins, vitamins, and minerals.⁶ In particular, we have shown that NZB/W mice fed near-isocaloric diets, varying only in the type and quantity of fat, showed marked differences in immune responses and severity of autoimmune disease manifestation.^{7–9} Invariably, the low fat diets reduced the symptoms of autoimmunity, whereas animals fed high levels of lipid generally showed severe disease.

Thus the aim of this work was to examine the contribution of dietary lipid to some of the less studied aspects of autoimmune disease in NZB/W mice and make a preliminary assessment of the role of nutrition in regulating the progression of these symptoms.

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Materials and methods

ANIMALS

Weaned female NZB/W mice (3–4 weeks of age) were purchased from the Jackson Laboratory, Bar Harbor, ME. Food and water were given freely.

DIETS

The diets selected for these experiments have been used previously in our laboratory,^{7–9} except that in this study the high saturated fat diet contained coconut oil instead of animal lard. This fat has a higher degree of saturation than lard. Each diet varied only in the fat concentration and degree of saturation and differed in energy value by no more than 10%. The experimental diets were obtained from Teklad Test Diets, Madison, WI, and were in pellet form. The diets were as follows: (a) high in saturated fat (7.7% coconut oil, 1.3% corn oil); (b) high in unsaturated fat (9% corn oil); and (c) low in fat (1.2% corn oil). The control diet was conventional rodent pellets (Ralston-Purina, St. Louis, MO), which contained 4.5% total fat consisting of approximately equal proportions of saturated and unsaturated fats. When the animals were killed the mean body weights of all animals on these diets did not differ significantly. This finding is consistent with previous reports.^{8,9}

SCHIRMER TESTS

Schirmer tests were performed using a modified version of the technique described by Hoffman *et al.*¹⁰ Mice were anaesthetised with a ketamine/xylazine cocktail (0.25/0.15 ml/kg animal weight), and one end of a 0.5 mm × 5.0 mm strip of Whatman No 1 filter paper was placed under the lower eyelid (Fig. 1). After two minutes the strip was removed and the wetted area measured under a dissecting microscope to the nearest 0.1 mm. Tests were performed twice within one week before the animals were killed.

HISTOLOGICAL STUDIES

At 7 months of age (the stage of active disease) the mice were anaesthetised with ether, killed by cervical dislocation, and bled via cardiac puncture. The left and right lacrimal (exorbital) and salivary (parotid) glands were excised and fixed in a 3.7% formalin solution, dehydrated by passage through a graded series of alcohol solutions, and embedded in a plastic medium (Polysciences Inc, Warrington, PA). Tissue sections (4 µm) were cut on a microtome, melted smooth onto a glass slide, stained for 15 seconds with methylene blue, and washed in cold buffer. Sections from each tissue were examined for infiltration of mononuclear cells by light microscopy

Fig. 1 Schirmer test as performed on an NZB/W mouse.

and graded on a positive/negative scale. This system was used to distinguish more accurately the destructive tissue infiltration from focal infiltration previously reported to occur in 25% of non-autoimmune female mice.¹⁰ Sections with focal and diffuse infiltrations affecting less than 10% of the exocrine tissue were scored as negative (Fig. 2a). The presence of abnormality, including multifocal infiltration and destruction of tissue architecture, affecting at least 10% of the tissue was scored as positive (Fig. 2b). Several sections from each tissue were examined to ensure an accurate evaluation. Slides were scored blind by two independent observers. In the event of disagreement between scorers the slide was reviewed until an agreed score was achieved. At the end of the study the code was broken and the results tabulated.

HAEMATOLOGICAL STUDIES

Packed cell volumes were determined after centrifugation. Reticulocyte count was obtained by adding two drops of new methylene blue N solution (Harleco Product) to four heparinised capillary tubes of blood. The solution was mixed and allowed

Fig. 2a

Fig. 2b

Fig. 2 (a) Normal lacrimal gland tissue showing darkly staining zymogen granules in the serous cells and secretory ducts. (Methylene blue.) (b) Arrows indicate a region of severe mononuclear cell infiltration and destruction of tissue architecture in a section through the lacrimal gland of a mouse maintained on a high fat diet. Section shows typical acinar serous cells and secretory ducts. (Methylene blue.)

to stand for 10 minutes. Slide smears were made and reticulocytes noted by the presence of basophilic material. Counts were performed blind by two independent observers. Indirect Coombs' tests for antierythrocyte antibodies were performed by standard methods. Briefly, erythrocytes were obtained from Swiss-Webster mice and after three washes in normal saline were inoculated with varying concentrations of heat inactivated test or control serum for 30 minutes at 37°C. After a further three washes the presence of bound mouse immunoglobulin was determined by adding goat antimouse IgG (Cappel Laboratories, Malven, PA) diluted 1:20 with normal saline. Agglutination was measured on a scale of 1-4+.

STATISTICAL ANALYSES

Data analysis was performed with a Statview 512 software package on an Apple Macintosh. Differences in populations having Gaussian distribution were assessed by a Student's *t* test. Data that were non-Gaussian—for example, reticulocyte counts, were normalised by log₁₀ transformation before analysis. Statistical significance was set at the 5% level.

Results

MEASUREMENT OF LACRIMAL GLAND SECRETION

Table 1 shows that the mean length of filter paper wetting (expressed in mm) in the mice fed the low fat diet was significantly greater ($p<0.001$, Student's *t* test) than in those mice fed either the high saturated or unsaturated fat diets. Although ocular wetting by animals fed the control diet was also greater than by mice fed either of the high fat diets, the differences were not statistically significant.

Table 1 Measurement of lacrimal gland secretion

	Diet group			
	HSF*	HUF*	LF*	Pellet*
Number of animals tested	20	20	20	24
Schirmer test (mm)†	2.2 (0.73)	2.2 (0.97)	3.3 (0.78)	3.0 (1.6)

All animals were 7 month old females.

*HSF=high in saturated fat; HUF=high in unsaturated fat; LF=low in fat; Pellet=conventional rodent diet (control).

†Figures represent mean length (SE) of wetting by ocular secretion as measured with a Whatman No 1 filter strip.

Statistically significant differences ($p<0.001$, Student's *t* test) were noted between animals in the low fat diet group and those fed either of the high fat diets. Confidence was set at the 95% level.

Table 2 Incidence of abnormal lymphocytic infiltration in lacrimal and parotid glands

	Diet group			
	HSF*	HUF*	LF*	Pellet*
Number of tissues tested	17	17	17	20
Number (%) positive	8 (47)	7 (41)	4 (23)	5 (25)

*HSF=high in saturated fat; HUF=high in unsaturated fat; LF=low in fat; Pellet=conventional rodent diet (control).

HISTOLOGIC EVALUATION OF EXORBITAL AND PAROTID GLANDS

Table 2 summarises the scoring of exocrine gland disease from each diet group. The incidence of destructive infiltration of the exocrine glands was nearly twice as high in those mice fed either the high saturated fat (47%) or the high unsaturated fat (41%) diets than in those fed the low fat diet (23%) or in the control (25%) group.

PACKED CELL VOLUMES, RETICULOCYTOSIS AND ANTIERYTHROCYTE ANTIBODIES

Figure 3 shows average packed cell volumes for each diet group taken at 4 months of age for reference.

Fig. 3 Differences in packed cell volumes in both 4 and 7 month old NZB/W mice. Vertical lines represent standard error. Each group comprised the following number of animals: low fat 8 (4 mo), 16 (7 mo); pellet/control 8 (4 mo), 13 (7 mo); high unsaturated fat 8 (4 mo), 22 (7 mo); high saturated fat 8 (4 mo), 18 (7 mo). At 7 months differences between low fat and high saturated fat fed animals were significant ($p=0.0073$).

Fig. 4 Reticulocyte counts from 4 and 7 month old NZB/W mice fed diets varying in fat content. Vertical lines represent standard error. Each group comprised the following number of animals: low fat 8 (4 mo), 16 (7 mo); pellet/control 8 (4 mo), 13 (7 mo); high unsaturated fat 8 (4 mo), 22 (7 mo); high saturated fat 8 (4 mo), 18 (7 mo). Only differences between low fat and high saturated fat fed animals were significant ($p < 0.001$).

and again at 7 months of age. At 7 months a substantial drop in packed cell volume was observed in those mice maintained on the control, high unsaturated fat and, most notably, the high saturated fat diets. Those animals fed the low fat diet showed only a slight decrease in packed cell volume. In the 7 month old animals the difference between the mean packed cell volumes of the low fat and high saturated fat groups was 0.06; this difference was significant ($p = 0.0073$). For comparison purposes, a normal packed cell volume for inbred, non-autoimmune BALB/c is 0.48.¹¹

Reticulocyte counts (Fig. 4) at 4 months of age showed no marked difference between the diet groups. At 7 months, however, reticulocyte counts increased dramatically in animals from all diet groups (again this effect was most pronounced in the high saturated fat group) except for those animals maintained on the low fat diet, where no change was observed. There was a significant ($p < 0.001$) difference between reticulocyte values from the low fat and high saturated fat fed groups at 7 months of age. A normal reticulocyte value for non-autoimmune BALB/c3 mice is 2.9%.¹¹ We were unable to detect anti-red blood cell antibodies in the sera of any of our test mice by the indirect Coombs' test, though control sera obtained from NZB mice (which

develop marked haemolytic anaemia in comparison with the NZB/W mice) consistently gave scores of 3-4+.

Discussion

Previous studies by our group^{1 7-9 12} and others (reviewed in reference 6) have indicated that dietary fat can dramatically affect the development of autoimmune disease in susceptible strains of mice. The present results suggest that dietary manipulation can also modulate the appearance of Sjögren's syndrome-like immunopathology and haematological abnormalities which develop in NZB/W mice.

The connection between exocrine disease and exocrine fluid production has yet to be established. Scoring of gland disease in all diet groups was apparently associated with measurements of tear production. These findings suggest that the Schirmer test commonly used in humans may be a useful indicator of exorbital gland function in the mouse. Although exact identification of the infiltrating cells cannot be made from Figs 2a and 2b, a detailed examination and identification of lacrimal and parotid gland infiltration in NZB/W mice has been previously described by Kessler *et al.*⁴ Thus low power magnification was used to illustrate the extensive destruction of tissue architecture rather than identification of the inflammatory cells. As the mean animal weights from each of the four diet groups did not differ significantly, body (and thus organ) size is unlikely to be responsible for differences in the Schirmer test results.¹⁰ The mechanism(s) by which a low fat diet partially protects the animals from exocrine dysfunction remains speculative. Although it is possible that changes in fat intake may directly alter the metabolism of the secretory cells in the lacrimal gland, we believe that it is more likely that the dietary manipulations act through the immune system and regulate the inflammatory processes that occur in these animals. Specifically, we believe that the lipid content of the diet influences the migration of mononuclear cells into the exocrine tissues of these animals and thus has a direct bearing on the development of the autoimmune disease. As mentioned above, previous experimentation by our group has shown that both qualitative and quantitative changes in dietary fat intake can profoundly affect a broad range of humoral and cellular factors that are associated with autoimmune disease in NZB/W and MRL/lpr mice, and it would seem logical that the immunopathology of the lacrimal gland falls into the same category. Nevertheless, the precise mechanism by which alterations in the rate of progression of Sjögren's

syndrome occur is unclear. The effects of dietary fat on the immune system are extremely complex and lymphocyte function can be modulated in several ways, including via arachidonic acid metabolism, alterations in the fatty acid composition of the cell membrane, and plasma lipoproteins.^{13 14} Extensive studies are necessary to determine the exact manner by which dietary fats influence autoimmune disease.

The array of autoantibodies associated with murine autoimmune disease includes anti-red blood cell antibodies, which mediate their effects via type II hypersensitivity.⁵ The ability of dietary lipids to ameliorate the severity of immunohaemolytic anaemia was shown by the preserved packed cell volume and near-normal reticulocyte count in those mice fed the low fat diet. Conversely, increases in reticulocyte counts and marked drops in packed cell volumes were seen in those mice fed diets higher in lipid, especially the high saturated fat group. These data are consistent with an earlier study performed by Fernandes *et al*,¹⁶ who showed that NZB mice had a late onset of haemolytic anaemia when fed a low fat/high protein diet in comparison with counterparts maintained on a high fat/low protein regimen. Although we were unable to show the presence of autoantibodies by the Coombs' test, the combination of the low packed cell volumes and increased reticulocyte counts clearly suggests the existence of a haemolytic process in these animals. Thus we attribute this apparently negative finding to the fact that most of the antierthrocyte antibodies, which in the NZB/W mouse are produced in relatively small quantities in comparison with the NZB parent,³ are fully absorbed onto circulating red blood cells. It is conceivable that we might have detected such antibodies using the direct Coombs' test, but the logistics of the experiment did not permit the procedure. We do not exclude the possibility that the loss of red blood cells in these animals may be because of haematuria or mechanical angioathic haemolysis due to vasculitis. Such possibilities can still be attributed to the broad effects of the autoimmune disorder(s) present in these animals and thus warrant further investigation.

The results from this study support the use of the NZB/W mice as a model of human systemic lupus erythematosus and Sjögren's syndrome, and confirm the dramatic effect of lipids on immune function. Interestingly, human Sjögren's syndrome is usually unresponsive to conventional non-steroidal anti-inflammatory drugs, and in some cases these agents may have adverse effects.¹⁷ Further investigations may help to determine the efficacy of nutritional

intervention as an adjunctive therapeutic regimen in patients with diagnosed Sjögren's syndrome and systemic lupus erythematosus.

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References

- 1 Levy J A. Viral, anti-viral, cellular and nutritional factors associated with autoimmunity in mice. In: Krakauer J, Cathcart M K, eds. *Immunoregulation and autoimmunity*. North Holland, New York: Elsevier, 1980: 117-28.
- 2 Theofilopoulos A N, Dixon F J. Murine models of systemic lupus erythematosus. *Adv Immunol* 1985; **37**: 269-390.
- 3 Morrow W J W, Isenberg D A. *Autoimmune rheumatic diseases*. Oxford: Blackwell Scientific Publications, 1987.
- 4 Kessler H S. A laboratory model for Sjögren's syndrome. *Ann Pathol* 1968; **52**: 671-85.
- 5 Bloch K J, Buchanan W W, Whol M J, Bunin J J. Sjögren's syndrome: a clinical, pathological, and serological study of cases. *Medicine (Baltimore)* 1965; **44**: 187-231.
- 6 Homsy J, Morrow W J W, Levy J A. Nutrition and autoimmunity: a review. *Clin Exp Immunol* 1986; **65**: 433-88.
- 7 Levy J A, Ibrahim A B, Shirai T, *et al*. Dietary fat affects immune response, production of antiviral factors, and immune complex disease in NZB/W mice. *Proc Natl Acad Sci USA* 1989; **79**: 1974-8.
- 8 Yumura W, Hattori S, Morrow W J W, Mayes D C, Levy J A, Shirai T. Dietary fat and immune function: II. Effect on immune complex nephritis in (NZB×NZW)_{F1} mice. *J Immunol* 1985; **135**: 3864-8.
- 9 Morrow W J W, Ohashi Y, Hall J, *et al*. Dietary fat and immune function: I. Antibody responses, lymphocyte and accessory cell function in (NZB×NZW)_{F1} mice. *J Immunol* 1985; **135**: 3857-63.
- 10 Hoffman R W, Alspaugh M A, Waggie K S, Durham J, Walker S E. Sjögren's syndrome in MRL/l and MRL/n mice. *Arthritis Rheum* 1984; **27**: 157-65.
- 11 Green E R, ed. *Biology of the laboratory mouse*. New York: McGraw-Hill, 1966: 352.
- 12 Morrow W J W, Homsy J, Swanson C A, Ohashi J, Estes A, Levy J A. The influence of dietary fat on autoimmune disease in MRL/lpr/lpr mice. *Immunology* 1986; **59**: 439-43.
- 13 Morrow W J W, Levy J A. Dietary regulation of the autoimmune process in murine lupus. *Immunology Today* 1987; **4**: 249-50.
- 14 Morrow W J W, Homsy J, Swanson C A, Levy J A. The influence of nutrition on experimental autoimmune disease: an overview with particular emphasis on the effects of dietary fat. In: Cunningham-Rundles S, ed. *Nutritional regulation of immune responses*. New York: Marcel Dekker (in press).
- 15 Miale J B. *Laboratory medicine and hematology*. 4th ed. St Louis, Mosby, 1972: 1204.
- 16 Fernandes G, Yunis E J, Smith J, Good R A. Dietary influence on breeding behavior, hemolytic anemia and longevity in NZB mice. *Proc Soc Exp Biol Med* 1972; **139**: 1189-96.
- 17 Horrobin D F. Essential fatty acid metabolism in diseases of connective tissue with special reference to scleroderma and Sjögren's syndrome. *Med Hypotheses* 1984; **14**: 233-47.