PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 477–482 (1979)

Correlation of Dairy Food Intake with Human Antibody to Bovine Milk Xanthine Oxidase[1] (40474)

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A novel theory has recently been proposed by Oster (1), which suggests that bovine milk xanthine oxidase (BMXO) may produce the initial atherosclerotic lesion by depleting the plasmalogen content of myocardial and arterial tissue. Several investigations have been done during recent years that add support to Oster's theory (2–5); however, conclusive evidence has not been obtained. In a recent FASEB review (6) of the significance of BMXO in the etiology of atherosclerosis, it was concluded that further investigation is warranted in this area.

One of the points which needs to be established in relation to this theory is whether any correlations can be made between dairy food consumption and levels of BMXO in the blood of humans. This study was concerned with measuring the concentrations of antibody to BMXO in human sera, relating this to the amount of dairy food consumption, and attempting to determine if the amount of circulating immunoglobulins (IgA, IgG, and IgM) correlated with BMXO titers.

Materials and methods. Subjects. A group of 94 employees of ICI Americas Inc., who were taking part in an ongoing company employee health program, voluntarily participated in this study. This group included 88 men and 6 women ranging in age from 21–65 and 20–48, respectively.

Eighteen of the 94 participants suffered from what might be considered chronic ailments which included (number of subjects) kidney stones (3), gastric ulcer (1), angina pectoris (1), thyroidectomy (1), gout and/or hypothyroidism (2), spastic colon (1), asthma (2), elevated blood sugar (2), removed gall bladder (1), hypertension (2), colitis (1), and a combination of skin cancer, colitis and allergies (1).

Serum collection. Each subject had fasted for 14–15 hr prior to blood sampling. Blood samples were collected in 10 ml B-D disposable vacutainer tubes containing no additives. One half of each serum sample was divided into three aliquots and was frozen at −20° until it was tested for antibodies to BMXO and for immunoglobulin concentrations. These sera remained frozen for several months until the study was completed. The other portion of the serum was used to determine clinical chemical values and β-lipoprotein electrophoretic patterns, immediately after collection.

Questionnaires. Questionnaires were used to obtain estimates of the quantities and frequency of consumption of various types of fluid milk (i.e., pasteurized homogenized whole milk, powdered, chocolate, etc.). Consumption of other dairy products such as ice cream, milk shakes, various cheeses, and yogurt were also surveyed. A brief description of any chronic illnesses or special diets was also requested. Other pertinent information gathered included age, sex, weight, height, and types of medication of lowering cholesterol or triglycerides and smoking habits.

Clinical Chemical and β-lipoprotein Analyses. Sixteen serum clinical chemistries (7–13) and a β-lipoprotein electrophoresis (14) were determined for each subject in this study.

Serum analysis for BMXO antibodies. Serum antibodies to BMXO were detected by the passive hemagglutination technique described by Campbell (15), using tanned sheep erythrocytes. All human and rabbit sera were absorbed with sheep red blood cells (SRBC) in equal volumes. All controls involving XO treated SRBC and human or rabbit serum

[1] Published with the approval of the Director of the Delaware Agricultural Experiment Station as Miscellaneous Paper No. 819, Contribution No. 41, of the Department of Animal Science and Agricultural Biochemistry, University of Delaware, Newark.

0037-9727/79/040477-06$01.00/0

were negative. Normal rabbit serum (NRS, 1:100 in Abbott's physiologic saline) was used to dilute the human sera. A 1:40 dilution of the human sera was found to be an optimal concentration corresponding to the concentration of purified BMXO that was used (0.0062 mg/ml containing 21.8 ImU activity) in this procedure. Subsequently, serial twofold dilutions of the 1:40 sera were made. The sheep blood used was obtained fresh from castrated males, and was preserved in a 1:1 ratio with Alsevers solution. The human sera were tested twice, at different times under similar conditions. Geometric means were then calculated for the two replicates for each of the sera tested (16).

The BMXO used was prepared from fresh cow's milk by an improved method (17, 18) yielding enzyme of the highest purity ever reported without the use of proteolytic and lipolytic enzymes, butanol, and other harsh organic solvents. The final BMXO preparation is over 4800 fold purified, had an average protein flavin ratio (E_{280}/E_{450}) value of 4.1 (the most sensitive indicator of BMXO purity), one symmetric peak by column chromatography, analysis by polyacrylamide disc gel electrophoresis demonstrated a single band, and one symmetric peak by ultracentrifugation.

Quantitations of immunoglobulin levels. All of the sera collected were assayed for immunoglobulin (Ig) A, G, and M[2] concentrations by a modified single radial immunodiffusion method described by Mancini *et al.* (19), and Fahey and McKelvey (20).

For serum IgA quantitation, human IgA antiserum was diluted 1:15 in 0.01 *M* saline buffered with 0.03 *M* phosphate (PSB), pH 8.0. A 3% Oxoid agar was prepared by bringing 1.5 g of agar to a total volume of 50 ml with PSB, and heating without boiling. Both agar and antiserum were equilibrated in a 56° water bath for 10–15 min and combined in a 1:1 ratio by adding the agar to the antiserum and mixing gently with a glass rod. Ten ml of the antiserum-agar mixture was applied to Miles disposable diffusion plates and the agar was allowed to harden. Wells (3 mm dia) were cut into the agar gel using Miles Template Kit. The human sera to be tested for IgA were diluted 1:16 with PS.

Conditions for IgG quantitations were similar to those for IgA quantitations except that test sera were diluted 1:32 with PS. Antiserum for IgM quantitations was diluted 1:30 with PS and the test sera were diluted 1:2 with PS. Reference serum for standardizing the quantitation of human IgA, IgG, and IgM was purchased from Meloy Laboratories and was diluted 1:32, 1:64, and 1:128 in PS for IgA; 1:64, 1:128, 1:256, and 1:512 for IgG; and undiluted, 1:2, 1:4, and 1:8 for IgM.

Thirteen μl of the reference and test serum dilutions were pipetted into the antigen wells using a micro pipettor and disposable tips. The test plates were incubated at 4° for 3 days, and the precipitin rings viewed using indirect light. The ring diameters were measured with vernier calipers. All Ig quantitations were done twice, at different times, but under similar conditions.

Statistical analysis. The data were analyzed by stepwise multiple regression to determine which parameters contributed to the BMXO titer. This regression analysis was done in two ways: (a) In the accepted fashion by analyzing the data as a single group, and (b) by an experimental variation of the stepwise multiple regression.

Following the hemagglutination assay the subjects were divided into 5 arbitrary groups which were based on the range of geometric BMXO titer means. These groups were (subjects per group): 0 (21), 7–80 (15), 104–160 (16), 221–640 (29), and 891–5120 (13) hemagglutinating units (HAU).

The data were analyzed by an IBM 370/145 computer using the stepwise multiple regression analysis contained in the Statistical Analysis System (SAS) computer program package based on a book by Draper (21), designed and implemented by A. J. Barr and J. H. Goodnight, and made available through the Department of Statistics of the University of North Carolina.

Results. Antibodies to BMXO were found in the sera of 73 of the 94 individuals tested, and the mean antibody titers ranged from 7 to 5120 HAU. The data are represented by the mean and standard error of the mean in Figs. 1 and 2. As the mean consumption of

[2] All antisera were prepared in rabbits and were purchased in lyophilized form from Miles Laboratories, Elkhart, Indiana 46514.

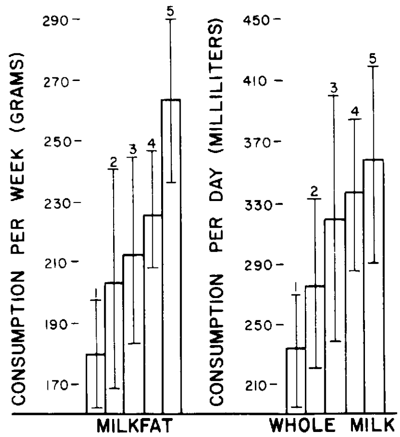

FIG. 1. Increase in antibody titers to bovine milk xanthine oxidase with increased milkfat and volume of whole milk consumed with standard error of the mean for each group. The numbers 1–5 pertain to the subjects as they were arbitrarily divided into the following titer groups (number per group), based on the range of geometric BMXO titers: 1 = 0 (21); 2 = 7–80 (15); 3 = 104–160 (16); 4 = 221–640 (29); and 5 = 891–5120 (13) hemagglutinating units.

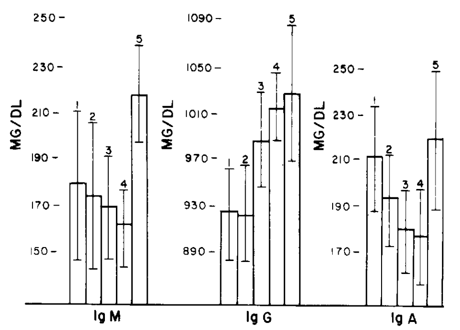

FIG. 2. Levels of serum immunoglobulins M, G, and A as they relate to each of the levels of antibodies to bovine milk xanthine oxidase. Numbers 1–5 are defined in the caption of Fig. 1. The standard error of the mean for each group is indicated.

milkfat and the volume of whole milk increased, so did the BMXO antibody levels (Fig. 1). Mean Ig values obtained were within normal limits. However, the results of the three serum Ig assayed showed a trend toward increased BMXO titers with lower IgA and IgM levels in all titer groups except the 891-5120 HAU group in which Ig levels and BMXO titers were highest (Fig. 2). The opposite was true with serum IgG where the trend was toward increased BMXO titers with higher IgG levels.

When all of the data were analyzed using stepwise multiple regression (Table I), lower serum glucose, high milk shake consumption, abnormal β-lipoprotein patterns, the incidence of tonsillectomy at the age of 10 or later, drinking milk before retiring, and milk consumption early in life showed a combined correlation of $r = 0.64$ with the presence of BMXO antibody titers which was highly significant ($P < 0.0001$).

Statistical results obtained from the experimental variation of the stepwise multiple regression analysis, when subjects were divided into 5 arbitrary groups, are presented in Table II. This was done to see which parameters would correlate with specific BMXO levels.

Discussion. Recently, Clark *et al.* (22) found that serum xanthine oxidase activity increased in rats 2 hours after intubation with processed cow's cream (half and half). They concluded that active BMXO was absorbed from the gut or that the cream may have stimulated endogenous xanthine oxidase activity. In another recent study, Ho and Clifford (23) estimated that of 100 mg of xanthine oxidase in fresh raw milk (500–700 ml of bovine milk), 41 mg survived processing, 27 mg entered the intestine active, and 20 ng were absorbed enzymatically active. Al-

TABLE I. RESULTS OF STEPWISE MULTIPLE REGRESSION ANALYSIS OF BOVINE MILK XANTHINE OXIDASE ANTIBODY VS. INDEPENDENT VARIABLES OBTAINED BY QUESTIONNAIRE.

Variable entered[a]	Cumulative R^2 values	Cumulative r values	Cumulative probability[b]
Glucose (−)	.139	.37	.0017
Milk shakes (+)	.228	.48	.0002
Lipoprotein electrophoresis (+)	.288	.54	.0001
Tonsils removed at age 10 or later (+)	.337	.58	.0001
Milk consumed before retiring (+)	.377	.61	.0001
Milk consumed early in life (+)	.410	.64	.0001

[a] The significance level for entry into the model was 50%; the significance level for staying in the model (partial F statistic) was 10%.

[b] This is the level of significance for each of the six multiple regression models after each new parameter was included into the model.

(+) = positive correlation; (−) = negative correlation.

TABLE II. STEPWISE MULTIPLE REGRESSION ANALYSIS OF FIVE BOVINE MILK XANTHINE OXIDASE TITER GROUPS VS. INDEPENDENT VARIABLES OBTAINED BY QUESTIONNAIRE.

Titer Group (HAU)[a]	Parameters included in the statistical model (Level of statistical significance)[b]	Cumulative R^2 values	Cumulative r values
7–80	Smoking (+)	0.51	0.71
	($p < 0.03$)		
104–160	IgM (−)	0.35	0.59
	Colds in winter and fall (−)	0.62	0.78
	Smoking (−)	0.82	0.91
	Butter (+)	0.91	0.95
	Pasteurized cheese (+)	0.988	>0.99
	IgG (+)	0.993	>0.99
	Vol. skim milk (−)	0.996	>0.99
	Adenoids removed (−)	0.999	>0.99
	Diastolic blood pressure (+)	0.9997	>0.99
	Total protein (+)	0.9999	>0.99
	Sour cream (+)	0.9999	>0.99
	B1 cardiovascular Risk[c] (+)	1.00	1.00
	($p < 0.0002$)		
221–640	IgM (+)	0.39	0.63
	Tonsils removed (+)	0.72	0.85
	Milk consumed before retiring (+)	0.84	0.92
	Colds in summer and spring (+)	0.89	0.94
	Systolic blood pressure (−)	0.91	0.95
	($p < 0.0002$)		
891–5120	B1 cardiovascular risk[c] (+)	0.60	0.77
	SGOT (+)	0.79	0.89
	Hematocrit (+)	0.89	0.95
	Smoking (−)	0.96	0.98
	Glucose (−)	0.99	>0.99
	Diastolic blood pressure (−)	0.999	>0.99
	IgA (−)	0.999	>0.99
	Calcium (−)	1.00	1.00
	($p < 0.0002$)		

[a] HAU = hemagglutinating units.
[b] Level of statistical significance of the regression analysis for each group of parameters.
[c] Individuals falling into this category were classified as moderate risks for cardiovascular disease, based on a cardiovascular risk profile.
(+) = positive correlation; (−) = negative correlation.

though the absorbed amount of active BMXO by this estimate appears to be nutritionally negligible, on a lifetime basis this quantity may be biologically very important.

Due to the practical problems when humans are the test subjects, the dairy food intake data in this study are based on estimates obtained by questionnaires and as such they do not represent exact dairy food consumption. Nonetheless, these estimates are useful to obtain an indication of any possible relationship of dairy food consumption and serum antibody to BMXO. The apparent dose response observed between consumption of milkfat and whole milk and BMXO titers (Fig. 1), tend to support the possibility of dietary BMXO entering the blood stream via absorption or persorption (24) from the gut. Seventy-eight percent (73 subjects) of the individuals tested had a titer to BMXO while 22% (21 subjects) did not. There are several possible explanations why the 21 subjects in titer group 1 did not have measurable BMXO titers. This group had the lowest dairy food consumption of the 5 groups (Fig. 1). In addition, group 1 had the highest level of serum IgA and IgM in four of the five titer groups (Fig. 2), suggesting that this group had a potentially high immunological protection. Finally, the sera of the subjects in this group may indeed contain antibody to BMXO, the level of which may have been lower than the sensitivity of the detection method used.

Other variable factors that may be playing a role in the uptake of dietary BMXO from the gut include the following. First, since the amount of active BMXO in any dairy product varies widely from manufacturer to manufacturer (because of the processing methods used) (25), the amount, type, and brand of dairy product consumed would determine the quantity of active or heat-inactivated BMXO available. For example, due to the processing condition, butter contains practically no BMXO activity (25). Nonetheless, butter still contains the inactivated BMXO which has been found to be antigenic when administered parenterally to animals (26–28). A second consideration would be the concentration of intestinal secretory Ig that would influence BMXO absorption-persorption. Animal studies have shown that decreased levels of secretory IgA and altered gut function can lead to increased uptake of macromolecules (4), which could find their way into the blood through the lymphatics (1). A third factor would be the levels of serum antibody that would be needed to inactivate BMXO once in the blood. A fourth factor that could influence BMXO uptake and/or its presence in the circulation would be the functional capabilities of secretory and circulating Ig as well as the reticuloendothelial cells of the liver, which would determine the ability of the body to remove BMXO from the blood. Since smoking has been implicated as a risk factor in heart disease, it would be interesting to explore the possible effects of smoking on the functional capabilities of these immune mechanisms.

Oster (29) has shown that individuals with clinically manifested atherosclerosis had higher titers to BMXO than did individuals with no clinical evidence of atherosclerosis. An overlap of BMXO titers in both atherosclerotics and nonatherosclerotics was shown. Since both active and inactivated BMXO are antigenic (26–28), this overlap may be due to the combined antigenicity of the two forms of the enzyme. Furthermore, only the active form of the enzyme has been implicated as inducing a cardiovascular lesion. The contribution of each of these enzyme forms to the titer needs to be resolved to determine how much of the antibody is produced to active BMXO. Therefore, a reliable assay needs to be developed to differentiate antibody to active BMXO from antibody to inactivated BMXO.

Since abnormal β-lipoprotein patterns, type II or low density lipoprotein (LDL) and type IV or very low density lipoprotein (VLDL) have been associated with atherosclerosis (30), the contribution of abnormal β-lipoprotein patterns to the statistical correlation with the presence of serum antibody to BMXO, could be a potential indicator in identifying early atherosclerosis and related heart diseases. A study recently completed by Ross *et al.* (31) indicates that BMXO is present in liposomal structures in homogenized pasteurized cow's milk. These structures need to be characterized further to determine if they are themselves LDL or VLDL, or if they contain LDL and/or VLDL which have been associated with atherosclerosis.

Finally, the possiblity of shared antigens between BMXO and human liver xanthine oxidase is likely, however, an immune response to such antigens would constitute an autoimmune reaction of serious consequences to the individual. It is, therefore, reasonable to believe that the Ab measured in this study were to the BMXO and not to endogenous sources.

Summary. Seventy-three out of 94 human subjects were found to have antibodies to BMXO. Individuals with no BMXO titer had the lowest dairy food consumption of the population tested and there appears to be an inverse relationship between increased dairy fat consumption and decrease in IgA and IgM concentrations in titer groups 1–4. When divided into arbitrary groups according to the geometric mean titer, BMXO titers were higher as the milkfat and whole milk consumption increased (Fig. 1). Multiple regression analysis of the data indicated highly significant correlations ($P < 0.0001$) between milkshake consumption, milk consumption in early life, abnormal lipoprotein patterns, low serum glucose level and the incidence of tonsillectomy at age 10 or later with the presence of antibodies to BMXO. These correlations and the observation that higher milkfat and whole milk consumption resulted in higher BMXO titers suggest the uptake of dietary BMXO from the gut.

The authors thank Dr. H. Freedman for his critical evaluation of this paper and Dr. R. Frelick for his interest and support of this project. The authors also acknowledge the contributions from the following individuals:

Drs. D. H. Sussdorf, J. Dean, M. Tripp, and J. Butler, Mr. F. Baker, Mses. L. Varland, C. Dougherty, D. Biddle, K. Ryan, D. Robinson and E. Wu.

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Received March 17, 1978. P.S.E.B.M. 1979, Vol. 160.