

## THE USE OF ERYTHROCYTES AS BIOMARKERS WITH A TIMELINE

Many research groups have been looking for the signal for clearance of RBCs from circulation using density gradients as the procedure to separate the RBCs by age. This was summarized in my review (*Aminoff 1988*). Inadequacy of density gradients to separate RBCs of different age led us to use flowcytometry to monitor changes in RBC surface at the cellular level. This was achieved by washing the RBCs with hypotonic saline, with minimum lysis, 0.63%, resulting in a monomodal peak, with the same 0.63% saline in the flow cytometer flow through. Viewing RBCs in a flow cytometer, we demonstrated that they decrease in cell size in a time dependent manner, with the Forward Light Scatter as an indicator of cell size and age. The largest RBCs represent the youngest cells, and the smallest RBCs the oldest cells. It became apparent from these observations that the decrease in cell size and loss of sialic acid residues were not stochastic, as previously believed, but rather a result of a well-orchestrated sequence of events in the life of RBCs in circulation—120 days in humans. We found that the 2-6 linked sialic acid residues were lost first, followed by the 2-3 linked sialic acid residues of glycophorin on the cell surface. The data was confirmed and, so far, not refuted. This suggested that we could use RBCs to follow changes in the body with time. Advantages and disadvantages of using RBCs as biomarkers are discussed by Aminoff et al (*Aminoff et al 1992*). The demonstrated physiological sequence of events immediately indicated the potential of using the flow cytometer to monitor the autobiography of RBCs in circulation, restricted only by the availability of a suitable fluorescent probe to reveal the story.

Feasible under normal physiological conditions, then why not also under pathological conditions? With further extrapolation of the concept, it became apparent that RBCs also could be used as biomarkers of exposure to extraneous chemicals that could form adducts with components on membrane surface lipids or proteins.

The objective of the below findings is to describe the use of erythrocytes in a flow cytometer to identify biomarkers for multiple diseases and pathogens, including Alzheimer's Disease (AD), and for hemodialysis patients before and after dialysis.

To test the hypothesis, we followed the effects of pesticides on acetylcholine esterase, (ACHE) – an enzyme on the RBC surface. RBCs also can be used for RBC-cytoplasmic contained structures, like choline. It has been shown that the choline content of RBCs changes before and after hemodialysis. To confirm this report, we chose to follow the changes in RBC-choline content before and after hemodialysis. We monitored the choline content of RBCs using a cell sorter in different segments of RBCs, from the largest to smallest RBC as indicators of cell size and hence age (choline assay kit from ABCAM, Inc.). The RBCs previously were washed with isotonic saline (0.63%), with the circulating fluid in the flow cell sorter also adjusted to 0.63%. With the help of department colleague Dr. Peter Freddolino, we monitored the hemoglobin content of the RBCs of each segment in a spectrophotometer as the indicator of the mean cell

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size of the RBCs of each segment. We were successful in demonstrating the reported difference in choline content of RBCs in patients before and after hemodialysis.

Having demonstrated that we could monitor differences on RBC cell surfaces as well as in the cytoplasm, we applied this approach to more current critical neurodegenerative diseases (e.g. Alzheimer's and Parkinson's) which also affect RBCs. Alzheimer's was a focus because a colleague was afflicted with the disease. Dr. Henry Paulson, Director, Michigan Alzheimer's Disease Center, was intrigued by my ideas and was prepared to let me test my approach in his department with the help of Francisco Padron, one of his undergraduate students.

We initiated preliminary experiments to test the potential reagents and their appropriate concentrations, with RBCs from healthy donors to serve as controls: Eosin maleimide (Sigma Aldridge) was used to react with Band 3, the anion exchanger on the surface of RBCs. Thioflavin T (Sigma Aldridge) was used to react with the amyloid binding to the RBC surface; Goat-anti-IgG and Goat-anti-IgM (Thermo-Fisher) were used to detect the autoantibodies that might attach to the affected RBCs.

We tested RBC from 7 healthy volunteer controls, M/F of varying ages, with optimal concentration of EMA/PBS (EMA detects anion exchanger and is the significant biomarker for Alzheimer's Disease.) The FITC fluorescence of the 7 donors, ages 26-92, was consistent, with no differences between male and female or age.

With the possibility that the beta amyloids would cover the anion exchanger, we tested the effect of trypsin on RBCs from a healthy donor and found that ThT (thioflavin-T) reacts with both RBCs and the trypsin treated RBCs, indicating that trypsin did not affect the ACHE on RBCs. Additionally, 0.01% ThT is quite effective with regard to the intensity of fluorescence and, moreover, there was little or no hemolysis as compared to the 0.04% reagent; trypsin treatment appears to affect the reactivity with EMA, indicating that approximately 50% of Band 3 was destroyed by the 0.5 hr treatment with trypsin; and that the trypsin treatment of the RBCs appears to decrease the reactivity with GAH-IgG, but not with the GAH-IgM, which we found rather surprising.

Having tested the blood from healthy controls, we now were ready to test the RBCs from Alzheimer's patients.

### Summary

Our studies to date demonstrate the advantages of RBCs as biomarkers for Alzheimer's and Parkinson patients, despite the fact that they cannot be frozen and must be used within 48 hours (ideally within 24 hours.). Blood for the assays is easily obtainable in clinic and is far less laborious than undergoing an MRI. The blood will provide information for a period of at least 120 days. Adducts on cell surface, as well as enzymes such as acetyl choline esterase (ACHE), can be monitored using a regular flow cytometer. With the use of the flow cell separator, any changes in RBC cytoplasm, such as choline, also can be monitored – again using isotonic saline.

## REFERENCES

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