Studies of Blood Lipid Fractions of Quiescent Rheumatic Fever Patients and Their Siblings

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The maturation of the rheumatic process in man requires a summation of several factors. As pointed out by McKusick (1), "presumably, genetic factors determine the qualitative and/or quantitative nature of the host response to repeated streptococcal infections. The mechanism of this gene action is, of course, of great interest but is unknown." In studying the roles of the genes and nutrition on blood fat content in man, Osborne et al. (2) concluded: "By this method it has been possible to demonstrate that both genetic and environmental factors may produce measurable variations in the serum lipid levels of healthy adults." The present study is designed to determine whether rheumatic fever is associated with abnormalities in serum lipids, which might be due to genetic or environmental factors.

Todd, Coburn, and Hill (3) showed that rheumatic children, in contrast to their siblings, failed to develop the expected rise of antistreptolysin S following hemolytic streptococcal pharyngitis. Subsequently, antistreptolysin S was shown to be streptolysin inhibitor (SSI) by Stollerman et al. (4, 5), who demonstrated that the inhibitor was associated with alpha and beta lipoproteins. Later Stollerman et al. (6) pointed out that the fall in serum levels of streptolysin S inhibitor during the rheumatic attack indicates a disturbance of lipoprotein metabolism in rheumatic fever. They found in acute rheumatic fever that: (a) the SSI level falls, (b) there is a positive correlation between the serum SSI and phospholipid concentration, and (c) this relationship is best illustrated by the lecithin fraction.
This finding was similar to the observation on vitamin A metabolism in acute rheumatic fever made by Shank, and his collaborators (7). In a study by Chang et al. (8), of the serum lipid levels of clinically quiescent rheumatic subjects it was found that these rheumatic children in good health had statistically significant lower fasting blood lipid levels than their nonrheumatic siblings living under similar environmental conditions. The purpose of this report is to point out that the quiescent rheumatic child maintains a blood lecithin level significantly lower than its nonrheumatic sibling.

Children clinically free of rheumatic activity for at least one year, and their siblings below age 14 living under similar environmental conditions, were included in this study. Of 80 rheumatic families under investigation 29 were selected for three chemical determinations on the basis of there being (a) one rheumatic fever child apparently free of disease activity for at least one year, (b) one full sibling of similar age who had escaped rheumatic manifestations, and (c) both eating approximately the same meals, even though the quantities of individual foods consumed may have varied. This study is limited to differences in plasma lecithin and sphingomyelin levels of rheumatic subjects and presumably nonrheumatic siblings. Attempts to obtain accurate values for the ethanolamine moiety of cephalin to date have been unsuccessful.

PROCEDURE

In order to assay lecithin, sphingomyelin, and cephalin with economy of available amounts of specimen and laboratory time, the phospholipid extraction procedure of Folch and Van Slyke (9), as modified by Axelrod (10), was adopted so that lecithin and sphingomyelin could be estimated on extracted aliquots by the procedure of Levy, Appleton, et al. (11), and the ethanolamine moiety of cephalin by the procedure of Axelrod et al. (10).

PHOSPHOLIPID EXTRACTION

REAGENTS

1. Dialyzed iron containing 5% Fe₂O₃ (Amend Drug Co.).
2. Magnesium sulfate solution; 50 Gm. of MgSO₄·7H₂O dissolved in 50 ml. of water.
3. 0.5 N HCl in 50% ethyl alcohol.
4. Absolute ethyl alcohol.
5. Absolute ether.
2 ml. of plasma and 30 ml. of distilled water are pipetted into a 40-ml. conical graduated glass-stoppered centrifuge tube. 2.5 ml. of the dialyzed iron solution and 1.3 ml. of the magnesium sulfate solution are then added. The tube is shaken and centrifuged for 10 minutes at 3500 rpm. The supernatant fluid is then removed by decantation. The precipitate is washed and centrifuged three times, using 30 ml. of water and 1.3 ml. of the magnesium sulfate solution each time. After the last washing the precipitate is suspended in 8 ml. of absolute ethyl alcohol, and absolute ether is added to the 20 ml. mark. The lipids are extracted from the precipitate by shaking for five minutes. The tubes are then centrifuged at 3500 rpm for 10 minutes. A 15-ml. aliquot of the alcohol-ether supernatant fluid is transferred to a 60-ml. glass-stoppered bottle. The solution is evaporated to dryness in a stream of air over a steam bath. The bottles are then cooled and 0.1 ml. of 0.5 N HCl in 50% ethyl alcohol is added, followed by 20 ml. of petroleum ether, and shaken for 10 minutes. An 8-ml. aliquot of the petroleum ether extract is used for the determination of lecithin and sphingomyelin, and a 10-ml. aliquot of the petroleum ether extract is used for the determination of phosphatidyl ethanolamine.

ESTIMATION OF LECITHIN AND SPHINGOMYELIN (11)

The extracted phospholipids are subjected to two alkaline hydrolyses. The condition of the first hydrolysis permits complete hydrolysis of lecithin but leaves sphingomyelin unhydrolyzed. Following this hydrolysis the choline liberated from the lecithin is determined on the acidified supernatant (12). The precipitate is washed and the sphingomyelin fraction is subjected to strong alkaline hydrolysis. Sphingomyelin choline is determined on the resulting hydrolysate.

REAGENTS

1. Potassium hydroxide, 1N.
2. Potassium hydroxide, 3.6N.
3. HCl, 4N.
4. Acid-acetone, reagent acetone, and concentrated HCl (9:1, v/v).
6. For the determination of choline the reagents used are as described under the heading "Precipitation and Estimation of Choline as Periodide" (12).
PROCEDURE

The 8-ml. petroleum ether extract is evaporated on a steam bath in a stream of air. During the evaporation the tube is shaken to redissolve the lipids that adhere to the walls of the tube above the level of evaporation. At the end of the evaporation the test tube is heated several minutes longer on the steam bath to drive off the last traces of petroleum ether. One ml. of 1N KOH is added to the residue, and the tube is covered with a glass marble and placed in a constant temperature water bath set at 37°. After 10 minutes the tube is removed and carefully shaken to distribute the solids as a homogeneous suspension. The tube is then returned to the water bath for 18 hours, after which it is cooled to room temperature. One ml. of acetone:HCl (9:1) and a small amount of Hyflo-Supercel are added, and the contents mixed by shaking. The tube is centrifuged for 10 minutes at 3500 rpm, and 0.5 ml of the supernatant fluid is removed by carefully inserting a capillary tube below the surface layer of the floating particles and above the precipitate at the bottom of the tube, and transferred to a 15-ml. graduated tube. The acetone is evaporated by warming over a steam bath in a stream of air until approximately 0.2 ml. remains. The solution is diluted exactly to 1.5 ml. with distilled water, centrifuged for 10 minutes at 2500 rpm, and 0.5 ml of the solution is transferred to a special tube for the determination of choline as described by Appleton et al. (12).

The original tube containing the precipitate is recentrifuged for 10 minutes at 3500 rpm, and the supernatant fluid is carefully decanted. The precipitate is washed twice with 2-ml. portions of acetone:water (1:1), centrifuging for 10 minutes between each washing. The tubes are warmed gently on a steam bath in a stream of air until all of the acetone and most of the water are evaporated. The precipitate is then suspended in 1 ml. of 3.6N KOH and the tube is covered with a glass marble and placed in a water bath kept exactly at 100° for four hours. It is important that the 100° temperature of the water bath is maintained throughout the entire hydrolysis procedure (9). The tubes are cooled to room temperature, and are acidified by the addition of 1 ml. of 4N HCl. The tubes are centrifuged for 10 minutes at 3500 rpm, and 0.5 ml. of the solution is transferred to the special choline determination tube for the sphingomyelin choline analysis. The determination of choline by the procedure of Appleton et al. (12) is made by measuring choline- ennea-iodide in a solution of ethylenedichloride with a Bausch & Lomb Spectronic 20 spectropho-
tometer at a wavelength of 365 m\(\mu\). Standard choline solutions were run through the entire precipitation procedure with the unknown specimens. For the final calculations of lecithin and sphingomyelin, 1 mg. of lecithin was used as equivalent to 112 \(\mu\)g. of choline chloride, 1 mg. of sphingomyelin as equivalent to 153 \(\mu\)g. of choline chloride. The molecular weight (11) of lecithin (\(\text{C}_{43}\text{H}_{98}\text{NO}_{9}\text{P}\)) was taken as 793, the molecular weight (12) of sphingomyelin (\(\text{C}_{47}\text{H}_{77}\text{N}_{2}\text{O}_{7}\text{P}\)) as 832, and that of ethanolamine as 61.

**ESTIMATION OF PHOSPHATIDYL ETHANOLAMINE**

The measurement of phosphatidyl ethanolamine was based essentially on the procedure of Axelrod et al. (10). The petroleum ether extract, 10 ml. obtained as described under the heading *Phospholipid Extraction*, was evaporated to dryness. The lipid residue was then subjected to alkaline hydrolysis and the ethanolamine liberated was reacted with dinitrofluorobenzene to form a yellow-colored complex. The yellow dinitrofluoro-ethanolamine complex was separated from the excess reagent by chloroform extraction. The chloroform was evaporated to dryness, and the residue dissolved in petroleum ether. The yellow complex was then returned to an aqueous phase for reading by extracting the petroleum ether solution with acid.

**REAGENTS**

1. 2N NaOH.
2. 2N HCl.
4. Dinitrofluorobenzene reagent, 0.1 ml. of dinitrofluorobenzene (Mann Chemical Company) dissolved in 2 ml. ethyl alcohol.
5. 2.5% NaHCO\(_3\).
6. Chloroform, reagent grade.

**PROCEDURE**

The 10-ml. aliquot of the petroleum ether lipid extract is evaporated to dryness on a steam bath in a stream of air. During the evaporation the tube is shaken to redissolve the lipids that adhere to the walls of the tube above the level of evaporation. When the last trace of the petroleum ether is removed, 1 ml. of 2N NaOH is added to the tube and the tube is covered with a glass marble and placed in a boiling-water bath for 30 minutes. At this time the tube is shaken to dislodge any solid particles, and the hydrolysis is continued at
100° for one and a half hours. The tube is then cooled and 1 ml. of 2N HCl and a small amount of Hyflo-Supercel are added, and the tube is centrifuged for 10 minutes at 3500 rpm. After 1.5 ml. of the supernatant fluid is put into a glass-stoppered 15-ml. centrifuge tube, 0.1 ml. of the dinitrofluorobenzene reagent and 1 ml. of 2.5 per cent NaHCO₃ are added. The tube is stoppered and placed in a water bath at 75-80° for one hour. After cooling, 8 ml. of chloroform are added and the tube is shaken for 10 minutes. The layers are separated by centrifuging and the supernatant aqueous phase is removed by aspiration.

Six ml. of the chloroform extract is then transferred to a 40-ml. glass-stoppered centrifuge tube, and evaporated almost to dryness on a steam bath, utilizing a stream of air. Ten ml. of petroleum ether and 4 ml. of 5N HCl are then added, and the tubes are shaken for five minutes. Three ml. of the acid phase is transferred to a cuvette and read at 420 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer. A reagent blank run through the entire procedure is used for the zero setting.

**CALCULATIONS**

The following formulas are for a 2-ml. plasma specimen and the working aliquots described in the text.

\[
\text{mMol/L lecinthin} = \frac{\text{O.D. unk.}}{\text{O.D. std.}} \times \text{con. std. (15 μg.)} \times \frac{500}{112} \times \frac{56.1}{793} \tag{1}
\]

\[
\text{mMol/L sphingomyelin} = \frac{\text{O.D. unk.}}{\text{O.D. std.}} \times \text{con. std. (15 μg.)} \times \frac{500}{153} \times \frac{18.7}{332} \tag{2}
\]

\[
\text{mMol/L ethanolamine} = \frac{\text{O.D. unk.}}{\text{O.D. std.}} \times \text{con. std. (30 μg.)} \times \frac{500}{8.79} \times \frac{61}{61} \tag{3}
\]

**EXPERIMENTAL**

Children clinically free of rheumatic activity for one year, and their siblings below the age of 14 years living under similar environmental conditions, were included in this study. Of 80 rheumatic families under investigation, 29 were selected for the three chemical determinations on the basis of there being (a) one rheumatic-fever child apparently free of disease activity for one year, and (b) one sibling of similar age, who had escaped rheumatic manifestations.

Preliminary observations showed that the accuracy of the methods used on random duplicate samples of plasma before and after meals was ±7% for lecinthin and ±14% for sphingomyelin. Altogether, 29 plasmas from 29 rheumatic subjects were tested and 31 plasmas from
siblings considered nonrheumatic. The age mean of the former was 10.8 years (range 6-14), and of the latter, 9 years (range 5-13). Each group contained about 60 per cent females. Blood samples from rheumatic children and siblings were taken at the same time, between 2 and 3 P.M., centrifuged together, stored at $-20^\circ$, and tested in parallel. The medical histories of the children were unknown to the analyst until the study had been completed and analyzed statistically.

**RESULTS**

**LECITHIN**

Twenty-one rheumatic children had a mean lecithin value of 2.76 mMol./L. with a Standard Deviation of ±0.78. Thirty-one siblings had a mean lecithin value of 3.15 mMol./L. with a Standard Deviation of ±0.56. The evaluation of the differences between the means gave $Z = 2.2$ and a $p$ value of 0.03 which showed that the difference between the means was statistically significant. Evaluation of direction of differences showed that nine of the rheumatic fever children had higher lecithin values than their siblings. Nineteen rheumatic fever children had lower lecithin values than did their siblings, and one rheumatic fever child had the same value as its sibling. For this group chi-square $= 2.9$ (with one degree of freedom).

**SPHINGOMYELIN**

Rheumatic fever children had a mean sphingomyelin value of 0.23 mMol./L. with a Standard Deviation of ±0.08. Their siblings had a mean sphingomyelin value of 0.25 mMol./L. with a Standard Deviation of ±0.14. The evaluation of the difference between the means gave $Z = 0.7$ and a $p$ value of 0.48. These data showed that the differences between the means were not statistically significant. Evaluation of the direction of the differences showed that 16 of the rheumatic fever children had higher sphingomyelin values than did their siblings, and 13 rheumatic fever children had lower sphingomyelin values than their siblings. For these data chi-square $= 0.1$ (with one degree of freedom).

Attempts to obtain accurate values for the ethanolamine moiety of cephalin to date have been unsuccessful.

**DISCUSSION**

Previous reports showed that among disease states studied in hospital wards for adults, only rheumatic fever had a statistically significant low fasting blood lecithin level. For example, Appleton and
Steele found mean lecithin plasma levels to be 2.03 mMol./L. in normal adults, 7.14 in nephrotics, 7.24 in patients with diabetes melitus, 8.04 in biliary cirrhosis, but only 1.45 in patients with active rheumatic fever (13). The present findings show that, among children exposed to the same environmental diet of the home, there were differences in blood lipids between those who had manifested and those who were apparently unable to develop rheumatic fever, following group A streptococcal infections of the upper respiratory tract. A key problem in rheumatic fever remains: Are blood lipid values of susceptible children lower than those of the nonsusceptible prior to onset of the rheumatic state? In determining whether this metabolic association is a precursor or a sequela of rheumatic fever, the plasma lecithin determination may serve as a useful guide. For this purpose it would be necessary to screen a large number of underprivileged children living in an environment where a high incidence of rheumatic fever can be reasonably anticipated. This must await the development of a simple and rapid method for determining blood lecithin values.

SUMMARY

Three phospholipid fractions have been determined in plasmas of quiescent rheumatic children and their siblings of similar ages.

The method for determining blood lecithin gave reproducible results, and the lipid fraction appeared indicative of difference in levels of blood lipids between rheumatic and nonrheumatic children.

In this series of lecithin determinations only one rheumatic subject had a high normal level and only one sibling (presumably nonrheumatic) had a low level.

The difference in mean nonfasting lecithin plasma levels between rheumatic subjects and siblings was 0.36 mMol./L. Similarly, the lecithin level of each of the nonrheumatic siblings in this series averaged 0.41 mMol./L. higher than that of the corresponding quiescent rheumatic child. In both of these differences P < 0.05.

REFERENCES