Processing Human Samples, Utilizing Isolated Components to Conduct Isohemagglutination Assay and Cell Culture Experiment

Adriana Mar, Kim Derkatz, Bruce Motyka, Stephanie Tollenaar, and Dr. Lori J. West
Department of Pediatrics, University of Alberta, Edmonton, Alberta

Background Information

Classification of human blood is dependent on the presence or absence of distinguishing proteins called antigens, which are found on the surface of red blood cells (RBCs). The ABO blood groups were discovered by Karl Landsteiner in 1901. His findings provided rationale for the deaths of numerous people upon receiving blood transfusions. Blood is made up of four primary elements - plasma, RBCs (erythrocytes), white blood cells (leucocytes), and platelets. There are two types of antigens, A and B, and four distinct blood groups - A, B, AB, and O. Corresponding antibodies are located in the blood plasma. Agglutination occurs when the antibodies of one blood type react with the antigens of another blood type. Therefore, matching blood types of donors and recipients has been a key factor for many years for successful blood transfusions and organ transplantations.

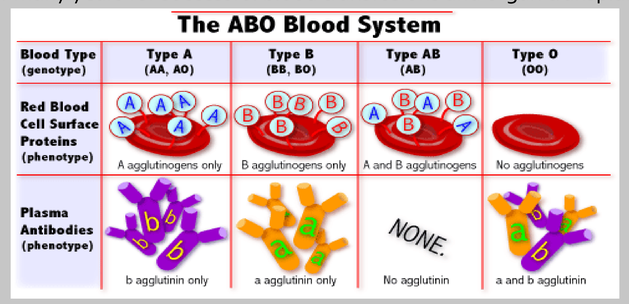


Figure 1: ABO Blood System

However, an exception to the ABO compatibility rule is seen in the case of infants. This is due to their lack of ABO antigen-specific antibodies because of their immature immune systems. These infant-specific characteristics reduce the risk of hyper-acute antibody-mediated rejection, a response which is a major concern in adult procedures. Successful ABO- incompatible heart transplants have been conducted on infants, qualifying this as valid clinical practice. Problems such as insufficient donor organs can be alleviated as compatibility is not necessary, thereby creating an improved donor pool. As they age, these patients have been observed and monitored to understand the mechanism responsible for the success of this procedure. During maturation, the recipients do not develop antigen-specific antibodies to the donor organ blood groups. Assays that qualify B-cell tolerance show nonexistent or very low levels of donor blood group specific antibodies. Further research is being conducted to fully comprehend the mechanism of B-cell tolerance. If infant ABO-incompatibility tolerance can be clearly understood, it may be applicable to adult transplantations, and therefore provide many solutions to problems concerning this patient group.

Isolation of Human PBMC's and Plasma

Peripheral blood samples are processed in order to isolate peripheral blood mononuclear cells (PBMCs) and plasma serum. Plasma is used in isohemagglutination assays to measure antibody titers, and PBMCs are used for phenotypical and functional analyses.

Upon receiving a blood sample, the vacutainer tubes are centrifuged to separate the plasma from the other blood components.

Isolation of Human PBMC's and Plasma

The plasma is isolated and frozen at -80° C. The remaining sample is re-suspended in R-10 media made of RPMI plus 10% fetal bovine serum (FBS), and layered atop ficoll then spun for 30 minutes. The ficoll permits the further separation of the blood into PBMCs and RBCs as shown in Figure 2. The PBMC layer along with the extra media is extracted using a pipette. RBCs are kept for blood typing if necessary. The isolated PBMCs are washed 2 times and then a sample is taken for counting purposes.

Calculations are made to determine the total number of cells, their viability, and the amount of freeze media required to reach a specific cell concentration. The freeze media is composed of FBS and 10% dimethyl sulfoxide (DMSO). These substances allow the majority of cells to remain alive upon being frozen in liquid nitrogen. The cells are resuspended in this media and frozen in the liquid nitrogen tank.

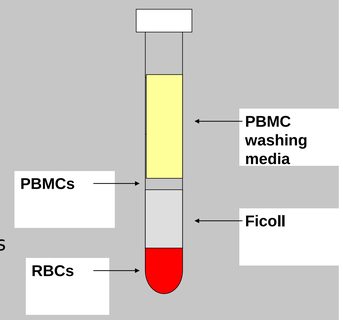


Figure 2: Blood sample after ficoll spin

Blood Typing

The four different blood types - A, B, AB, or O are determined using an individuals' RBCs with A, B, and D (Rh) antibodies. When antigens, present on the RBCs, are exposed to subsequent antibodies, agglutination occurs showing which antigen is present on the cells and thereby asserting the blood type of the individual. Anti-A, anti-B and anti-D are placed in three separate wells and a 20% solution of RBCs is added to each one. The plate is shaken on the plate shaker, left for 5- 10 minutes, then shaken again and analyzed. In Figure 3, agglutination can be seen in wells containing anti-A, anti-B and anti-D. This shows that this person is AB +. If the person was O +, there would only be agglutination in the last well and the first two would have a cloudy appearance.

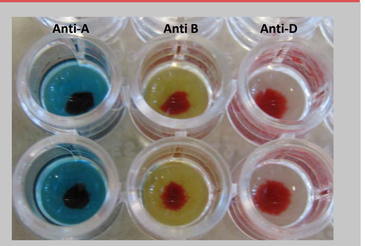


Figure 3: Blood typing

Isohemagglutination Assay

An isohemagglutination (iso) assay is performed to determine the levels of anti-A and anti-B titers in a sample. A 96-well U-bottom plate is used in this assay. 50µL of 1x phosphate buffered saline (PBS) is added to columns 2-12. 100 µL of plasma is added to column 1. A two-fold serial dilution of plasma is performed (undiluted→ 1:2048). A 1:4 diluted mixture of A and B RBCs are then added to all wells and the plate is mixed on the plate shaker. The plate incubates at room temperature for 1 hour, is shaken and then put into the ImmunoSpot scanner, which takes detailed pictures of each individual well and composes a larger picture of the entire plate (Figure 4). This picture is used to analyze the antibody titers of the plasma samples.

Isohemagglutination Assay: Results

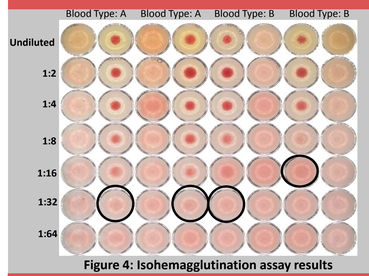


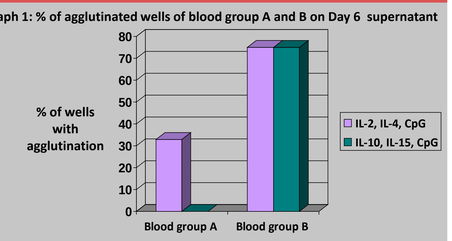
Figure 4: Isohemagglutination assay results

Figure 5: Results from iso assay of day 6 supernatant from A and B blood group cells

Cytokine Results in the most Agglutination

In the West lab, assays and experiments are being conducted to better understand the mechanism behind ABO -incompatible transplantation. This experiment uses CpG (sequence of nucleotides seen as foreign DNA, signaling cells to produce various proteins). CpG stimulates memory B cells to differentiate into antibody secreting B cells. Cytokines, such as interleukins, aid in this process. Various interleukins were tested for proliferation and differentiation effectiveness. IL-2, IL-4, CpG (combo 1) and IL-10, IL-15, CpG (combo 2) were tested. PBMC cells from blood type A and B persons were incubated for 4 days with combo 1 and 2. The media was changed on day 4 and supernatant was isolated and frozen.

Graph 1: % of agglutinated wells of blood group A and B on Day 6 supernatant



Cells incubate further until day 6 when iso's were ran on day 4 and day 6 supernatants (Figure 5). The results are shown on graph 1. There is not a significant difference between the effectiveness of the two combinations of cytokines. However, it does seem that the blood group B cells reacted more significantly to the stimulation. Since only two individuals' cells were tested, no serious conclusions can be made. Further experimentations must be done to obtain conclusive results.

* The cells from this experiment were used in an ELISpot assay to quantify the number of antibody secreting B cells.

Summary

During the time I have spent in the lab, I have learned how samples are processed and used within the lab. I have had the chance to process volunteer blood samples in order isolate plasma and PBMCs. I have used these components to conduct assays and an experiment. Plasma from the volunteer samples was used in isohemagglutination assays, and PMBCs were used in culture to conduct an experiment comparing different cytokines. From beginning to end, I have been exposed to how human samples are utilized in the lab. I was able to understand how the knowledge I received applied to the lab's research, and how that research contributes to advancements in the medical world.

Acknowledgments

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