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Measurement of the Phagocytic Activity of Human Peripheral Blood Using a Highly Sensitive Fluorometric Detection Device Without Hemolysis

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Abstract. *Background/Aim: Phagocytes recognize pathogens that enter the body as well as other abnormal and foreign materials that may exist within an organism (such as dead cells, oxidized lipids, and denatured proteins), and phagocytose and eliminate them to maintain a healthy state. In a previous study a simple prototype device was used, under development by Hamamatsu Photonics (Prototype), that detects fluorescence to determine the phagocytic activity of the murine macrophage cell line J774.1. The present study aimed to determine whether it was possible to detect phagocytic activity in a slight amount of human peripheral blood without using hemolysis. Materials and Methods: Three microliters of human peripheral blood was drawn from the fingertip and mixed with 30 μg of pH-sensitive fluorescent particles. The fluorescence intensity of the human peripheral blood sample was then measured using the Prototype in development, cultured for 2 h at 37˚C, and then re-measured. The phagocytes were observed under fluorescence microscopy and the phagocytosis rate of CD11b-positive cells was verified with a flow cytometer. Result: The phagocytic activity of non-hemolyzed human peripheral blood was measured using the Prototype under development; fluorescence after phagocytosis was detected. Furthermore, this was confirmed by both fluorescence microscopy and flow cytometry. The precision of the measurements of human peripheral blood phagocytic activity was verified with the Prototype using samples from three healthy individuals. The relationship between blood sugar levels and phagocytic activity before and after meal times was determined. Concerning exercise, phagocytic activity tended to decrease, although salivary amylase level increased in the healthy individual examined after exercise. Conclusion: The simple Prototype can measure phagocytic activity in a small amount of peripheral blood without hemolysis. The device allows for rapid and minimally-invasive detection of changes in phagocytic activity, which has conventionally been difficult. These findings provide promising evidence that assessment of individual phagocytic capacity can be made easier using this novel device.*

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Leukocytes, such as macrophages and neutrophils, are well-known phagocytes. They engulf and digest foreign materials, such as bacteria, viruses, denatured proteins and lipids, and apoptotic cells (1-4). It is thought that these phagocytes help maintain an individual's health through the elimination of these foreign bodies (5). With a decrease in phagocytic capacity due to aging or stress, foreign bodies increase within an organism; this can trigger chronic inflammation, ultimately resulting in the onset of lifestyle-related diseases (6).

The phagocytic capacity is reduced in patients with chronic diseases (7). Low phagocytic activity has been reported in patients with hyperglycemia and diabetes (8); macrophage phagocytic activity of the alveoli is also reduced in patients with chronic obstructive pulmonary disease (9). Low macrophage phagocytic activity has also been shown in a cellular co-culture with extracellular matrix treated with cigarette smoke (10). These reports suggest that the foreign

body elimination by phagocytes is extremely important in maintaining homeostasis and that it might be possible to evaluate overall health by assessing phagocytic activity.

A simple and minimally-invasive method of assessing phagocytic activity is desirable in order to evaluate overall daily health. However, the current method to evaluate phagocytic activity uses fluorescent latex beads or particles, such as Zymosan, subsequently counting the number of cells that ingested the beads under a microscope or analyzing the phagocytic rate with flow cytometry. In addition, flow cytometry requires the hemolysis of several milliliters of blood in order to isolate leukocytes and requires specialized techniques for analysis. Research on diurnal fluctuations in human phagocytic activity requires repeated measurement each day in the same individual to assess their health condition, which places a great burden on participants. For this reason, limited studies have been conducted on this topic.

We have been investigating a simple prototypic device under development by Hamamatsu Photonics K.K. (Prototype) that detects microscopic amounts of fluorescence (11) and reported on a simple method of assessing the phagocytic activity of J774.1 cells (12). These cells phagocytosed pH-sensitive fluorescent particles to lower the pH level inside the phagosomes, thus making it possible to detect fluorescence at high sensitivity. This allowed for a sensitive assessment system that is able to assess phagocytic activity from as few as 10^4 cells without removing particles. This method is also extremely rapid in comparison with quantification by flow cytometry or counting cells under a microscope. The fluorescence measurement of this Prototype was able to be stably completed in only 5 sec for 10 counts (12).

The present research aimed to verify whether it is possible to measure phagocytic activity, which normally requires hemolysis because red blood cells obstruct such measurement, by taking advantage of the characteristics of the developed device. Furthermore, because phagocytic activity can be measured with this system using small amounts of human peripheral blood, we measured the difference between phagocytic activity before and after meals and after exercise in a few healthy individuals to demonstrate that fluctuations in phagocytic activity throughout the day could be measured.

Materials and Methods

Cell culture. The murine macrophage-like cell line J774.1 was obtained from Riken Cell Bank (Tsukuba-shi, Tsukuba, Japan). Cells were maintained at 37˚C in an atmosphere of 5% CO_2-95% air in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich).

Human peripheral blood sampling. The participants were healthy individuals who were able to engage in daily tasks with no impairment. They were provided with a thorough description of the nature of the test, methods, measurement items, and the results to be obtained before sampling of their blood in order to obtain their consent for participation. Approximately 10 μl of peripheral blood from the fingertip was drawn using a blood collection Mult-Lancet device (BD Microtainer Contact-Activated Lancet; BD, Tokyo, Japan). Ethical approval for the study was obtained from the Ethical Committee of Linking Setouchi Innate immune Network (Takamastu, Japan) (approval number: 2016-02).

Evaluation of J774.1 cell phagocytic fluorescence intensity using the Prototype and a flow cytometer. pH-sensitive green fluorescence labeling phagocytosis particles (GE particles, 10 μg) (pHrodo; Green-*Escherichia coli*; Molecular Probes, Carlsbad, CA, USA) were added to 2×10^4 J774.1 cells/100 μl in each test. As a negative control, phagocytosis inhibitor cytochalasin D (CyD) (Wako, Osaka, Japan) was added until reaching 20 μM and the total amount was 200 μl. The samples were reacted for 2 h at 37˚C in a CO_2 incubator. After the phagocytic reaction, the samples were transferred to a glass tube (micro tube; Maruemu Corporation Inc., Osaka, Japan) and 1300 μl of phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) were added and measured it for 10 counts (0.5 sec/count) with the Prototype. Those collecting cells that were measured with the Prototype were measured with a Beckman Coulter Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA) and using Kaluza software (Beckman Coulter, Indianapolis, IN, USA) to measure the phagocytic rate.

Evaluation of phagocytic activity of human peripheral blood using the Prototype, flow cytometry, and fluorescence microscopy. A volume of 1500 μl solution containing 30 μg of pH-sensitive fluorescent particles was heated to 37˚C with a block heater. During the measurement, 3 μl of human peripheral blood were added and the fluorescence intensity was measured immediately with the Prototype for 10 counts (0.5 sec/count). These values were defined as the negative control. After phagocytosis for 2 h at 37˚C, the fluorescence intensity was again measured with the Prototype for 10 counts (0.5 sec/count). The difference in the fluorescence intensity obtained by subtracting the negative control value from the second measurements were defined as the phagocytic fluorescence intensity (PFI) (12). The phagocytes in human peripheral blood were labelled with $CD11b^{APC}$ antibodies by adding GE particles, and after hemolysis, the phagocytic rate of CD11b-positive cells was verified with flow cytometry. The phagocytosed samples were observed under a fluorescence microscope to confirm findings.

Verification of measurement stability with the Prototype using peripheral blood from healthy individuals. Three healthy adults (two women and one man) with a mean age of 31 years (range=28-42 years) volunteered samples of blood. The blood samples of each were drawn at around the same time, and measurements were then performed.

Verification of changes in phagocytic activity before and after meals using peripheral blood from healthy individuals. Peripheral blood sample were drawn from the volunteers described above at 11:30 hours before their meal and after their meal. A part of the peripheral blood was used to measure the blood sugar level using a glucometer

(ACUU-CHEK; Roche-dcjapan, Tokyo, Japan). Samples were taken for a total of 8 days. Phagocytic activity was determined as described above.

Verification of changes in phagocytic activity before and after exercise using peripheral blood of healthy individuals. This part of the study comprised one healthy adult (male), aged 42 years. After a total of three sessions of medium-load exercise consisting of 60 min of running, the phagocytic activity and salivary amylase level before and after exercise were measured. Similarly, phagocytic activity and salivary amylase level were measured after the same time lapse without exercise. The methods used were similar to those mentioned above for taking the blood samples and performing measurements.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6.02 for Windows (Graph Pad Software, Inc., La Jolla, CA, USA) and JMP 13.1 for windows (SAS, Cary, NC, USA). All results are expressed as the mean standard deviation (SD). When the difference based on one-way analysis of variance (ANOVA) was significant, Student's t-test was used for paired comparisons. The results were considered to be significantly different at $p<0.05$.

Results

Results of measurements by Prototype and flow cytometry of phagocytosis of GE particles by J774.1 cells and human peripheral blood and observations using fluorescence microscopy. The phagocytic activity of J774.1 cells was confirmed using the Prototype (Figure 1A). The GE particles were added to J774.1 cells (2×10^4/test) and phagocytosis compared to the same sample treated with CyD. The PFI for the sample without CyD increased by 1,838. To verify this fluorescence intensity derived from phagocytosis, the phagocytic rate was verified with flow cytometry (Figure 1B). In the sample without CyD, a phagocytic rate of 45% was measured, which confirmed fluorescence due to phagocytosis using flow cytometry.

The fluorescence intensity of phagocytic activity was measured with the Prototype after 2-h culture of GE particles with 3 μl of human peripheral blood (Figure 1C). On comparison with the fluorescence intensity before phagocytosis, the fluorescence intensity increased after 2 h. To confirm the increase in fluorescence intensity of the human peripheral blood sample was due to phagocytosis, the phagocytic rate of CD11b-positive cells was measured using flow cytometry. Compared to that at 0 h, a 98% phagocytic rate was confirmed (Figure 1D). The sample materials before and after the phagocytic reaction were observed at ×400 magnification (Figure 1E), and confirmed that the fluorescence originated from the fluorescent particles taken into the cells over the 2 h incubation (Figure 1F).

Confirming the accuracy of the Prototype using human peripheral blood from healthy individuals. Human peripheral blood was used from three healthy individuals and the accuracy of the Prototype was investigated. Ten counts for each of three healthy persons were obtained and the mean, standard deviation, minimum and maximum values, and coefficients of variation calculated for each. Results are shown in Table I. The mean PFI for the three individuals were 907, 1,145, and 1,080. The coefficients of variation for each individual were 17.03%, 15.46%, and 18.66%, respectively.

Table I. *Prototype phagocytic fluorescence intensity as measured for three healthy persons (n=10/individual)*.

Participant	Mean	Max	Min	SD	CV
A	907	1183	710	154.4	17.03%
B	1145	1471	896	177.0	15.46%
C	1080	1346	733	201.6	18.66%

CV: Coefficient of variation; SD: standard deviation.

Assessment of correlation of post-prandial blood sugar levels and phagocytic activity using human peripheral blood from healthy individuals. The blood sugar level within 30 min after a meal was tested for correlation with the phagocytic activity using the blood samples taken 30-60 min after and before the meal were compared (Figure 2). A strong negative correlation for participants A and B between the postprandial blood sugar level and peripheral blood phagocytic activity was shown, whereas a slightly strong positive correlation was shown for participant C (Figure 2).

Changes in salivary amylase level and phagocytic activity before and after exercise using human peripheral blood from a healthy individual. Figure 3 shows the salivary amylase level and phagocytic activity changes (over three tests) of one individual before and after exercise. Although no significant changes in the salivary amylase level and phagocytic activity were observed when the individual was not exercising, the salivary amylase level significantly increased after performing heavy exercise, whereas phagocytic activity tended to decrease (Figure 3).

Discussion

A previous study reported that phagocytic activity of cultured J774.1 cells can be easily assessed and using a small volume using the Prototype device developed by Hamamatsu Photonics K.K. (12). The Prototype was adapted from a fluorescence detection device that uses a small amount of whole blood to assess neutrophil activity to measure phagocytic activity. The present study demonstrated that it was possible to assess phagocytic activity with only 3 μl of

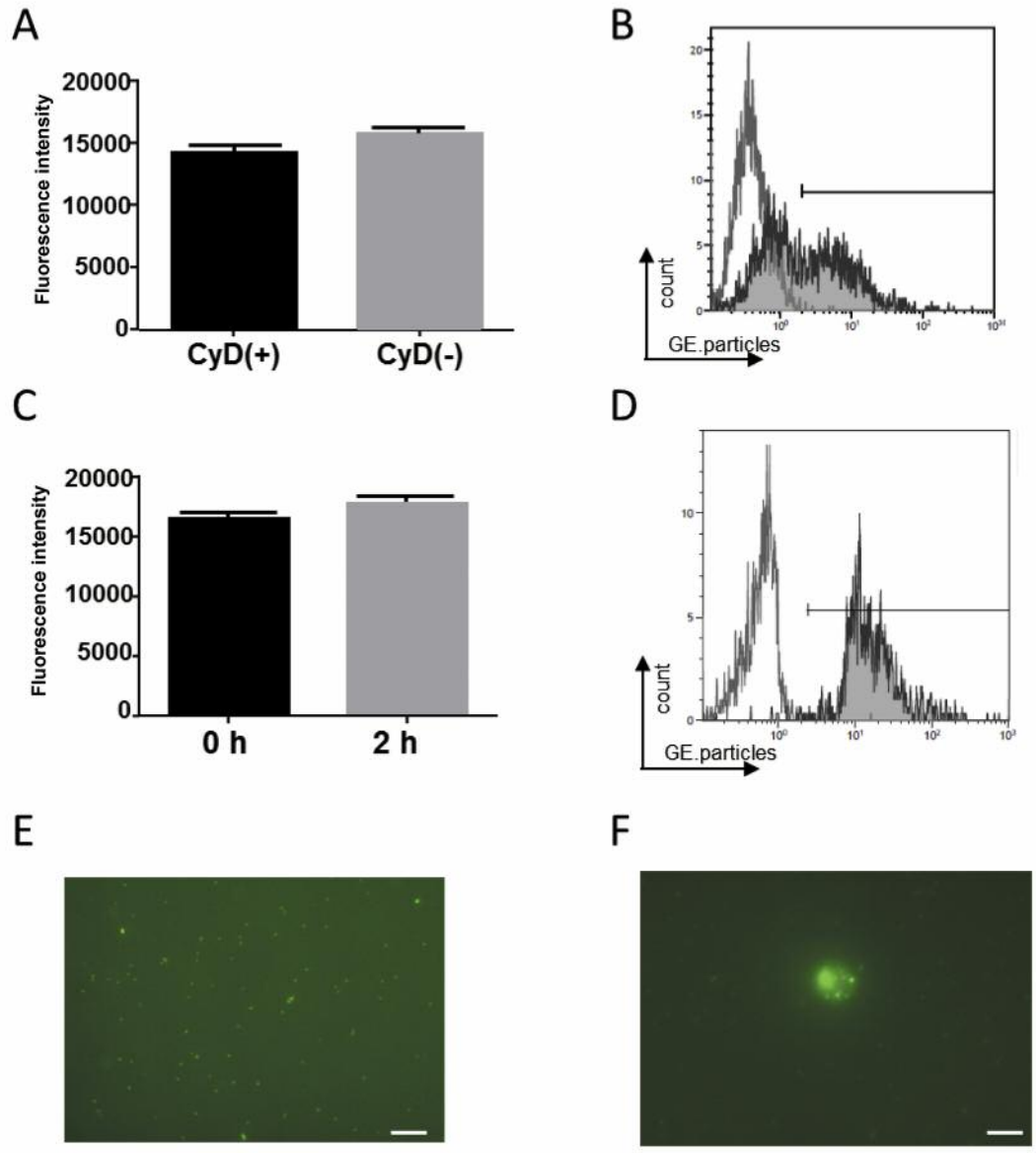

Figure 1. *Measurement of phagocytosis by J774.1 and human peripheral blood using the Prototype, flow cytometry, and fluorescence microscopy. A: Phagocytic activity measurements with the Prototype after phagocytosis by adding 2×10^4 J774.1 cells to GE particles (10 μg). Cell fluorescence intensity is shown with (+) and without (−) adding the phagocytosis inhibitor cytochalasin D (CyD) B: Phagocytic activity measurements using flow cytometry. The peak on the left indicates fluorescence of cells with CyD, and that on the right indicates that of cells without CyD. C: Phagocytic activity of 3 μl of human peripheral blood with GE particles. The fluorescence activity was measured by the Prototype before (0 h) and after 2 h of reaction at 37°C (phagocytosis). D: Phagocytic activity measurements using flow cytometry. The peak on the left represents the fluorescence intensity before phagocytosis and that on the right represents that after phagocytosis. Observation under fluorescence microscopy before (E) and after (F) phagocytosis confirmed Prototype findings of phagocytosed blood peripheral blood cells. Bar=20 μm.*

human peripheral blood and without hemolysis. The phagocytic rate of CD11b-positive cells in the same sample of human peripheral blood was confirmed by flow cytometry (Figure 1).

In the present phagocytosis detection system, pH-sensitive fluorescent particles were phagocytosed, taken into phagosomes, and emitted fluorescence when the pH level lowered to approximately 4.5 with the formation of phagosomes. In fact, this was confirmed by observing fluorescence being emitted within the phagosomes using a fluorescence microscope (Figure 1E-F). The major problem in the present study was that the detection of fluorescence

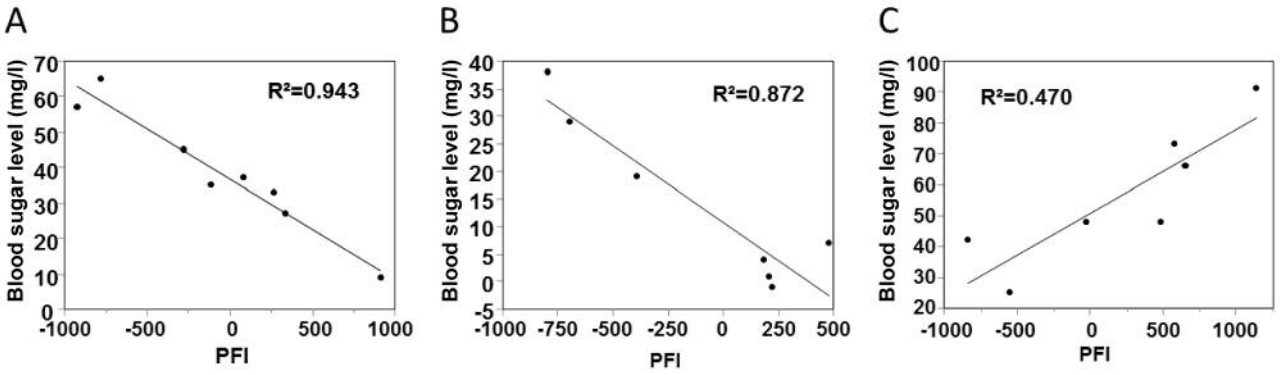

Figure 2. *Correlation of changes in blood sugar level (before and 30 min after a meal) and changes in phagocytic activity as shown by phagocytic fluorescence intensity (PFI) (before and within 30–60 min after a meal). The differences in blood sugar level (that measured 30 min after a meal subtracted from that before the meal and that measured within 30-60 min after a meal subtracted from that before the meal) were analyzed in three healthy individuals in a distribution chart with an approximate curve (A: n=8, B: n=7, C: n=7).*

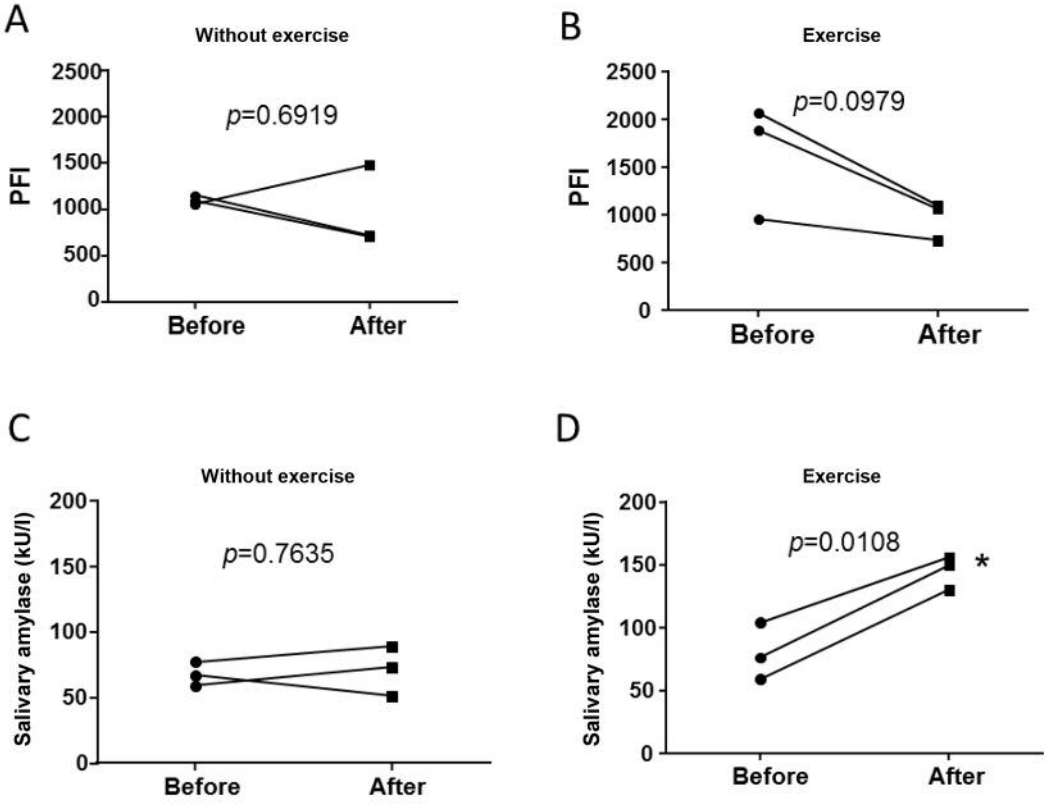

Figure 3. *Changes in phagocytic activity and salivary amylase level with and without exercise. A total of three sessions of 60 min of running was conducted by one volunteer. Phagocytic activity was measured before and after no exercise (A) and exercise (B). Amylase level was measured before and after no exercise (C) and exercise (D). *Significantly different from the negative control (before) (n=3).*

was disturbed by the presence of red blood cells that were not removed. For this reason, the total activity is affected by individual differences, which requires setting the optimal conditions for each person in order to make it possible to take measurements in many individuals. In the present study, we preliminarily studied the human peripheral blood of several individuals and provisionally set the quantity of blood used to 3 μl, which made it possible for detection of

phagocytosis; however, in the future, the optimal conditions that will allow the use of the device for a larger numbers of individuals needs to be investigated.

The addition of CyD as a phagocytosis inhibitor to J774.1 cells, which was used as a negative control for phagocytosis, made evaluation possible (12). However, when CyD was added to the peripheral blood sample in the present study, a high concentration was required to inhibit phagocytosis, and at this concentration, CyD caused a modification in the morphology of the phagocytes. Furthermore, through flow cytometric analysis, it was not possible to use CD11b-positive cells as the negative control, as these cells died (not shown). Therefore, the difference between fluorescence intensity was defined before phagocytosis and 2 h after phagocytic reaction as the phagocytic intensity.

To examine the stability of measurements using the present device, the measurements on a small number of healthy individuals were obtained (Table I). The results demonstrated large coefficients of variation in phagocytosis values. An acknowledged drawback of the currently used methods is the fact that when taking microscopic measurements, the glass tube surface is uneven, which introduces variation in fluorescence intensity from assay to assay. For consistent results, the glass tube needs to have a uniform surface. In the future, improvements in these respects, such as improved sensitivity of the device and development of containers with low variability in fluorescence intensity, are warranted.

In the present study, two tests were used to show that a simple, self-blood sampling method could be used for measuring phagocytic activity (Figures 2 and 3). Firstly, the correlation between postprandial blood sugar levels and changes in phagocytic activity were examined in three healthy volunteers. The results showed a negative correlation between the postprandial blood sugar level and phagocytic activity in participants A and B. For participant A, when the difference in blood sugar level before and 30 min after a meal was <37 mg/dl, the difference in phagocytic activity before and within 30-60 min after a meal was >0. indicating increased phagocytic activity. However, a difference in blood sugar level of >37 mg/dl led to a more negative correlation with phagocytic activity. The same variation was observed for participant B; however, the difference in blood sugar levels at the boundary in phagocytic activity before and after a meal was 7 mg/dl. On the other hand, participant C demonstrated completely opposite correlation to that of participants A and B. In participant C, the higher the blood sugar level, the higher the phagocytic activity was. There have been past studies which showed that blood sugar levels and phagocytic activity negatively correlated (16). Our finding suggests that the correlation was dependent on the individual.

As another example, the changes in salivary amylase level and phagocytic activity before and after three sessions of heavy exercise were examined in one healthy volunteer (Figure 3). As lactic acid and salivary amylase levels are known to increase after exercise, they can be used as markers for exercise loading (13). The salivary amylase level significantly increased after exercise in our volunteer; therefore, we judged that the individual was under an exercise-loaded condition. Phagocytic activity tended to decrease before and after exercise, suggesting that phagocytosis was lower in this individual immediately after heavy exercise. However, some studies have reported that exercise increases phagocytic activity (14, 15).

Using the Prototype, phagocytic activity without subjecting individuals to any major stress through daily follow-up observations was measured easily. We believe that the follow-up of individual phagocytic capacities could be applied to new assessment systems of health.

Disclosures

The Authors have no financial conflicts of interest in regard to this study.

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