What's the ABO Story?
Human blood components: isolation & analysis

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Introduction

In 1901, Karl Landsteiner discovered the ABO blood groups, a discovery which eventually won him the Nobel Prize in 1930. This discovery shed light upon the deaths related to blood transfusions that are now known as incompatible. It also provided the necessary background information that would be needed in the future to carry out successful organ transplants. One of the first rules of organ transplantation: the ABO blood type of the graft must be compatible with the recipients blood type in order to prevent hyperacute antibody-mediated rejection. Unfortunately, this complicates the donor-recipient matching process. In addition to matching organ size, blood type must also be considered. Many people die every year waiting for organs and many viable donor organs are wasted.

ABO Blood Groups

	Type A	Type B	Type AB	Type O
RBCs/ endothelium Cell Surface Antigens: A antigen B antigen				
Plasma Antibodies Anti-A antibodies Anti-B antibodies			NONE	

Table 1. Human blood group system

It was later discovered that infants have an immature immune system and do not produce antibodies until later in life. Antibodies are secreted by B cells of the immune system and are responsible for the initiation of events which can lead to hyperacute rejection in the case of incompatible organ transplantation. Infancy, was therefore seen as an opportune time to perform ABO-incompatible (ABOi) heart transplantation. The first successful intentional ABOi heart transplant was performed in 1996[1]. The mechanisms behind the maintenance of the transplant (tolerance) are still not fully understood. Dr. West's lab focuses on transplant immunology – trying to determine the immunological mechanisms behind ABOi transplantation.

One of the human projects in the lab focuses on the B cell and its surface molecule, CD22. CD22 is an inhibitory molecule involved in B cell inactivation[2]. Signaling through CD22 may result in diminished antibody production and therefore may play a role in B cell tolerance to the ABOi graft.

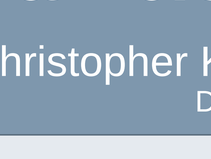

Figure 1. B cell and its surface molecules

In order to better study the immune system it is necessary to isolate and investigate various components of the whole blood including: plasma, RBCs and PBMCs (peripheral blood mononuclear cells).

Methods

Blood Processing
Blood is collected, and placed into the centrifuge. The plasma layer is removed from the top of the blood and stored at -80°C for later use. The blood is diluted to twice its original volume using R-10 media, layered atop Ficoll and placed back into the centrifuge. The blood separates into four distinct layers **(Fig. 2)** during its Ficoll spin. The washing media and PBMC layers are removed from the Ficoll and set aside. The Ficoll is aspirated from the RBC pellet and discarded. The RBCs are washed and set aside for later use. The PBMCs are washed twice, and suspended in a 10% Dimethyl sulfoxide (DMSO)/fetal bovine serum (FBS) freeze media which is stored in liquid nitrogen for later use.
After processing whole blood, three products are isolated including plasma, PBMCs, and RBCs.

Isohemagglutinin Assay – use of plasma
Undilute blood plasma is placed into two wells of the first row of a 96 well plate. The other 11 rows are filled with phosphate buffered saline (PBS). Serial dilutions of the plasma are performed using a multi-channel pipette. A and B type RBCs are added to the plate (A RBCs added to the first column and B RBCs added to the second column). The plate is shaken and then incubated at 20°C for one hour. After the incubation period, the plate is shaken again and read on the ImmunoSpot plate reader (see results: **Fig. 3**).

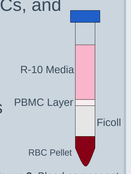

Figure 2. Blood component separation after Ficoll spin

Methods (con't)

Blood Typing – use RBCs
A 20% RBC/PBS solution of the human sample is added to 3 wells of a 96 well plate. In addition to the RBCs, one drop of Anti-A antibody is added to the first well, Anti-B into the second well and Anti-D into the third well. The plate is shaken and then incubated at 20°C for 5-10 minutes. The plate is shaken again and read. This process will allow one to determine the blood type of the sample.

Phenotypic Analysis of B cells – use of PBMCs
Preserved PBMCs are thawed and washed twice. The cells are then suspended in FACs Staining Buffer (FSB). Cells are added to a 96 well plate and then stained with specific fluorescent antibodies (see panel: **Table 2**). The plates are incubated in the dark for 30 minutes at 4°C and then washed twice in FSB to remove unbound antibodies. Each well is topped up to a final volume of 150uL. Finally, the samples are run on a MACSQuant flow cytometer to identify the phenotype of the B cells.

Tube	Violblue		FITC		PE		PerCP-eFluor 710		PE-Cy7		APC		APC-H7		Sample
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	unstained
2	2 - CD19	2.5µL undil			-	-	-	-	-	-	-	-	-	-	single stain
3	-	-	3 - CD22	10µL at 1:2	-	-	-	-	-	-	-	-	-	-	single stain
4	-	-	-	-	4 - IgM	10µL undiluted	-	-	-	-	-	-	-	-	single stain
5	-	-	-	-	-	-	5 - CD27	2.5µL 1:2	-	-	-	-	-	-	single stain
6	-	-	-	-	-	-	-	-	6 - CD38	2.5µL at 1:2	-	-	-	-	single stain
7	-	-	-	-	-	-	-	-	-	-	7 - IgG	10µL at 1:2	-	-	single stain
8	-	-	-	-	-	-	-	-	-	-	-	-	8 - IgD	2.5µL at 1:2	single stain
9	CD19	5µL undil	CD22	20µL at 1:2	IgM	20µL undiluted	CD27	5µL 1:2	CD38	5µL at 1:2	IgG	20µL at 1:2	IgD	5µL at 1:2	sample
10	CD19	5µL undil	CD22	20µL at 1:2	IgM	20µL undiluted	CD27	5µL 1:2	CD38	5µL at 1:5	CD43	20µL at 1:5	IgD	5µL at 1:2	sample
11	CD19	5µL undil	FITC iso-control	0.5µL at 1:2	PE iso-control	0.3µL undiluted	PerCP-eFluor 710 iso-control	0.3µL undiluted	PE Cy-7 iso-control	5µL at 1:2	APC iso-control	10µL at 1:2	APC Cy-7 iso-control	0.625µL at 1:2	iso controls

Table 2. Antibody staining panel. Antibodies, their volumes and concentrations that were used in phenotypic analysis of B cells.

Results

Isohemmaglutinin Assays

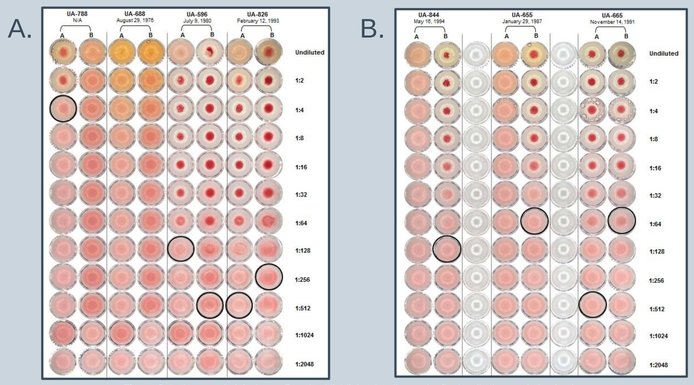

Figure 3. Isohemmaglutinin Assay Results. Highlighted wells are the final wells that agglutination is present.

Blood Typing

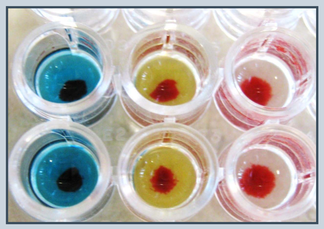

Figure 4. The left wells are filled with Anti-A. The centre wells are filled with Anti-B. The right wells are filled with Anti-D. In this sample, all six wells display agglutination. This concludes that A antigens, B and RH antigens are all present on the surface of the RBCs and this sample is blood type AB+

Results (con't)

Phenotypic analysis of B cells

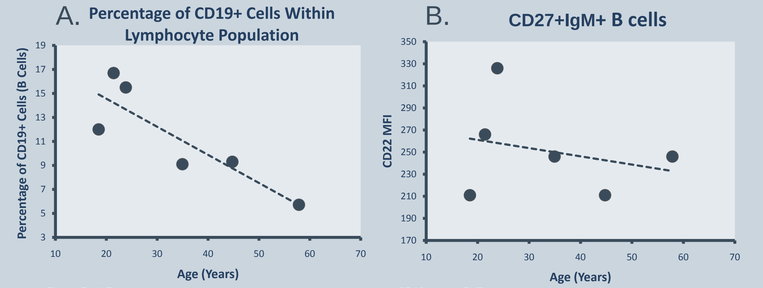

Figure 5. A) There is a negative correlation between age and percentage of CD19+ cells. B) There is a negative correlation between age and the Median Fluorescence Intensity (MFI) of CD22 in the CD27+IgM+ B cell subset.

Summary

ABOi heart transplantation in infants and young children alleviates transplant wait lists and the wastage of donor organs. However, further investigation is necessary to better understand the immunology involved in tolerance after ABOi transplantation.
Initial investigation begins with whole blood separation into various components including plasma, RBCs and PBMCs. In depth analysis of these components will aid in elucidating the mechanisms at work. A better understanding of B cell tolerance in ABOi heart transplantation may allow us to extend the window of time in which this procedure can safely be performed.

Literature Cited

1. West LJ, Pollock-BarZiv SM, Dipchand AI, Lee KJ, Cardella CJ, Rebeyka IM, Benson LN, Coles JG: ABO-incompatible heart transplantation in infants. *The New England Journal of Medicine.* 2001;344(11): 793-800.
2. Duong BH, Tian H, Ota T, Completo G, Han S, Vela JL, Ota M Kubitz M, Bovin N, Paulson JC, Nemazee D. Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. *J Exp Med.* 2010;207(1):173-87.

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