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A and B antigen levels acquired by group O donor-derived erythrocytes following ABO-nonidentical transfusion or minor ABO-incompatible haematopoietic stem cell transplantation

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Running title: A and B antigens on group O donor cells

Abstract

Background and Objectives: ABO-incompatible haematopoietic stem cell transplantation (HSCT) presents a challenge to blood component transfusion. The aim of this study was to investigate the weak blood group A or B antigen expression by donor-derived group O red blood cells (RBC) observed following transfusion or minor ABO-incompatible HSCT. In addition, *in vitro* experiments were performed to elucidate possible mechanisms underlying this phenomenon.

Materials and Methods: A sensitive flow cytometry assay for the semi-quantification of RBC A/B antigen levels was used to assess patient samples and evaluate *in vitro* experiments.

Results: Analysis of blood samples from patients, originally typed as A, B and AB but recently transplanted or transfused with cells from group O donors, revealed A antigen expression on donor-derived RBC, ranging from very low levels in non-secretor individuals, to almost subgroup A_x-like profiles in group A secretors. B antigen expression was less readily detectable. *In vitro* experiments, in which group O donor RBC were incubated with i) group A/B, secretor/non-secretor donor plasma or ii) group A/B donor RBC in the absence of plasma, supported the proposed adsorption of A/B-antigen-bearing glycolipids from secretor plasma but also indicated a secretor-independent mechanism for A/B antigen acquisition as well as direct cell-to-cell transfer of ABO antigens.

Conclusion: The *in vivo* conversion of donor-derived blood group O RBC to ABO subgroup-like RBC after transfusion or minor ABO-incompatible HSCT raises the question of appropriate component selection. Based on these data, AB plasma should be transfused following ABO-incompatible HSCT.

Key words: ABO blood group antigen, red blood cell, haematopoietic stem cell transplantation, glycosphingolipids, flow cytometry

Introduction

Current guidelines for transfusion support in ABO-incompatible haematopoietic stem cell transplantation (HSCT) prescribe blood components which are compatible with both the recipient and the donor ABO groups in order to avoid hemolysis of any transfused red blood cells (RBC), the residual recipient RBC and the engrafting donor-derived RBC. From the time of complete donor erythropoietic engraftment, the recipient is generally recommended to receive blood components of donor ABO group (Apperley *et al.*, 2012; Fung *et al.*, 2014).

The stably engrafted HSCT recipient is, however, a (complete) chimaera of donor ABO blood group, intrinsically synthesized and expressed on RBC and platelets (Oriol *et al.*, 1981; Mollicone *et al.*, 1988), and recipient-group ABH antigens, ubiquitously distributed in tissues, and also in body fluids of secretors (Matsui *et al.*, 1999; Ravn *et al.*, 2000; Mueller *et al.*, 2011). Furthermore, donor-derived blood cells may exhibit low levels of recipient-group A/B antigens (Swanson *et al.*, 1971; Needs *et al.*, 1987). In the setting of a minor ABO-incompatible HSCT, where group O donor haematopoietic stem cells (HSC) are transplanted to a group A recipient, the switch to donor-group transfusion support upon complete donor engraftment will expose the recipient to antibodies directed against blood group A epitopes, expressed on vascular endothelium, carried by soluble structures in plasma and, possibly, present on circulating group O HSC-donor-derived blood cells. Extending the concept of ABO-compatible transfusion support to include the life-long chimaeric ABO status of the recipient would, thus, suggest possible implications for blood component selection.

Accumulating data indicate that infusion of plasma which contains ABO antibodies may cause rapid clearance of platelets, formation of immune complexes, endothelial cell damage and multiple organ dysfunction (Heal *et al.*, 1987; Heal *et al.*, 1993; Benjamin *et al.*, 1999; Lapierre *et al.*, 2005). Also, donor-derived ABO antibodies from passenger lymphocytes of minor ABO-incompatible HSC graft origin may induce destruction of transfused group O

RBC, possibly mediated through the adsorption of either soluble A/B antigens, A/B-antigen/antibody immune complexes or complement components onto the RBC surface (Gajewski *et al.*, 1992; Tiplady *et al.*, 2001; Worel *et al.*, 2002).

At the molecular level, the *ABO* gene encodes two different glycosyltransferases, which add either *N*-acetylgalactosamine (GalNAc) or galactose (Gal) to the H antigen precursor structure, forming A or B antigens, respectively. In haematopoietic tissue, the *FUT1* (or *H*) gene encodes a 2-α-fucosyltransferase, which synthesizes H antigen mainly on type 2 oligosaccharide acceptors, linked to proteins (approximately 90%) and lipids (approximately 10%) in the RBC membrane. In epithelial cells, the *FUT2* (also known as the secretor) gene governs the synthesis of H antigen, preferentially on type 1 precursor chains. It also determines the presence of type 1 ABH antigens in secretions, found in approximately 80% of people, so-called secretors(Daniels, 2013) (Clausen *et al.*, 1989; Morgan *et al.*, 2000; Ravn and Dabelsteen, 2000).

The acquisition *in vivo* of A/B antigens by group O RBC following transfusion or HSCT has previously been observed, using standard serological techniques (Renton *et al.*, 1962; Swanson *et al.*, 1971; Needs *et al.*, 1987; Wichmann *et al.*, 1999), and has been attributed to the adsorption from plasma onto RBC of glycosphingolipids with A/B specificity expressed on type 1 oligosaccharides (Garretta *et al.*, 1974; Tilley *et al.*, 1975; Clausen *et al.*, 1985; Jovall *et al.*, 1987). In the current study, we used a sensitive flow cytometry assay optimised in our laboratory for the semi-quantification of low levels of A/B antigens (Liu *et al.*, 2007; Hult *et al.*, 2010) to assess the extent of antigen acquisition by donor-derived group O RBC in clinical patient samples, following transfusion or HSCT. *In vitro* experiments that mimic the *in vivo* situation were performed to address the previously proposed adsorption of A/B-antigen-bearing glycosphingolipids onto RBC, and to investigate the hypothesized conversion

of H substance on the RBC surface by enzymatic action of ABO glycosyltransferases in plasma.

Material and methods

Patients

As part of the clinical monitoring, patients (n=11) of different ABO blood groups, transplanted with HSC from ABO-incompatible donors of group O or A, were evaluated by extended blood group analysis. Ethylenediamine tetracetic acid (EDTA)-anticoagulated blood samples drawn for routine blood grouping or pre-transfusion testing were analyzed and anonymized data included in the study. In addition to their HSCT, seven patients had received blood transfusions from group O blood donors within the last three months prior to testing (range: 3 - 23). Pre-transplant DNA samples were used to establish the patients´ secretor status by *FUT2* genotyping and for *ABO* genotyping of selected patients. At the time of investigation, all patients were complete donor chimaeras as assessed by computer records of short tandem repeat-polymerase chain reaction analysis (data not shown).

EDTA samples were also collected from non-transplanted patients (n=15) of group A, B or AB, who had been transfused with RBC units from group O donors. The number of units received within the last three months prior to testing varied considerably (range: 1-36. Again, *FUT2* genotyping was performed to establish the patients´ secretor status.

Donors

Anonymized blood samples from ABO-typed donors (n=61) collected in acid-citrate-dextrose (ACD) tubes at routine donation were used for *in vitro* experiments. No extra samples were taken for this study. Secretor status was determined by Lewis blood group phenotyping. Donors whose secretor status could not be concluded by RBC typing only, i.e. Le(a–b–), were not included. The age of the donor ACD samples varied but none of the samples used

were older than one week at the start of each experiment. Donor RBC and/or plasma were used.

Serology

ABO and Lewis phenotyping was performed with established and validated methods used at the Department of Clinical Immunology and Transfusion Medicine in Lund, Sweden.

ABO *and* FUT2 *genotyping*

Genomic typing of the *ABO* and *FUT2* loci were performed with established and validated methods used at the Nordic Reference Laboratory for Genomic Blood Group Typing (NRLGBT) in Lund, Sweden. Polymerase chain reaction with allele-specific primers (PCR-ASP) was used (Hosseini-Maaf *et al.*, 2007).

Flow cytometry

Flow cytometry was performed as previously described (Liu, Sulzenbacher *et al.*, 2007; Hult and Olsson, 2010). Samples of the following ABO phenotypes (genotypes) were included as controls in each run: A_2, B, O, A_x (*ABO*AW30.01/O.01.01*) and B_w (*ABO*BW.03/O.01.01*). ABO terminology according to ISBT is used throughout this paper (www.isbtweb.org).

In vitro experiments

Group O RBC were mixed with either plasma or RBC from donors of ABO groups A_1, A_2, or B, both secretors and non-secretors (Fig.1). Group O RBC mixed with autologous group O plasma or RBC, were included in each experiment as negative controls.

Investigating plasma-to-cell transfer of ABO antigens

ACD tubes were centrifuged at 1500 x g for 10 minutes, to separate plasma and RBCs. Washed, packed group O RBC were suspended 1:5 in group A_1/B/O plasma in appropriately labelled glass tubes, sealed and placed in either 4°C, room temperature (RT), 37°C or 37°C with constant mixing, for up to 16 days.

Papain-treated RBC were used in two experiments. Papain-treatment was performed according to standard blood bank practice (Fung *et al.*, 2014). In preparation for flow cytometric analysis, 25 µL RBC from every tube were transferred to a new glass tube, washed three times in PBS and 10 µL packed RBC were re-suspended in 400 µL PBS.

Investigating cell-to-cell transfer of ABO antigens

ACD tubes were centrifuged at 1500 x g for 10 minutes, plasma and RBC were separated. RBC were washed three times in PBS. Packed group O RBC were mixed 1:4 with packed A_1/A_2/B/O RBC in 1 mL Eppendorf tubes, sealed and placed in an incubator with constant gentle mixing at 37°C, for up to four days.

In one experiment, cell-cell mixes diluted with 200 µL of PBS or A/B plasma to a hematocrit of approximately 45%, aiming to create a more *in-vivo*-like environment, were incubated in parallel with the undiluted cell-cell mixes. Cells were prepared for flow cytometric analysis as described above.

Detection of A and B glycosyltransferase activity in plasma

Twenty-five microliters of a 10 % suspension of washed group O RBC were incubated with 190 µL of plasma from group A_1/A_2/B/O, non-secretor individuals at 37°C for up to 48 hours under the following conditions: 0.35 mM UDP-GalNAc (Uridine 5'-diphospho-*N*-acetylgalactosamine disodium salt, Product#U5252, Sigma-Aldrich, Sweden) or UDP-Gal

(Product# U4500, Sigma-Aldrich, Sweden); 10 mM $MnCl_2$ (BioChemika, product#48975, Sigma-Aldrich, Sweden) in a total volume of 250 µL (adapted from Schenkel-Brunner *et al.*, 1969). In one experiment, inactivation of glycosyltransferase activity in plasma was attempted with three different approaches: i) Heat inactivation using ACD plasma incubated for 10 minutes at 56°C; ii) using plasma from blood drawn in EDTA tubes, and iii) using ACD plasma supplemented with an excess of EDTA. EDTA is a well-known chelating agent and complex binding of the Mn^{2+} ions required for ABO transferase activity was anticipated. Cells were prepared for flow cytometric analysis as described above.

Results

Transplanted patients

A total of 11 patients who had been transplanted with ABO-incompatible HSC were tested. Seven patients were also transfused with group O RBC. Repeat samples were available from four patients. The results are summarized in Table 1.

When donor-derived group O RBC were tested with anti-A, an increase in mean fluorescence intensity (MFI) compared to group O RBC controls were observed in all patients but one, who had received multiple blood transfusion on consecutive days just before blood sampling. The A antigen levels ranged from low but well detectable to higher, almost subgroup A_x-like profiles. The mean MFI values with anti-A were higher in secretors than in the only group A non-secretor patient available for testing (Fig. 2a and b). Two patients, who were originally group A secretors and had similar transplant and transfusion histories but very different MFI, were genetically defined as *ABO*A2.01/O.01.02* and *ABO*A1.01/O.02*, respectively (Fig. 2e). Two group B secretor patients and one group AB secretor patient showed no detectable B antigen on donor-derived group O RBC (Fig. 2d), although the group AB patient exhibited a weak A antigen expression (Fig. 2c).

Non-transplanted but transfused patients

A total of 15 patients, secretors and non-secretors of group A, B or AB transfused with RBC from group O donors were tested. Repeat samples were available in six patients. The results are summarized in Table 2. All blood group A patients showed an increase in MFI, greater in group A secretors (Fig. 2f), than in group A non-secretors (Fig. 2g). In transfused group B secretor patients, as opposed to the single group B non-secretor tested, a slight rise in MFI was observed compared to group O RBC controls. A particularly informative flow cytometric pattern was observed in a group A secretor patient (Fig. 2h). Three distinct cell populations

were displayed, with MFI values corresponding to A antigen levels of i) normal group A RBC, ii) donor-derived group O RBC circulating in group A secretors and iii) the group O RBC control. This patient had been transfused with ten units of group O RBC within the last three weeks, including one unit just prior to blood sampling, indicating that time in circulation for transfused group O RBC correlates with A antigen levels adsorbed. In one group A secretor patient, transfused with three group O RBC units in five consecutive days, repeat samples were available at two, four and eight weeks and a rise in MFI of the donor-derived O RBC over time was observed.

In vitro experiments

Investigating plasma-to-cell transfer of ABO antigens

In an attempt to mimic *in vitro* the circulating donor-derived group O RBC following transfusion or minor ABO-incompatible HSCT *in vivo*, normal group O RBC were mixed with secretor or non-secretor plasma from group A_1 and B donors. Group O plasma was used as a negative control.

When tested with anti-A, all group O RBC incubated with group A_1 plasma at 37ºC or RT showed an increase in MFI over time compared to the group O plasma control (Fig. 3a). A greater increase in MFI, corresponding to a higher level of A antigen, was observed when group O RBC were incubated with group A_1 secretor plasma compared to group A_1 non-secretor plasma. Incubating RBC/plasma mixes with or without constant mixing (at 37ºC) did not influence the increase of MFI, whereas incubation at 4ºC gave no rise in MFI compared to the O plasma control. Group O RBC incubated with group B secretor or non-secretor plasma and tested with anti-B showed no rise in MFI over time compared to the group O plasma control (Fig. 3b).

Investigating cell-to-cell transfer of ABO antigens

Washed and packed group O RBC, mimicking donor-derived group O RBC, were mixed with washed and packed RBC from group A_1, A_2 and B, secretor and non-secretor donors and incubated at 37ºC with constant mixing. Group O RBC were used as a negative control.

When group O RBC incubated with group A_1 RBC were tested with anti-A, a rise in MFI was observed equivalent to the MFI seen in the naturally-occurring ABO subgroup A_x. When group O RBC incubated with group A_2 or B RBC were tested with anti-A or anti-B, respectively, the rise in MFI was not as apparent as in group O RBC/A_1 RBC mixes but still clearly detectable.

A difference in MFI between cell-cell mixes including secretors *vs.* non-secretors was also clearly shown for group A_1 and B mixes (Fig. 4a and b). No apparent difference was seen with group A_2 secretors versus non-secretors.

Attempting to mimic a more physiologic RBC concentration environment, PBS or group A/B non-secretor plasma was added to the RBC mixes. The rise in MFI was not as dramatic as in the undiluted RBC mixes but still detectable when PBS was added. When non-secretor AB plasma was added to the group O RBC/A_1 RBC mixes the rise in MFI was very discrete (Fig. 4c) and with the group O RBC/A_2 or B RBC mixes a rise in MFI was not detectable.

Functional detection of A and B glycosyltransferase activity in plasma

Substrate (UDP-GalNAc for A-glycosyltransferase [GTA] and UDP-Gal for B-glycosyltransferase [GTB]) and $MnCl_2$ were added to group O RBC mixed with group A_1, A_2 or B non-secretor plasma and incubated at 37ºC. When incubated with the appropriate A or B antigens were readily detectable on the group O RBC after 4 hours. MFI was higher for group O RBC incubated with group A_1 and B plasma than for group O RBC incubated with group A_2 plasma (Fig. 4d-f). The MFI increased with time and after 48 hours group O RBC

incubated with group A_1 and B plasma expressed A and B antigen at a level almost equivalent to normal group A_2 and B RBC. When group O RBC were incubated with group B plasma, UDP-GalNAc and $MnCl_2$ they showed a very slight rise in MFI compared to the O plasma control, when tested with anti-A. The low levels of A antigen detected on the group O RBC were consistent with the small amounts of A antigen normally detectable on common group B RBC (Goldstein *et al.*, 1989).

When either of the reagents (donor substrate, $MnCl_2$ or A/B plasma) was excluded or group O plasma was used instead of group A/B plasma no change in MFI compared to the group O control RBC was observed. Complete inactivation of the glycosyltransferases was achieved only by the addition of an excess of EDTA to ACD plasma. Inactivated A_1 plasma was incubated with group O RBC, UDP-GalNAc and $MnCl_2$ and no A antigens could be detected with anti-A.

Discussion

In this study, we demonstrate and semi-quantify the *in vivo* conversion of donor-derived blood group O RBC to ABO-subgroup-like RBC following transfusion to ABO-nonidentical recipients or after minor ABO-incompatible HSCT. Utilizing a sensitive flow cytometry protocol, donor group O RBC were found to express variable levels of acquired antigen, ranging from very small amounts in non-secretor individuals to subgroup A_x-like profiles, in group A_1 secretors. Our findings support the major role of A/B antigen adsorption from secretor plasma, but also indicate that secretor status is not an absolute prerequisite for the *in vivo* conversion of group O RBC.

Detectable levels of antigen adsorbed from secretor plasma onto group O RBC may increase over time as demonstrated in the *in vitro* experiments (Fig. 3a) and illustrated by the characteristic flow cytometric patterns of group A secretor individuals repeatedly transfused with group O RBC (Fig. 2f). Also, the time course of antigen adsorption onto RBC may be influenced by inhibiting factors in plasma (Tilley *et al.*, 1975), suggesting competition by plasma lipoproteins for lipid uptake which may have influenced the flow cytometric pattern observed in Fig. 4c.

The marked difference in detectable levels of adsorbed antigen between secretor individuals of different ABO blood groups reflects a variable amount of soluble antigen available in plasma, governed by the complex interaction between the *ABO* and *FUT1* gene, the secretor gene (*FUT2*) and the Lewis gene (*FUT3*), and is influenced by allelic variants at these loci. The enzymes encoded by *ABO* and *FUT3* compete for the common type 1 H acceptor substrate to produce A/B antigens or difucosylated Le^b antigen (Watkins *et al.*, 1988; Henry *et al.*, 1995). The enzyme produced by the A^1 allele of the *ABO* locus is more efficient than its A^2 allelic variant in competing with the Lewis enzyme for type 1 H acceptor substrate. Accordingly, among group A secretors of the Le(a-b+) phenotype, levels of soluble A or

ALe^b antigen are higher in A_1 than A_2 individuals (Holburn *et al.*, 1974; Tilley *et al.*, 1975; Achermann *et al.*, 2005). In two secretor patients genetically defined as *ABO*A1.01/O.02* and *ABO*A2.01/O.01.02*, respectively (Fig. 2e), we found a clear difference in A antigen levels on donor-derived group O RBC, corresponding to the quantitative difference in A_1- and A_2-glycosyltransferase activity when it comes to synthesis of A type 1 (Schachter *et al.*, 1971; Schenkel-Brunner *et al.*, 1973). A possible effect of secretor gene zygosity was suggested by the finding in our study that patients who typed homozygous for c.428G in their *FUT2* genes (i.e. homozygous for active secretor alleles, previously referred to as *Se*), expressed comparatively high levels of original blood group A/B antigens on donor-derived group O RBC. In another study, however, levels of soluble ABO blood group substance in plasma were not influenced by the homozygous expression of the A^1, A^2 or *Se* alleles, respectively (Achermann *et al.*, 2005).

In non-secretor plasma, A/B antigens are found, albeit in small quantities, on type 2 carbohydrate chains linked to glycoproteins and glycosphingolipids, and are presumed to emanate from endothelial or haematopoietic cells (Hostrup, 1962; Holburn 1974; Tilley *et al.*, 1975; Le Pendu *et al.*, 1982). Lactosylceramide, which is the major glycosphingolipid precursor on RBCs (Clausen *et al.*, 1989), is also expressed in endothelial cells. In plasma, glycosphingolipids are associated with lipoproteins and may, thus, be exchanged between cells and lipoproteins, or vice versa, via shedding and/or receptor-mediated endocytosis (Chatterjee, 1998). In the recipient of a minor ABO-incompatible HSCT (group O donor to group A recipient, Fig. 2b), the endothelium is, thus, a plausible source of type 2 A antigen available for transfer to transfused group O RBC. In a transfused group A, non-secretor patient (Fig. 2g), the haematopoietic tissue may contribute to the total amount of lipid-linked type 2 A antigen available for uptake. A possible mechanism also for direct membrane glycolipid exchange between adjacent RBC was suggested by the *in vitro* experiments (Fig. 4

a-c), most prominent when RBC were close to each other. Among the plasma glycoproteins known to carry A/B antigens on *N*-linked oligosaccharide chains, endothelium-derived vWF and a minor portion (10%) of α_2-macroglobulin (Matsui *et al.*, 1993; Matsui *et al.*, 1999) circulate in plasma independently of secretor status. Available data, however, suggest that these glycoproteins are not of significant importance to *in vivo* RBC A/B antigen expression (Kirschkamp *et al.*, 2008; Santizo *et al.*, 2009).

The possible mechanism of H substance conversion on the surface of donor-derived group O RBC, by enzymatic action of GTA/GTB in non-secretor plasma, was hypothesized and successfully demonstrated *in vitro* by adding an excess of the appropriate donor sugar substrate. The A/B-enzymes are present in plasma irrespectively of secretor status, with only a minor part originating from haematopoietic tissue (Schachter 1971; Yoshida *et al.*, 1980), and are generally considered incapable of enzymatic activity on the RBC surface, due to insufficient extracellular concentrations of donor-sugar nucleotides (Varki *et al.*, 1999). Also, in the recipient of a minor ABO-incompatible HSCT, inhibiting antibodies directed against endogenous GTA/GTB may completely abolish enzymatic activity in plasma (Barbolla *et al.*, 1988). However, recent reports have shown evidence for extrinsic glycosylation driven by circulating extracellular glycosyltransferases where the donor sugar substrates are provided by activated platelets (Lee *et al.*, 2014; Lee-Sundlov *et al.*, 2017).

The difficulties in this study to detect adsorption of B antigen from secretor plasma by donor group O RBC following transplantation (Table 1, Fig. 2d) or transfusion (Table 2), as well as in the *in vitro* experiments (Fig. 3b), may either suggest that the GTB is a poorer competitor to the Lewis enzyme for H type 1 substrate in secretions than GTA, or that BLe^b antigen is preferentially formed but not detected. Notably, a reduced level of A antigen on donor group O RBC was observed in group A_1B secretors compared to group A_1 secretors (Table 1 and 2),

indicating GTB activity resulting in conversion of H type 1 antigen. The monoclonal antibodies used in this study, anti-A clone ES-15 and anti-B clone 9621A8, were specifically selected for their high sensitivity and ability to detect native ABO subgroups (Hult *et al.*, 2010) and remnant A/B antigens after exoglycosidase treatment (Liu *et al.*, 2007) by flow cytometry. However, in more recent experiments, using clone ES-15 and clone 9621A8 in flow cytometry evaluation of RBC modified with synthetic glycolipid A/B constructs (KODE technology), we found that a transformation with five times more B construct than A construct was required to create RBC with equivalent serological and flow cytometric features (Hult *et al.*, 2012). This phenomenon has shown to be consistent when either monoclonal or human polyclonal antibodies are tested against both natural and synthetic B antigen, and is believed to be due to the structure of the B antigen, which has an inherently lower affinity for specific antibody, in contrast to the A antigen, which has the *N*-acetyl protrusion on its terminal sugar. Notwithstanding, in contrast to the scarce amount of B antigen found to be adsorbed from secretor plasma, the co-incubation of group O and B RBC in the absence of plasma induced readily detectable levels of B antigen on donor group O RBC (Fig. 4b), suggesting a transfer of B type 2 glycolipids. Notably, this may point to a preference of the anti-B clone 9621A8 for type 2 based B antigen, although the degree of antibody specificity for type 1 versus type 2 oligosaccharides was not investigated in this study. However, the antibody is known to react in ELISA with synthetically produced group B oligosaccharides of both type 1 and 2 while BLe^b structures were apparently not tested (personal communication from the manufacturer of the reagent). It is possible that some of the antigen adsorbed onto donor cells is indeed of BLe^b type, which may explain why the anti-B used does not react readily.

The transient course of donor-derived A/B antibody titres and the apparent escape of endothelial cells from GvHD in minor ABO-incompatible HSCT, may be attributed to

antibody adsorption, induction of donor lymphocyte tolerance or endothelial cell accommodation (Takahashi, 2005; Stussi *et al.*, 2006). In contrast, repeat transfusion of donor ABO-type plasma and platelet components may cause immune-complex formation, multi-organ dysfunction and impaired survival (Benjamin *et al.*, 1999; Heal *et al.*, 2005). In this setting, circulating donor-derived RBC have an important role in CR1-receptor mediated clearance of immune-complexes (Reinagel *et al.*, 1997) but are, as shown here, also potential targets for direct ABO antibody binding and possibly hemolysis.

In this study, we demonstrate and characterize the *in vivo* conversion of donor-derived group O RBC to ABO subgroup-like RBC following transfusion or HSCT, utilizing a sensitive flow cytometer assay that enables the distinction between HSC-donor-derived RBC, expressing low levels of acquired A/B antigen, and reappearing RBC of recipient origin, which may herald relapse or HSC graft rejection (David *et al.*, 1999). Our findings confirm the major role of A/B antigen adsorption from secretor plasma, but also indicate an additional, secretor-independent mechanism for A/B antigen acquisition. In a clinical context, this study provides additional support for the proposed selection of plasma components compatible with both donor and recipient ABO blood group, (Heal *et al.*, 2005; O'Donghaile *et al.*, 2012) also beyond the time of complete HSC engraftment. It can be noted that certain source still recommend blood components of the donor's ABO group, once a routine grouping shows that the cells appear to type as the donor (Fung *et al.*, 2014) but our study shows that a highly sensitive flow cytometric analysis is valuable for establishing the recipient's true blood group status."

Author Contributions

AKH performed the experiments. AKH, JHD, JRS and MLO designed experiments and interpreted data. AKH, JHD and MLO wrote the paper, JRS reviewed and revised it, and all authors approved the final version.

Financial Disclosure Statement

The authors have no financial conflicts in relation to the presented study.

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Table 1. Semi-quantification of A/B antigen levels on donor-derived group O RBC following HSCT

Patients			Relative mean MFI[1] of donor-derived O RBC[2]	
Original ABO group and secretor status	HSCT with donor of blood group O	HSCT with donor of blood group A	Anti-A	Anti-B
A secretor	7[3]	-	2.9	-
AB secretor	1	-	2.6	1.0
B secretor	-	2	1.9	1.0
A non-secretor	1	-	1.5	-

HSCT = Haematopoietic stem cell transplantation; MFI = Mean fluorescence intensity

[1] Given as the ratio of mean MFI of donor-derived O RBC and mean MFI of O control RBC

[2] Blood group O RBC derived from donor of transplanted HSC or donor of transfused blood units. The number of units transfused within the last three months prior to testing ranged from three to 23 in seven of the transplanted patients.

[3] Up to four consecutive samples were available from four of the seven A secretor patients for analysis and inclusion in the calculation of relative mean MFI.

Table 2. Semi-quantification of A/B antigen levels on donor-derived group O RBC following transfusion

Patients		Relative mean MFI[1] of donor-derived O RBC[2]	
ABO group and secretor status	Numbers[3]	Anti-A	Anti-B
A secretor	7	2.3	-
AB secretor	1	1.9	1.1
B secretor	4	-	1.1
A non-secretor	2	1.7	-
B non-secretor	1	-	1.0

MFI = Mean fluorescence intensity

[1]Given as the ratio of mean MFI of donor-derived O RBC and mean MFI of O control RBC

[2] The number of units transfused within the last three months prior to testing ranged from one to 36

[3] Up to four consecutive samples were available from six of the 15 patients for analysis and inclusion in the calculation of relative mean MFI.

Figure 1

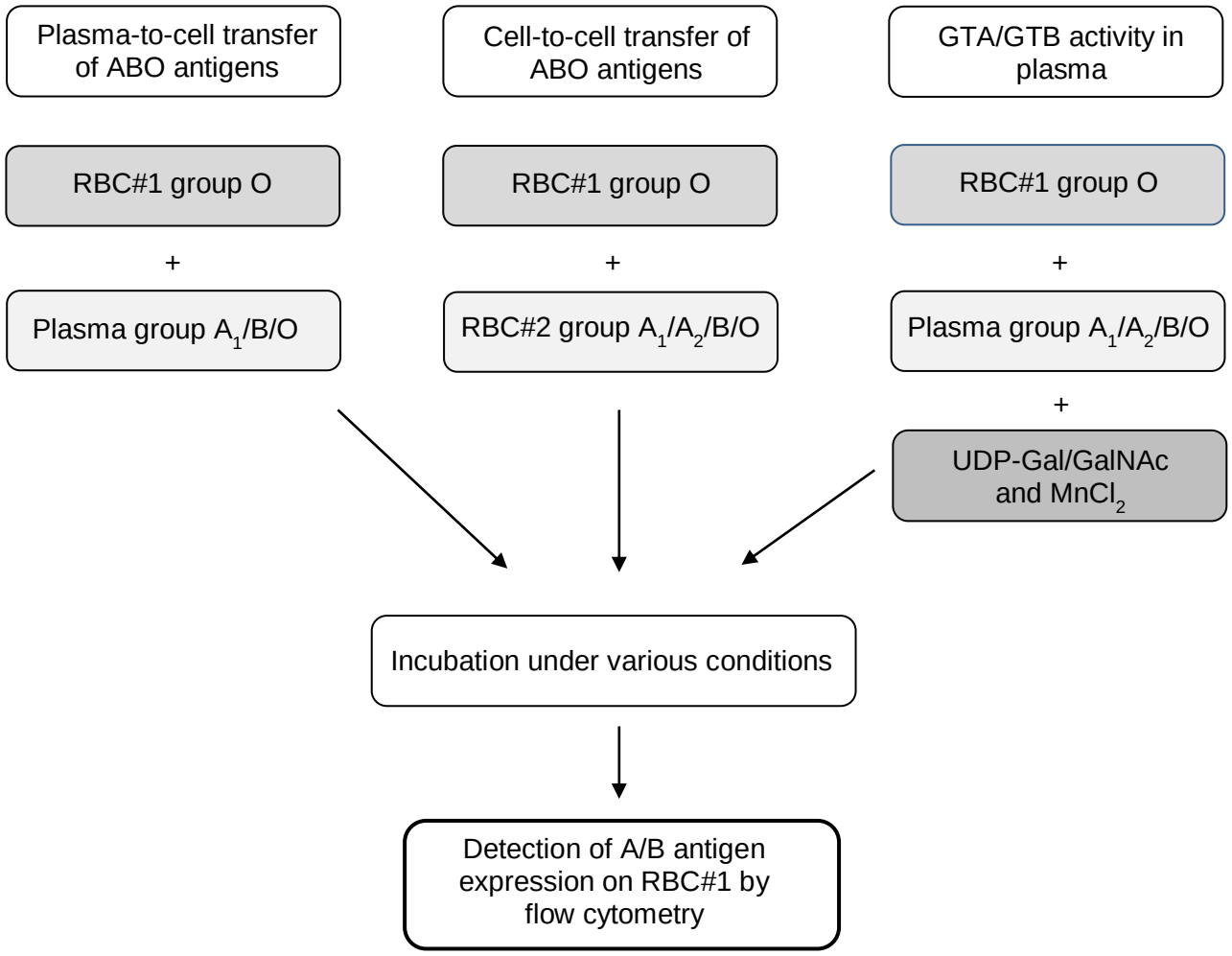
Plasma-to-cell transfer of ABO antigens
Cell-to-cell transfer of ABO antigens
GTA/GTB activity in plasma
RBC#1 group O
RBC#1 group O
RBC#1 group O
+
+
+
Plasma group A_1/B/O
RBC#2 group A_1/A_2/B/O
Plasma group A_1/A_2/B/O
+
UDP-Gal/GalNAc and $MnCl_2$
Incubation under various conditions
Detection of A/B antigen expression on RBC#1 by flow cytometry

Figure 2.

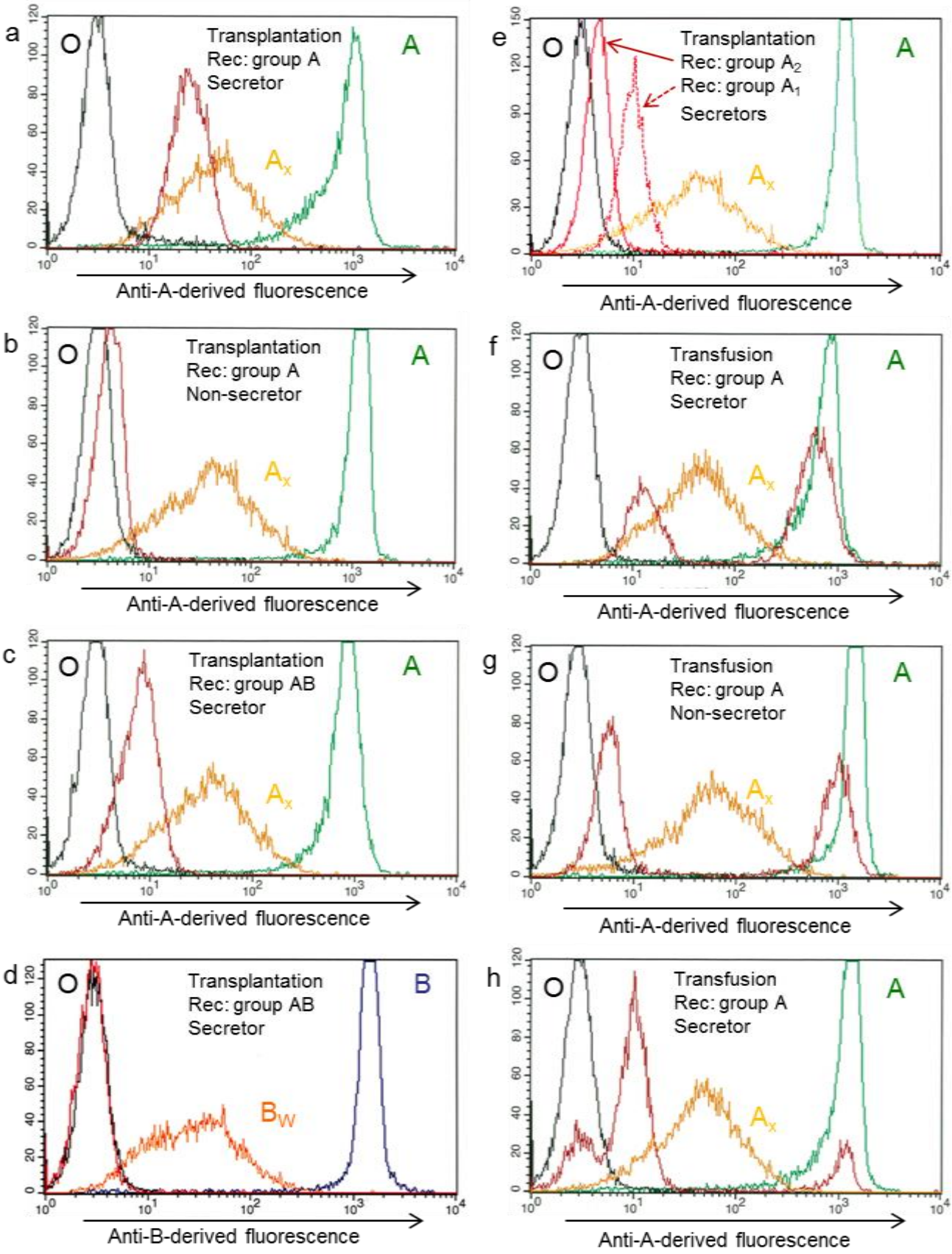
a
O
Transplantation
Rec: group A
Secretor
A
A_x
Anti-A-derived fluorescence
b
O
Transplantation
Rec: group A
Non-secretor
A
A_x
Anti-A-derived fluorescence
c
O
Transplantation
Rec: group AB
Secretor
A
A_x
Anti-A-derived fluorescence
d
O
Transplantation
Rec: group AB
Secretor
B
B_w
Anti-B-derived fluorescence
e
O
Transplantation
Rec: group A_2
Rec: group A_1
Secretors
A
A_x
Anti-A-derived fluorescence
f
O
Transfusion
Rec: group A
Secretor
A
A_x
Anti-A-derived fluorescence
g
O
Transfusion
Rec: group A
Non-secretor
A
A_x
Anti-A-derived fluorescence
h
O
Transfusion
Rec: group A
Secretor
A
A_x
Anti-A-derived fluorescence

Figure 3

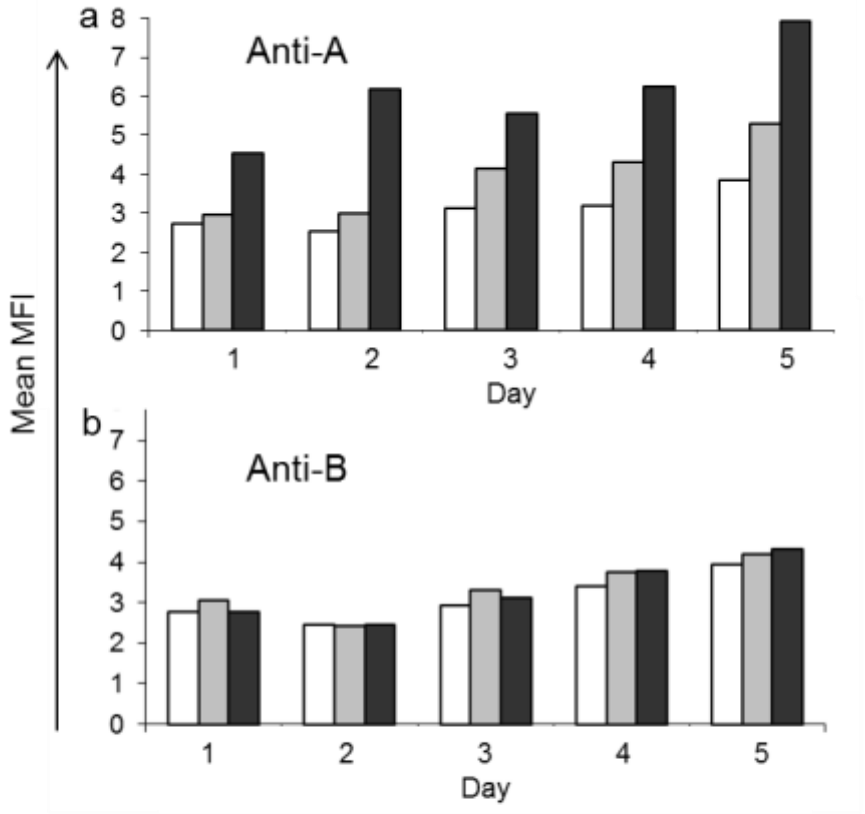
a
Anti-A
Mean MFI
0
1
2
3
4
5
6
7
8
1
2
3
4
5
Day
b
Anti-B
0
1
2
3
4
5
6
7
1
2
3
4
5
Day

Figure 4.

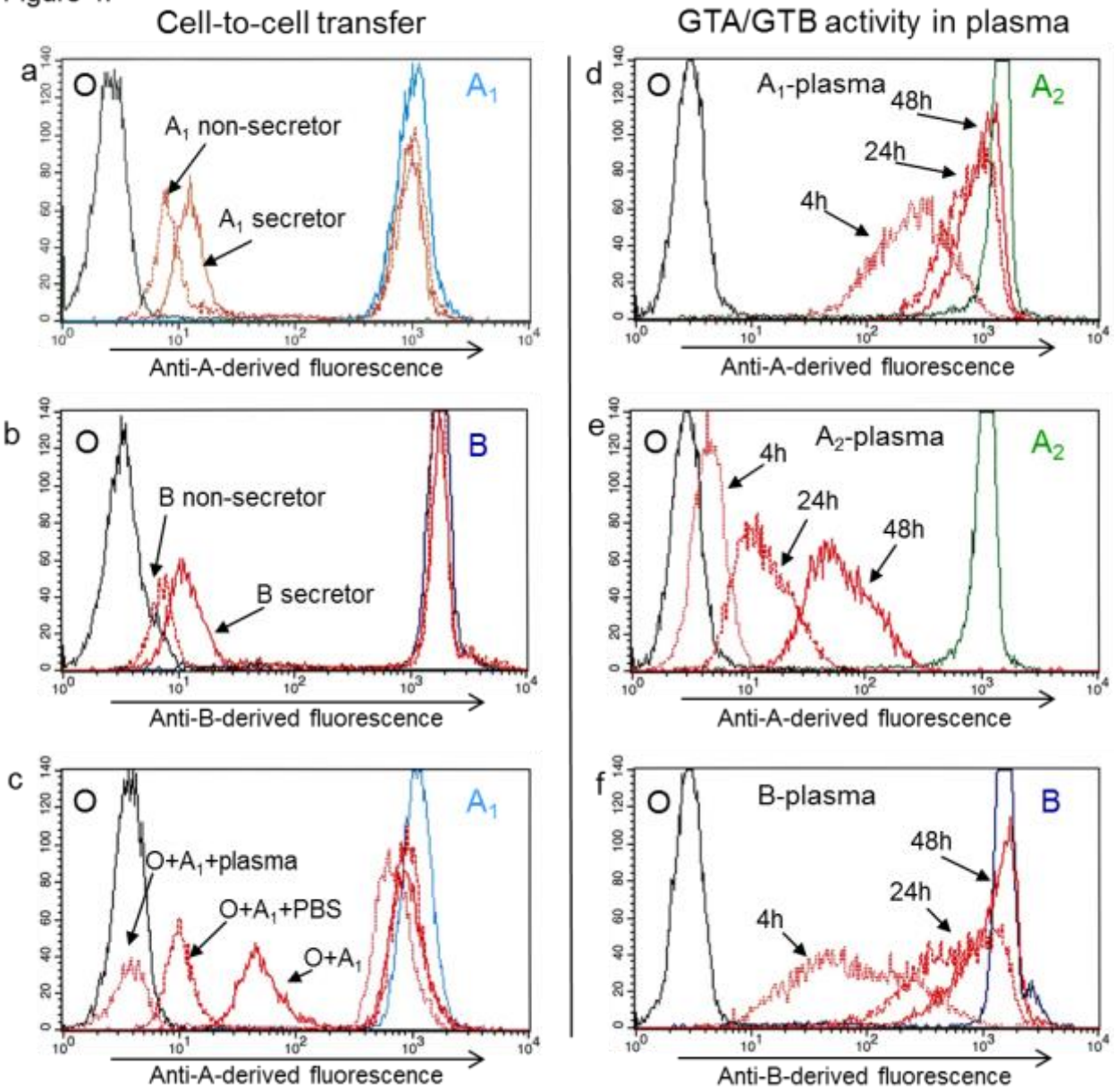
Cell-to-cell transfer
GTA/GTB activity in plasma
a
O
A1
A1 non-secretor
A1 secretor
Anti-A-derived fluorescence
b
O
B
B non-secretor
B secretor
Anti-B-derived fluorescence
c
O
A1
O+A1+plasma
O+A1+PBS
O+A1
Anti-A-derived fluorescence
d
O
A1-plasma
48h
24h
4h
A2
Anti-A-derived fluorescence
e
O
A2-plasma
4h
24h
48h
A2
Anti-A-derived fluorescence
f
O
B-plasma
48h
24h
4h
B
Anti-B-derived fluorescence

Figure legends

Fig. 1. Schematic overview of *in vitro* experiments.

Fig. 2. Flow cytometry testing of samples from recipients (Rec) of either HSCs or blood transfusions of blood group O. Histograms from flow cytometric testing of RBC samples. Colour code: red lines represent the patients' samples. Control RBC are included in each run. The x axis represent PE-derived fluorescence on a logarithmic scale while the y axis shows the number of cells on a linear scale.

(a) Post-transplant testing with anti-A of an A secretor patient who had received HSC from a group O donor**.** An almost A_x-like antigen level was detected on the donor-derived group O RBC. (b) Post-transplant testing with anti-A of an A non-secretor patient who had received HSC from a group O donor**.** Weak expression of A antigen was detected on the donor-derived group O RBC. (c and d) Post-transplant testing with anti-A and anti-B, respectively, of an AB secretor patient who had received HSC from a group O donor. Weak expression of A antigen on the donor-derived group O RBC was detected with anti-A but no B antigen could be detected with anti-B. (e) Two secretor patients, originally defined as A^2O^{1v} (solid red line) and A^1O^2 (dashed red line), respectively, with similar transplantation/transfusion histories but differing levels of detectable A antigen on the donor-derived group O RBC. (f) Post-transfusion testing with anti-A of an A secretor patient who had received blood transfusions from group O donors. (g) Post-transfusion testing with anti-A of an A non-secretor patient who had received blood transfusions from group O donors. (h) Post-transfusion testing with anti-A of an A secretor patient who had received blood transfusions from group O donors. Three populations of cells are seen, one expressing A antigen at a normal level and two populations of donor-derived O RBC

expressing no or low levels of A antigen. The patient had been transfused just prior to sampling.

Fig. 3. Flow cytometric testing of cell-plasma mixes. Two different group O RBC were incubated in parallel with either group O (□), A_1 or B non-secretor (▨) or A_1 or B secretor (■) plasma. Changes in mean MFI of the group O RBC are shown over time. (a) O RBC mixed with either O, A_1 secretor or A_1 non-secretor plasma incubated at 37°C and tested with anti-A. The rise in MFI is clearly seen for the O RBC/ A_1 secretor plasma mix. (b) O RBC mixed with either O, B secretor or B non-secretor plasma incubated at 37°C and tested with anti-B. There was no obvious rise in MFI in comparison to the O plasma control.

Fig. 4. *In vitro* experiments to examine cell-to-cell transfer of A/B antigens (a-c) and detection of GTA/GTB activity in plasma (d-f). Control RBCs are included in the flow cytometry testing and were run in parallel with the samples of interest. In the histograms the x axis represent PE-derived fluorescence on a logarithmic scale while the y axis shows the number of cells on a linear scale.

The cell-cell mixes were incubated at 37° for 24 h under constant mixing and subsequently tested with anti-A or anti-B. (a) Group O RBCs mixed with either A_1 secretor (solid red line) or A_1 non-secretor (dashed red line) RBCs. Clearly detectable levels of A antigen are seen on the RBCs originally typed as group O. A slightly higher expression is seen for O RBCs incubated with A_1 secretor RBCs in comparison to A_1 non-secretor RBCs. (b) Group O RBC mixed with either B secretor (solid red line) or B non-secretor (dashed red line) RBC and tested with anti-B. Clearly detectable levels of B antigen are seen on the RBCs originally typed as group O. A slightly higher expression is seen for O RBCs

incubated with B secretor RBCs in comparison to B non-secretor RBCs. (c) Flow cytometric testing of cell-cell mixes and with either PBS or A non-secretor plasma added. Different levels of A antigen were detected with anti-A in the different mixes. (d-f) Histograms from flow cytometric testing of RBC/plasma mixes with added substrate. Control RBCs are included in each run. All mixes show a rise in MFI over time.(d) O RBCs incubated with A_1 plasma, UDP-GalNAc and $MnCl_2$, tested with anti-A. (e) O RBCs incubated with A_2 plasma, UDP-GalNAc and $MnCl_2$, tested with anti-A. (f) O RBCs incubated with B plasma UDP-Gal and $MnCl_2$, tested with anti-B.