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RESEARCH ARTICLE

The Double Cause Hypothesis for Autoimmune Diseases is Supported in Diabetic Mice (NOD)

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ABSTRACT

Introduction: The lung surfactant dipalmitoylphosphatidylcholine (DPPC) leaks into the blood, settling on the luminal aspect of blood vessels to create active hydrophobic spots. Nanobubbles are formed at these spots from dissolved gas. We hypothesized that contact between a large molecule in the blood and a nanobubble at an active hydrophobic spot would disrupt the molecule's tertiary structure. An exposed epitope may then prompt an autoimmune response.

Methods: DPPC content was determined in the heart of diabetic and healthy non-obese diabetic (NOD) mice and two control mice strains (C57/BL6 and Swiss-webster).

Results: The hearts from NOD mice contained more DPPC (47.6 ± 3.7 SE mg/g) than the control mice (36.9 ± 2.2 SE mg/g) ($P < 0.018$).

Discussion: It is probable that leakage of large β-cell molecules is the difference between affected and non-affected NOD mice. The Double Cause Hypothesis (DCH) is supported and the further research and applicability is discussed.

Keywords: lung surfactant, nanobubbles, heart, Active Hydrophobic Spot

Introduction

ACTIVE HYDROPHOBIC SPOT = (AHS)

In the search for the hypothesized gas micronuclei from which bubbles evolve during decompression after diving, we succeeded in establishing the following chain of events: The lung surfactant dipalmitoylphosphatidylcholine (DPPC) leaks into the blood stream. Leaving the plasma, the DPPC settles on the luminal aspect of blood vessels to create an oligolamellar lining of phospholipids. We named this site an "active hydrophobic spot" (AHS). Nanobubbles are formed from dissolved gas at the AHS. During the dive, these nanobubbles become the gas micronuclei from which bubbles evolve on decompression (1). These AHS can be found on any type of blood vessel: arteries, veins, capillaries and heart chambers.

DOUBLE CAUSE HYPOTHESIS – (DCH)

Considering the possibility that the blood is thus faced with a constant gas phase contained in the nanobubbles, we proposed that this may also affect autoimmunity (2). The Double Cause Hypothesis – (DCH) suggests that the development of autoimmune disease may be due to two independent processes: 1. The leakage of large molecules (potential autoantigen and specific for each disease) into the blood. This molecule will change its tertiary structure at the gas/liquid interface and be transformed into an autoantigen. 2. The existence of many and large AHS. The DCH is appealing because, if proved correct, it would enable a number of prophylactic procedures. Sometime in the future, the elimination of plasma DPPC or the removal of the AHS may prevent the development of an autoimmune disease.

THE LEAKAGE OF LARGE MOLECULES

Recently, different studies have identified target cells as the origin of autoimmune diseases (3). Some target cells, hold genetic codes for excess protein production and inflammation. Certain overactive genes code for disease related proteins associated with multiple sclerosis and rheumatoid arthritis. Many target cells are located in glands which pump hormones and possibly other proteins directly into the blood and are rich in blood vessels. Eizirik et al., (4) suggested that inflammation might contribute to early induction and amplification of the immune assault against pancreatic beta cells. The increased vulnerability of women to autoimmune diseases is related to activation of genes on both X chromosomes and increased protein production (5). Women with lupus present activated genes on both X copies and their activity correlates with the severity of the disease. All of the above considerations lead to increased chances of releasing large molecules into the blood stream.

AHS VARIABILITY

There is large variability in the number and size of AHS in blood vessels of sheep, which corresponds to the variability of bubbling / non-bubbling divers (1). According to the DCH it would be expected that individuals with high levels of AHS would be susceptible to autoimmune diseases. A limited study of mice (8 vs. 5) pointed to an increased level of DPPC in the heart of mice affected by lupus (6).

STUDY FRAMEWORK

We know that the rate limiting factor for production of AHS is not spillage of DPPC from the lung into the plasma, but the amount which settles at the blood vessels. Our two reports on the concentration of DPPC in the plasma from diabetes type1 patients, did not reveal a significant difference from the control (7,8). This is contrasts with the amount of DPPC in the hearts of mice suffering from lupus (6). Some of the NOD mice were affected by diabetes type 1 and others remained healthy. If the difference between affected and non-affected is related to the level of settled DPPC, we would expect to find elevated DPPC in the hearts of affected mice compared to non-affected NOD mice. However, if high levels of DPPC is a trait of the NOD mice, and the difference between affected and non-affected is due to leakage of large pancreatic β cell molecules like GAD65 or proinsulin (9,10), we would expect the level of the settled DPPC to be high in NOD mice compared to control mice. In this study we sampled hearts from affected and non-affected NOD mice and two other types of control mice.

Methods

ANIMALS AND TREATMENT

Mice were bred in a specific-pathogen-free vivarium and were fed a standard laboratory diet and water ad libitum. The study involved non-obese diabetic (NOD), Swiss Webster, and C57BL/6 mice, and it was approved by the ethics committee of Bar-Ilan University.

GLUCOSE MEASUREMENTS

Twenty-week-old NOD/LtJ (JAX # 001976) males and females were monitored twice weekly for diabetes using a FreeStyle Freedom Lite blood glucose meter (Abbott Diabetes Care Inc., CA, USA) by drawing a single drop of blood from the tip of the tail. Diabetes was defined as blood glucose levels ≥ 250 mg/dL on two consecutive days. At the end of the experiment, blood glucose levels were

measured in all mice from all four groups before sacrificing.

PROTOCOL

Mice were sacrificed by dislocation. Excised hearts were carefully squeezed in saline to clear excess blood and stored at -20°C until analyses. When all 41 samples had been completed, they were transferred to the Western Galilee Medical Center for phospholipid extraction. Phospholipids were extracted using an accepted procedure, as described by the authors in a previous study (6). The N_2-dried phospholipids were kept at -20°C until delivery to the MIGAL laboratory in Kiryat Shmona for the determination of DPPC using liquid chromatography–mass spectrometry. Samples were analyzed as described in detail previously (6).

STATISTICAL ANALYSIS

As described in the introduction, A normality test (Shapiro-Wilk) and an equal variance test (Brown-Forsythe) were used for the concentration of DPPC in the diabetic and healthy NOD groups. The Student's *t*-test was then used for the equality/inequality of the results. The same procedure was done to compare the two control groups. When no difference was found in the previous tests, we compared the combined control groups to the combined NOD groups.

Results

The experimental mice are presented in Table 1. In seven of the diabetic NOD mice the glucose level was above the upper threshold and therefore the lowest value is given.

Table 1: Experimental mice data

Group	n	F/M ratio	Heart weight g	Glucose mg/g
NOD healthy	11	4/7	0.149 (0.040)	122 (25)
NOD diabetic	10	10/0	0.133 (0.102)	> 366
Control C57/BL6	10	1/9	0.145 (0.036)	144 (26)
Control Swiss-webster	10	7/3	0.158 (0.024)	117 (19)

Data represent mean (SD)

The concentration of DPPC in the four experimental groups is presented in Table 2 and Fig. 1.

Table 2: Concentration of DPPC in the hearts of mice.

Group	NOD diabetic	NOD healthy	Control Swiss-webster	Control C57/BL6
DPPC mg/g	44.2 (6.6)	50.6 (3.9)	33.0 (2.3)	40.7 (3.3)

Data represent mean (SE)

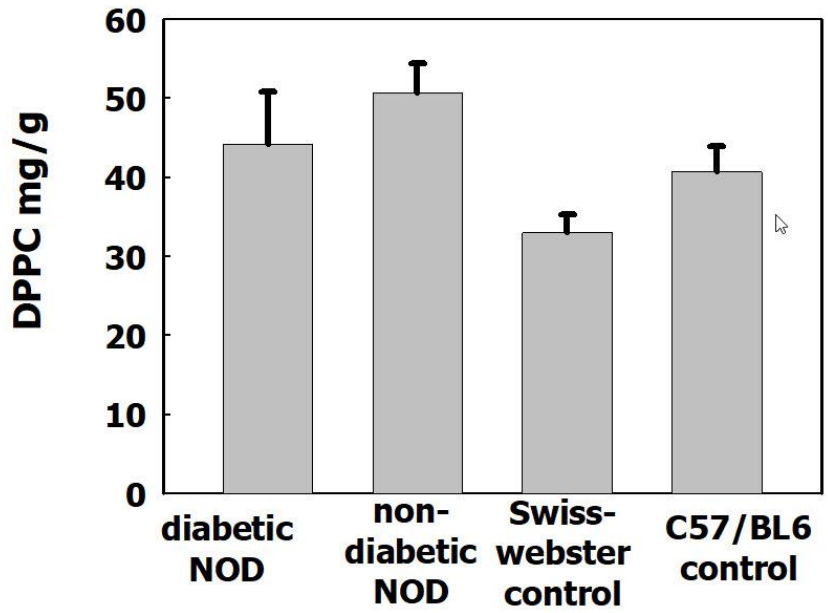

Figure 1–Concentration of DPPC in the four experimental group mean - bar + SE.

Evidently, the concentration of DPPC in the diabetic NOD mice was not above that of the non-diabetic NOD mice. A t-test for a difference between these two groups proved that there was no statistically significant difference between affected and non-affected NOD mice [Normality Test (Shapiro-Wilk): Passed (P = 0.310) Equal Variance Test (Brown-Forsythe): Passed (P = 0.827)]. The same test was employed for the two control groups with similar results: there was no statistically significant difference between C57/BL6 and Swiss-webster control groups [Normality Test (Shapiro-Wilk): Passed (P = 0.507) Equal Variance Test (Brown-Forsythe): Failed (P < 0.050). Equal Variances Not Assumed (Welch's t-test)]. We therefore, combined the two data sets from NOD mice and the two data sets from the control groups for further analysis. The concentration of DPPC in the NOD mice was 47.6 ± 3.7 SE mg/g and in the control groups 36.9 ± 2.2 SE mg/g. Statistical analyses showed a statistically significant difference between the input groups (P < 0.018) [Normality Test (Shapiro-Wilk): Passed (P = 0.112) Equal Variance Test (Brown-Forsythe): Failed (P < 0.050), Equal Variances Not Assumed (Welch's t-test): Two-tailed P-value = 0.0177]. The results are presented in Fig.2.

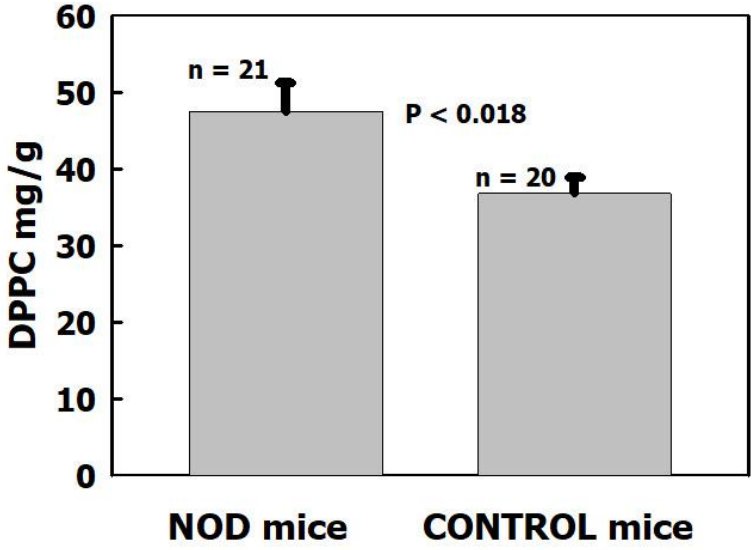

Figure 2: Concentration of DPPC in the hearts of NOD mice and control mice, mean - bar + SE.

Discussion

The concentration of DPPC in the hearts of NOD mice was greater than that in the control mice. As can be inferred from the Double Cause Hypothesis, the first cause of the development of diabetes type 1 is the elevated AHS in NOD mice as compared to control mice. It may well be possible that in the diabetic NOD mice, the other cause is a spillage of pro antigen molecules like insulin, GAD65, GAD67, proinsulin and RGIP (9, 10) into the blood. When one of these molecules gets at the AHS gas/liquid interface, it is recognized as an autoantigen. The present study's findings combined with the data from lupus infected mice (6) reinforce the DCH. Due to the widespread impact on treatment for autoimmune diseases that the DCH would have, this warrants further research. New treatment methodologies might become viable for autoimmune diseases, such as radioactive marking of the AHS to discover the risk before the appearance of the disease, or elimination of plasma DPPC or the AHS to prevent the development of autoimmune diseases.

Competing Interests: The authors declare no competing interests.

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Author Contributions: R.A. and R.P. contributed to the design and management of the study, and the analysis and interpretation of the data. N.A. performed mice follow-up for diabetes, collected the samples and extracted the phospholipids with R.A.
S.K. analyzed the DPPC.
R.A. wrote and edited the manuscript.
All authors have read and reviewed the manuscript.
R.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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