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Anti-A/B antibody depletion by semiselective versus ABO blood group-specific immunoadsorption

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Abstract

Background. Recipient desensitization using blood group (BG)-specific immunoadsorption (ABO-IA) has proven to enable successful kidney transplantation across major ABO barriers. In this context, the efficiency of non-antigen-specific (semiselective) IA adsorbers has not yet been established. The objective of our study was to quantify anti-A/B antibody depletion by protein A-, peptide ligand- and anti-human immunoglobulin-based semiselective IA in comparison to ABO-IA.

Methods. Eight ABO-IA-treated transplant candidates and 39 patients subjected to semiselective IA for a variety of different indications outside the context of ABO-incompatible transplantation were included. Antibody patterns (IgG, IgG1-4 subclasses, IgM, C4d-fixing reactivities) were analysed applying conventional agglutination testing and flow cytometry.

Results. As assessed by sensitive flow cytometric antibody detection, ABO-IA-based desensitization led to a profound even though often incomplete reduction of anti-A/B reactivities. Persistent complement- or non-complement-fixing reactivities, however, were not associated with transplant rejection or capillary C4d deposition. Single sessions of semiselective IA turned out to be more effective than ABO-IA in decreasing levels of anti-A/B IgG [median reduction to 28 versus 59% (ABO-IA) of baseline values, $P < 0.001$). In contrast, BG-specific IgM (74 versus 30%, $P < 0.001$) and IgG3 (72 versus 42%, $P < 0.05$) were reduced to a lesser extent, without differences between tested adsorber types. Analysis of four consecutive IA sessions revealed that inferior efficiency could not be overcome by serial treatment.

Conclusion. Our observation of limited adsorption capacities regarding distinct BG-specific Ig (sub)classes suggests caution in applying semiselective IA techniques in ABO-incompatible kidney transplantation.

Keywords: ABO incompatibility; immunoadsorption; kidney transplantation; recipient desensitization

Introduction

ABO incompatibility represents a major immunological barrier to transplantation [1]. In recent years, several plasmapheresis- or immunoadsorption (IA)-based desensitization protocols were reported to allow for successful ABO-incompatible kidney transplantation [1–3].

One major advantage of IA over plasmapheresis may be its capability to deplete large amounts of circulating antibodies without considerable losses of essential plasma constituents [4, 5]. Currently, blood group (BG) antigen-specific apheresis (ABO-IA) represents the IA technique of choice to remove anti-A/B antibodies [2, 6, 7]. However, ABO-IA adsorbers are licensed for single use only, and a need for repeated treatment implies excessive treatment costs (~30 000 USD for seven treatments). In this respect, the use of regenerative

semiselective IA techniques for non-antigen-specific immunoglobulin (Ig) depletion could represent an attractive less costly alternative (12 000–18 000 USD for a pair of reusable columns) [4, 5]. Another potential advantage may be that unselective antibody removal could be beneficial for transplant candidates with additional anti-human leukocyte antigen (HLA) sensitization [8, 9].

Previous studies have demonstrated efficient removal of isoagglutinins by semiselective IA in red cell aplasia following ABO-mismatched bone marrow transplantation [10]. However, there is so far only scarce data on ABO-incompatible kidney transplantation. Recently, Tyden *et al.* [4] reported the occurrence of antibody-mediated rejection (AMR) in a small series of ABO-incompatible transplant recipients subjected to IA with protein A. In one of three patients, antibody removal seemed incomplete using this modality [4]. This initial experience suggests that it may be first necessary to carefully dissect the capacity of this and of related techniques to remove BG-specific antibodies before considering their use in a clinical setting.

The present study was designed to clarify the impact of semiselective IA on anti-A/B reactivity patterns in direct comparison with ABO-IA. For detailed and sensitive assessment of antibody kinetics, we extended our analysis to flow cytometric detection of anti-A/B antibody binding and *in vitro* complement activation.

Materials and methods

Study design and patients

The present prospective study, which was designed to assess the extent of anti-A/B antibody removal by selective versus semiselective IA, was carried out in accordance with the Helsinki Declaration and approved by the ethics committee of the Medical University Vienna (EK Nr. 984/2009).

Comparator group. We included eight ABO-incompatible living-donor renal transplantation candidates subjected to serial sessions (5–10 treatment sessions >1–2 weeks) of ABO-IA (Glycosorb® ABO A- or B-columns; Glycorex Transplantation AB, Lund, Sweden) to reduce anti-A/B antibody titers to $\leq$1:8 [11]. All patients received a single dose of rituximab (375 mg/m^2 4 weeks before transplantation) and intravenous immunoglobulin (IVIG, 0.5 g/kg 4–7 days before transplantation; KIOVIG, Baxter Healthcare S.A., Lessines, Belgium). Basal immunosuppression [tacrolimus (initial trough levels: 12–15 ng/mL), mycophenolate mofetil (MMF; 2 × 1 g/day) and steroids] was initiated 2 weeks before transplantation. Seven patients were transplanted after successful desensitization (three living-related and four spousal transplants; A1 into O: $N = 3$, A2 into O: $N = 2$, A1 into B: $N = 1$, B into A1: $N = 1$). Clinical outcomes were excellent (100% 1-year graft survival; no rejection).

Study of single IA sessions. In this first analysis, the eight ABO-IA-treated transplant candidates were analysed in comparison to 30 patients subjected to one of three different semiselective apheresis modalities (10 subjects per modality): (i) IA with staphylococcal protein A (ProtA-IA; Immunosorba®, Fresenius Medical Care, Bad Homburg, Germany), (ii) IA with synthetic peptide GAM146 (GAM-IA; Globaffin®; Fresenius Medical Care) and (iii) IA with immobilized polyclonal sheep antibodies against human Ig (Ig-IA; Therasorb Ig®; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Semiselective IA was applied outside the context of ABO-incompatible transplantation [treatment of AMR (typical morphological features; capillary C4d deposition; Luminex-based solid-phase detection of donor-specific HLA class I and/or II antibodies) after ABO-compatible kidney ($n = 3$) or heart transplantation ($n = 1$); desensitization of allosensitized kidney transplant candidates: $n = 2$; systemic lupus erythemathosus: $n = 9$; myasthenia gravis: $n = 6$; multiple sclerosis: $n = 2$; other autoimmune diseases: $n = 7$]. At the time of IA treatment, 8 patients received immunosuppressive monotherapy (azathioprine or steroids), 10 patients dual therapy (steroids plus azathioprine, mycophenolic acid or cyclophosphamide) and four calcineurin inhibitor- or mammalian target of rapamycin inhibitor-based triple therapy.

Study of repeated IA. This analysis was designed to quantify antibody depletion by four repetitive IA sessions. Six ABO-IA-treated patients (two patients were excluded because of IVIG infusion already before the fourth treatment session and thus potential interference of infused anti-A/B antibodies with test results) were analysed in comparison to a group of nine patients subjected to treatment with either ProtA-IA ($n = 7$) or the related principle of GAM-IA ($n = 2$). Treatment indications were AMR ($n = 7$) or desensitization of sensitized kidney transplant candidates ($n = 2$).

IA therapy

Plasma (8 L per session) was separated by centrifugation (Cobe Spectra® Apheresis System) under regional citrate anticoagulation, with or without systemic heparin. For ABO-IA, separated plasma was passed through a single use absorber. For Prot-IA, GAM-IA and Ig-IA, plasma was pumped alternating through a regenerative twin column system using an Adasorb device (Medicap, GmbH, Ulrichstein, Germany).

Haemagglutination testing

Anti-A/B antibody titres were determined by direct agglutination (DA) at 22°C and indirect anti-human globulin testing (IAT) at 37°C using A1, A2 or B test red blood cells (RBC) (DiaMed, Cressier, Switzerland) and neutral gel cards or gel cards containing rabbit anti-human IgG, respectively (DiaMed). Tests were performed according to the manufacturer's instructions. The titre was recorded as the inverted value of the highest plasma dilution that gave a weak (1+) agglutination reaction. An influence of irregular red cell antibodies was excluded as detailed earlier [12].

Flow cytometry

RBC obtained from healthy volunteers (blood type A1, A2, B or O) were fixed in Karnovsky buffer to prevent agglutination [13–15]. Heparinized blood obtained from healthy volunteers was washed with phosphate-buffered saline (PBS), and 100 µL of RBC suspension (haematocrit 80–90%) were fixed for 30 min (4°C) in 4 mL Karnovsky buffer (0.2% glutaraldehyde and 1.8% formaldehyde in phosphate buffer, pH 7.4). RBC were washed and re-suspended in PBS containing 0.6% bovine serum albumin (BSA). All subsequent steps were performed at 4°C.

For detection of anti-A/B IgG and IgM, 5 µL fixed RBC (1% suspension) were incubated with 7 µL serum for 30 min. After washing with Ca^{2+}–Mg^{2+}-free Hank's balanced salt solution containing 0.1% BSA, RBC were stained for 30 min with saturating concentrations of DyLight 649-conjugated rabbit F(ab')$_2$ anti-human IgG Fcγ (Jackson ImmunoResearch Europe, Newmarket, UK) or DyLight 649-conjugated Fab goat anti-human IgM Fc (Jackson ImmunoResearch), respectively.

For IgG subclass detection, RBC were incubated with saturating concentrations of mouse anti-human IgG1 (clone HP 6069; Invitrogen GmbH, Lofer, Austria), IgG2 (clone HP 6002; Invitrogen GmbH), IgG3 (clone HP 6050; Southern Biotech, Birmingham, AL), or IgG4 (clone HP 6023; Millipore, Temecula, CA), respectively. After 30 min incubation, cells were washed and incubated for 30 min with DyLight 649-conjugated donkey F(ab')$_2$ anti-mouse IgG (H + L) (Jackson ImmunoResearch).

For detection of antibody-triggered C4d complement split product deposition, we applied a modified protocol. Following 30 min incubation of 5 µL fixed RBC with 7 µL test serum, 36 µL of a BG-AB serum obtained from a healthy male volunteer (source of intact human complement) were added. After another 30 min incubation cells were washed and stained with DyLight 549-conjugated rabbit anti-human C4d (Biomedica, Vienna, Austria) for 30 min, 4°C.

Samples were analysed using a BD FACSCanto™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Results were recorded as normalized mean fluorescence intensity [$MFI_{norm} = MFI_{(test\ RBC)} - MFI_{(type\ O\ control\ RBC)}$]. For IgG subclass binding and C4d fixation, test thresholds were defined according to MFI_{norm} obtained with three AB sera from healthy male volunteers. A test result was considered as being

positive if the MFI_{norm} was above the mean MFI_{norm} + 2 SDs detected for control sera.

For flow cytometric detection of anti-A/B IgG, IgG subclasses and IgM, sera were diluted 1:5 in PBS to prevent saturation and facilitate assessments of antibody levels in the range relevant for ABO-incompatible transplantation [16]. In addition, dilution prevented false low results caused by the prozone effect. Indeed, we frequently observed an increase in detected antibody levels following *in vitro* dilution of test sera. In parallel, in some patients, flow cytometric analysis of undiluted (but not 1:5 diluted) sera revealed an increase in antibody levels during IA therapy (data not shown). The prozone effect was not observed for *in vitro* C4d detection (data not shown). Here, we evaluated undiluted sera.

Immunohistochemistry

All ABO-incompatible kidney transplants underwent protocol biopsies one week post-transplantation. C4d was stained on paraffin sections [17]. Biopsies were classified as C4d positive if there was linear C4d deposition in at least 10% of peritubular capillaries.

Statistics

We applied Mann–Whitney *U*, Wilcoxon signed-rank, Fisher's exact or Spearman correlation tests, as appropriate. A two-sided P-value <0.05 was considered statistically significant. Statistical calculations were performed using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL).

Results

Anti-A/B antibody depletion by single sessions of selective versus semiselective IA

To assess BG-specific antibody depletion by a single session of selective versus semiselective IA (first session of a treatment cycle), we included 8 ABO-IA-treated transplant candidates and 30 patients treated with semiselective IA for indications outside the context of ABO-incompatible transplantation.

Baseline immunological results (evaluation of 73 individual anti-A/B reactivities) are shown in Table 1. A comparative analysis of agglutination- versus flow cytometry-based antibody detection revealed significant, although imperfect, correlations of IAT titers with IgG MFI_{norm} ($r = 0.795$, $P < 0.001$) and DA titers with IgM MFI_{norm} ($r = 0.627$, $P < 0.001$). IgG1 was the most common BG-reactive IgG subclass, followed by IgG3, IgG2 and IgG4 (Table 1). Only a minor proportion of anti-A/B reactivities (22%) had C4d-fixing ability. As shown in Table 1, among patients treated with semiselective IA the plasma volumes processed per body weight were slightly higher than those in ABO-IA-treated patients. With the exception of IgG3 (higher MFI_{norm} in ABO-IA-treated patients), baseline immunological results did not significantly differ between IA treatment groups (Table 1).

Antibody depletion by different IA techniques is illustrated in Figure 1. While ABO-IA did not, or only slightly, reduce total IgG and IgM concentrations {reduction to 94% [interquartile range (IQR): 89–99] and 93% (85–98)] of baseline levels}, semiselective IA reduced IgG to 37% (median, IQR: 26–49%) and IgM to 65% (58–75%) of pre-IA levels. ABO-IA more effectively reduced IAT and DA titres. Semiselective IA was more effective in depleting flow cytometric anti-A/B IgG [reduction to 28%

Table 1. Baseline (pre-IA) immunological data obtained in 38 patients subjected to single sessions of IA treatment

	ABO-IA (8 patients)	Semiselective IA (30 patients)	P-value
Processed plasma volume, mL/kg body weight, median (IQR)	101 (91–111)	117 (103–138)	0.04
Tested reactivities (*N*)	15	58	
Anti-A1, *n*	7	16	
Anti-A2, *n*	7	16	
Anti-B, *n*	1	26	
Total IgG, mg/dL[a], median (IQR)[b]	850 (679–1078)	627 (422–952)	0.3
Total IgM, mg/dL[a], median (IQR)[b]	53 (38–96)	81 (57–102)	0.5
IAT, median titre (IQR)[b]	1:8 (1:1–1:64)	1:8 (1:2–1:32)	0.8
DA, median titre (IQR)[b]	1:4 (1:1–1:8)	1:8 (1:2–1:16)	0.5
Flow cytometric anti-A/B reactivities			
IgG			
MFI_{norm}, median (IQR)[b]	23 837 (3677–88 498)	16 099 (3882–46 097)	0.6
IgM			
MFI_{norm}, median (IQR)[b]	5877 (2997–15 529)	3920 (1222–9901)	0.15
IgG1			
Positive result, *n* (%)	8 (53)	28 (48)	0.8
MFI_{norm}, median (IQR)[b]	5153 (2584–8060)	1418 (383–10 069)	0.09
IgG2			
Positive result, *n* (%)	4 (27)	17 (29)	>0.99
MFI_{norm}, median (IQR)[b]	21 375 (14 763–69 686)	18 656 (9747–45 454)	0.6
IgG3			
Positive result, *n* (%)	7 (47)	26 (45)	>0.99
MFI_{norm}, median (IQR)[b]	7692 (3872–34 127)	2113 (745–2969)	<0.001
IgG4			
Positive result, *n* (%)	1 (7)	5 (9)	>0.99
MFI_{norm}, median (IQR)[b]	2675	2262 (1389–2335)	[c]
C4d			
Positive result, *n* (%)	3 (20)	13 (22)	>0.99
MFI_{norm}, median (IQR)[b]	704 (1386–1682)	750 (410–1052)	0.19

[a]Ig concentrations were assessed by nephelometry (reference values for IgG: 700–1600 mg/dL; IgM: 40–230 mg/dL).
[b]Median and IQR are given only if sample sizes were ≥4.
[c]No statistical comparison because of insufficient samples sizes.

(IQR: 22–49) versus 59% (43–96) of baseline levels, P < 0.001] but less effective regarding anti-A/B IgM depletion [74% (IQR: 61–97) versus 30% (IQR: 22–45), P < 0.001]. Interestingly, there were no differences between the three tested semiselective adsorber types (Figure 1). Another remarkable finding was that ABO-IA also lowered ABO reactivities not directed against the BG antigens of applied columns [IgG: 80% (41–95); IgM: 74% (38–118) of baseline MFI_{norm}].

Semiselective IA was superior (GAM-IA) or at least equally effective (ProtA-IA, Ig-IA) with respect to BG-specific IgG1 depletion (Table 2). In contrast, semiselective

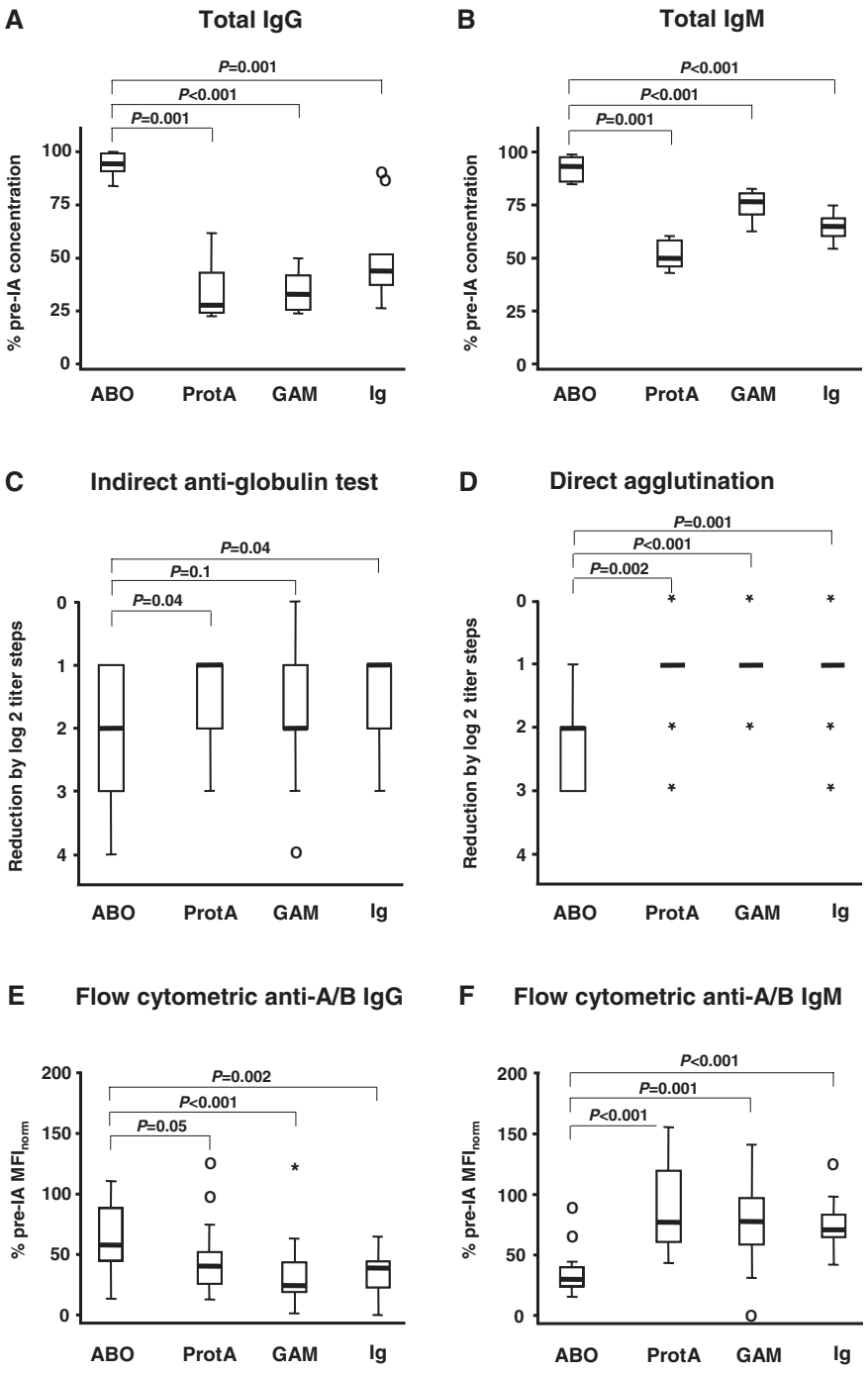

Fig. 1. Antibody depletion by single sessions of ABO-IA in comparison to semiselective IA modes. Levels of (**A**) total IgG and (**B**) total IgM were assessed by nephelometry (normal reference values for IgG: 700–1600 mg/dL; IgM: 40–230 mg/dL). Agglutination testing was performed using gel cards for (**C**) IAT or (**D**) DA. IgG (**E**) and IgM (**F**) anti-A/B reactivities were quantified by flow cytometry. Results are given as percentages of baseline (pre-IA) levels. Only ABO column BG-specific reactivities were considered for calculation and presentation of results obtained with ABO-IA. Results are presented as box plots indicating median, IQR and range. Mild outliers are indicated as open dots, extreme outliers as asterisks. For statistical comparisons, non-parametric testing (Mann–Whitney *U*-test) was applied.

adsorbers turned out to be less effective regarding IgG3 elimination. For IgG2, no significant differences were observed, and there were no major numerical differences between different adsorber types with respect to kinetics of IgG4 and C4d-fixing reactivities (Table 2).

Differing levels of immunosuppression among patients subjected to semiselective IA did not influence the extent of anti-A/B antibody depletion. Subjects receiving two or more immunosuppressive compounds did not differ from patients receiving one or no compound [anti-A/B IgG: reduction to 37% (median, IQR: 23–45) versus 27% (20–51); anti-A/B IgM: 72% (56–89) versus 75% (66–108), respectively].

Anti-A/B antibody depletion by repetitive treatment with selective versus semiselective IA

In a second analysis, we evaluated antibody depletion by four subsequent IA sessions (Supplementary table 1). Six ABO-IA-treated patients (two of the eight ABO-IA-treated patients were excluded from this analysis because of IVIG infusion before the fourth IA session) were analysed in comparison to nine patients subjected to semiselective IA. Again, repeated semiselective IA tended to be more effective than ABO-IA regarding anti-A/B IgG depletion but was significantly less effective in reducing BG-specific IgM and IgG3 (Supplementary table 1).

Anti-A/B antibody depletion upon ABO-IA-based recipient desensitization

Successful ABO-IA-based recipient desensitization (seven of the eight ABO-incompatible transplant candidates) was associated with a decrease in levels of flow cytometric donor BG-specific IgG and IgM reactivities to 31% (IQR: 15–108) and 21% (12–38) of baseline values. We observed a transient increase of anti-A/B reactivities following administration of IVIG, despite infusion during IA treatment. In 4 patients, anti-A/B IgG (12 tested BG-specific reactivities) was quantified shortly before and after IVIG infusion. Before treatment, IgG levels were 37% (median, IQR: 21–54), after IVIG treatment 56% (IQR: 29–155) of pre-IA baseline levels ($P = 0.01$). Immediately before transplantation, two patients still showed significant levels of BG-specific IgG subclass reactivity (Table 3). One of them tested positive for both IgG1 and IgG3, whereas another patient became weakly IgG4 positive, possibly due to IVIG treatment. A third patient had C4d-fixing reactivity without significant IgG subclass binding. Three of the seven ABO-incompatible transplants showed subclinical capillary C4d deposition in early protocol biopsies. They did not differ from C4d-negative patients regarding BG-specific IgG and IgM levels immediately before transplantation (not shown). The two individuals who tested positive for complement-binding IgG subclasses (IgG1 and 3) or *in vitro* C4d fixation were C4d negative (Table 3).

Discussion

The present study, which was designed to investigate the effect of different IA modalities on BG-specific reactivity patterns, revealed inferior anti-A/B IgM and IgG3 depletion by semiselective IA.

Regarding incomplete IgM elimination, our results are in accordance with earlier reports [18]. Interestingly, there were no meaningful differences between different types of semiselective adsorbers, a finding which may be in some contrast to one previous large study suggesting more pronounced total IgM depletion by Ig-IA [18]. However, there are also other studies that failed to demonstrate such differential effects [19, 20]. In contrast to IgM, semiselective IA was highly effective regarding depletion of anti-A/B IgG. Semiselective techniques were even more efficient than ABO-IA, but this may be at least partly explained by the slightly higher plasma volumes treated per body weight. Our finding of inferior IgG3 depletion by ProtA-IA and GAM-IA is in accordance with previous studies [20, 21]. However, we have no good explanation for a similar inferiority of Ig-IA, which may be in some contrast to earlier data [20].

Interestingly, differential effects of IA techniques on Ig (sub)class patterns did not result in major differences regarding complement-fixing abilities of detected reactivities. One possible explanation could be the predominance of complement-activating IgG1 (binding intensity and frequency), which may have overwhelmed differential effects on IgG3 and/or IgM.

In clinical routine, haemagglutination tests are widely used for anti-A/B antibody detection. Major pitfalls of agglutination testing may be marked inter-centre variabilities and the disadvantage of only semiquantitative estimation of

Table 2. Reduction of ABO-BG-specific IgG subclasses and C4d-fixing reactivities by single IA sessions

	IgG1	IgG2	IgG3	IgG4	C4d fixation
	Reactivity after IA treatment, median percentage of baseline MFI_{norm} (IQR)[a,b]				
Selective IA					
ABO-IA (8 patients)	56 (38–66)	29 (21–75)	42 (30–53)	81	57, 75, 88
Semiselective IA					
Prot-IA (10 patients)	51 (0–172)	42 (21–59)	70 (61–88)*	72, 90	73 (58–85)
GAM-IA (10 patients)	20 (7–40)*	25 (23–29)	80 (61–97)**	76	50 (46–54)
Ig-IA (10 patients)	20 (0–105)	29 (22–33)	67 (49–82)*	68, 92	90, 93

[a]MFI_{norm} values were recorded only for reactivities above the threshold defined in the methods section.
[b]Median and IQR are given only if sample sizes were ≥4.
*$P < 0.05$ (non-parametric comparison to the results obtained with ABO-IA).
**$P < 0.01$ (non-parametric comparison to the results obtained with ABO-IA).

Table 3. Donor-specific anti-A/B reactivity patterns in seven ABO-IA-treated recipients immediately before ABO-incompatible kidney transplantation

				IgG1		IgG2		IgG3		IgG4		C4d	
Protocol biopsy	Patient No.	Donor BG	IA sessions, *N*	Baseline →pre-Tx[a]	Pre-Tx MFI_{norm}	Baseline →pre-Tx[a]	Pre-Tx MFI_{norm}	Baseline →pre-Tx[a]	Pre-Tx MFI_{norm}	Baseline →pre-Tx[a]	Pre-Tx MFI_{norm}	Baseline →pre-Tx[a]	Pre-Tx MFI_{norm}
C4d-positive	3	B	5	Neg→neg	1134	Neg→neg	1466	Neg→neg	1157	Neg→pos	2419	Neg→neg	406
	5	A1	10	Pos→neg	2437	Pos→neg	3365	Pos→neg	2916	Neg→neg	7342	Pos→neg	396
	6	A1	6	Neg→neg	2886	Neg→neg	3976	Neg→neg	2287	Neg→neg	10 463	Neg→neg	604
C4d-negative	1	A1	8	Pos→pos	4848	Pos→neg	4503	Pos→pos	3726	Neg→neg	11 102	Pos→neg	440
	2	A1	6	Neg→neg	0	Neg→neg	3515	Neg→neg	224	Neg→neg	5810	Pos→pos	911
	4	A2	5	Neg→neg	545	Neg→neg	1814	Neg→neg	1612	Neg→neg	2003	Neg→neg	418
	7	A2	6	Pos→neg	1128	Neg→neg	1467	Neg→neg	933	Neg→neg	1742	Neg→neg	772

[a]Positivity was defined according to binding of AB control sera to test RBC as described in the 'Methods' section.

antibody levels [22, 23]. Recently, flow cytometric anti-A/B antibody detection was suggested to allow for more accurate and reproducible quantification of BG reactivities [14, 16]. In line with a previous study [16], we found significant correlations between flow cytometric IgG or IgM detection and the results of IAT or DA, respectively. However, as evidenced by r values <0.8, the level of test concordance was far from perfect. In this respect, a remarkable observation was the differing impact of semiselective IA on the results of IAT (less pronounced reduction in a comparison to ABO-IA) versus IgG detection by flow cytometry (more pronounced reduction). One can speculate that this phenomenon resulted from an influence of IgM-induced agglutination on the results of IAT.

However, there may also be some drawbacks with the use of flow cytometric antibody detection. It is important to note that changes in recorded MFI values may not simply reflect reductions in antibody concentrations but may be influenced by a variety of additional factors, such as individual levels of antibody avidity and affinity. Moreover, the definition of cut-off values may be a major challenge. While in earlier studies, one AB serum was used for negative control and/or threshold definition [13–15, 16], we have included three different AB control sera to better account for variations in background levels. Nevertheless, we are aware of the limitations of this approach and one can expect further improvement of test performance by including a larger number of control samples. A major technical caveat may be test artefacts caused by *in vitro* agglutination. However, there are several strategies to prevent agglutination, such as the use of dilute RBC [16] or, as in our study, fixation of RBC [13–15]. Moreover, evaluating undiluted sera, we found that test results were frequently affected by the prozone effect. This phenomenon has been described for a variety of different *in vitro* test systems including ABO and HLA antibody detection and was suggested to be caused by interfering IgM or C1 [24, 25]. Our results reinforce that strategies to overcome this phenomenon, such as standardized serum dilution (e.g. 1:5, as in our study), are necessary to avoid false low results. Another advantage of serum dilution may be that it prevents early saturation of high titre sera and allows for full assessments in the range relevant for ABO-incompatible transplantation [16].

A detailed knowledge of pathogenetic properties of antibody patterns may provide a useful basis for decision making regarding the choice of the IA device. For example, in dilated cardiomyopathy, a primordial role of IgG3 type autoantibodies has suggested the preferential use of IA schedules allowing for more efficient IgG3 elimination [20]. However, the relative contribution of different Ig (sub)classes to AMR after ABO-incompatible transplants is less well understood. In this specific context, the predictive value of baseline IgG and/or IgM ABO antibody levels has been controversially discussed. In an initial study of plasmapheresis-based desensitization, maximum pre-transplant IgG but not IgM agglutination titres were associated with rejection [26]. However, in a subsequent study of tacrolimus/MMF-treated patients, such associations could no longer be observed [27]. There is only scarce data regarding the role of anti-A/B antibody levels detected immediately before transplantation. Sensitive flow cytometric antibody

detection has uncovered persistent levels of pre-transplant reactivity (below the threshold defined according to agglutination testing) in our as well as in a previous study [15], without any impact on transplant outcomes. Interestingly, there was also no relationship between residual (complement-fixing) ABO reactivity and (subclinical) capillary C4d deposition, a common feature, which was earlier suggested to reflect a state of transplant accommodation [28].

Regarding ABO-IA, an interesting observation was a certain level of non-specificity, as suggested by some reduction also of reactivities to BG antigens not covered by the applied ABO-IA adsorber. A similar effect has been described in an earlier report and was suggested to result from antibody cross-reactivities [15].

There are several limitations to our study, including a marked heterogeneity regarding baseline immunosuppressive therapy between ABO-IA-treated and semiselective IA-treated patients. One can argue that differences in antibody depletion could at least partly be due to these imbalances. However, addressing this issue, we could not identify a major influence of concomitant immunosuppressive therapy on the kinetics of antibody levels. Another drawback of our study was that for some of the studied reactivities, such as IgG4, sample sizes were too small to allow for valid statistical comparison.

In conclusion, our present study demonstrates high efficiency of semiselective IA regarding elimination of BG-specific IgG, but inferiority with respect to IgM and IgG3 depletion. Our study cannot provide definite answers regarding prevention of AMR in ABO-incompatible transplantation. In this respect, a study limitation may be that we did not include patients subjected to standard or double-filtration plasmapheresis, techniques used at many transplant units, especially in the USA and Japan [1, 3]. However, our results suggest the need of caution in applying semiselective IA. While it is tempting to speculate that unselective IgG depletion could be advantageous for patients with additional HLA sensitization, one can expect that incomplete anti-A/B antibody depletion (IgM and IgG3) could in some instances necessitate additional measures (e.g. plasmapheresis) to achieve adequate antibody reduction.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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