Imlifidase-generated Single-cleaved IgG: Implications for Transplantation

Robert Bockermann, PhD,1 Sofia Järnum, PhD,1 Anna Runström, MSc,1 Tomas Lorant, MD, PhD,2 Lena Winstedt, PhD,1 Niklas Palmqvist, PhD,1 and Christian Kjellman, PhD1

INTRODUCTION

Highly sensitized patients with chronic kidney disease (CKD) harbor a broad spectrum of HLA-specific antibodies, which negatively impact the probability of finding a compatible organ. These patients are accumulating on kidney transplant waiting lists worldwide. Although recognition and prioritization within kidney allocations systems have led to improvement in rates of transplantation for highly sensitized patients, there remains a subset of very highly sensitized patients who are biologically incompatible with a high percentage of the donor pool and in whom a chance of a compatible organ offer is unlikely. In an adjusted negative binomial regression analysis, candidates with a calculated panel-reactive antibody (cPRA) of ≥99.9% had significantly lower transplant rates compared with nonhighly sensitized candidates, which has persisted despite substantial allocation priority.1-3 For some selected patients, participation in kidney paired donation programs may be an option4,5; however, only a small fraction of patients will be transplanted through these programs, and a large percentage of these patients will continue to accrue extended waiting times.6,7 In the absence of a compatible living donor, highly sensitized individuals may benefit from HLA-incompatible living or deceased donor transplantation given that preemptive desensitization protocols succeed in sufficiently reducing the level of circulating antibodies.

Background. Imlifidase is an immunoglobulin G (IgG)-specific protease conditionally approved in the EU for desensitization in highly sensitized crossmatch positive kidney transplant patients. Imlifidase efficiently cleaves both heavy chains of IgG in a 2-step process. However, low levels of the intermediate cleavage product, single-cleaved IgG (sclgG), may persist in the circulation. The study objective was to investigate Fc-mediated effector functions of sclgG and its potential impact on common clinical immunologic assays used to assess transplant eligibility. Methods. Imlifidase-generated sclgG, obtained by in vitro cleavage of HLA-sensitized patient serum or selected antibodies, was investigated in different complement- and FcγR-dependent assays and models, including clinical tests used to evaluate HLA-specific antibodies. Results. SclgG significantly reduced Fc-mediated effector function compared with intact IgG, although some degree of activity in complement- and FcγR-dependent models was still detectable. A preparation of concentrated sclgG generated from a highly HLA-sensitized individual gave rise to a positive signal in the anti–HLA IgG LABScreen, which uses anti-Fc detection, but was entirely negative in the C1qScreen. The same high-concentration HLA-binding sclgG preparation also generated positive complement-dependent cytotoxicity responses against 80%–100% of donor T and B cells, although follow-up titrations demonstrated a much lower intrinsic activity than for intact anti–HLA IgG. Conclusions. SclgG has a significantly reduced capacity to mediate Fc-dependent effector functions. However, remaining HLA-reactive sclgG in plasma after imlifidase treatment can cause positive assay results equivalent to intact IgG in clinical assays. Therefore, complete IgG cleavage after imlifidase treatment is essential to allow correct decision-making in relation to transplant eligibility.

(Transplantation 2022;106: 1485–1496).
donor-specific antibodies.\textsuperscript{5-7} The immunoglobulin G (IgG)-
degrading enzyme of \textit{Streptococcus pyogenes} (IdeS), with
the international nonproprietary name imlifidase, is a
35-kDa cysteine protease that with high selectivity cleaves
all 4 subclasses of human IgG just downstream of the hinge
region (between G\textsuperscript{236} and G\textsuperscript{237}), thus generating a F(ab\textsuperscript{'})\textsubscript{2}
and a homodimeric Fc fragment.\textsuperscript{5,6} In contrast to
the above methods, imlifidase provides an IgG antibody-free window based on its capacity to eliminate
the entire pool of plasma IgG, including extravascular
IgG.\textsuperscript{10,11} As part of the clinical development program at
Hansa Biopharma AB, to date, 46 HLA-sensitized patients
with CKD have been transplanted after treatment with
imlifidase.

Imlifidase cleaves the heavy chains of IgG in 2 separate
reaction steps.\textsuperscript{5,14} In the first reaction, one of the heavy
chains of the intact IgG molecule is rapidly cleaved result-
ing in the intermediate product, single-cleaved IgG (scIgG).
Due to a conformational change of scIgG, the second step
of the reaction, where the complete separation of scIgG
into F(ab\textsuperscript{'})\textsubscript{2} and Fc-homodimer is achieved, requires longer
time to completion compared with the rapid cleavage
observed in the first step.

During the initial dose-finding study in sensitized
patients with CKD,\textsuperscript{13} a single intravenous imlifidase infusion
of 0.25 mg/kg resulted in a near complete removal of circulating IgG and substantially reduced the levels of
HLA-specific IgG within 24 h among a wide range of
mean fluorescence intensity (MFI) levels evaluated with
single-antigen beads (SAB). In comparison, the imlifidase
0.12 mg/kg dose in 1 patient with pretreatment level of
anti-HLA antibody of 75 SABs with MFI\textsubscript{2} > 20,000 resulted in
the anti-HLA antibody signal remaining significant in
the LABScreen assay 24 h posttreatment (34 beads with
MFI\textsubscript{2} > 5000). Preliminary investigations indicated that this
remaining signal was attributed to residual scIgG.

As previously published data have mainly focused on
clinical outcomes from phase 2 studies, little has been
disseminated regarding the early preclinical and clinical
experiences. The purpose of this study was to (a) further
investigate the potential impact of scIgG in clinical assays
used to assess transplant eligibility, (b) investigate scIgG
with respect to potential residual Fc-mediated effector
functions, and (c) provide guidance on clinical assay use
and interpretation to facilitate clinical decision-making fol-
lowing desensitization with imlifidase as the drug advances
from investigational to commercial use.

MATERIALS AND METHODS

Cells, Animals, and Human Serum

Human lymphoma cell lines Raji [ACC-319] and Daudi
[ACC-78] were acquired from DSMZ. Human monocytic
leukemia cell line THP-1 was donated from Dr Maria
Allhorn, Lund University. Human erythrocytes were
pelleted from heparinized blood collected from healthy
donors. Cultured cells were maintained at 37°C in
a humidified CO\textsubscript{2}-incubator in R10 (RPMI 1640, 10% FBS
and PEST) (Raji and Daudi) or R10 supplemented with
10 mmol/L HEPEs and 0.05 mmol/L 2-mercaptoethanol
(THP-1).

Female BALB/c mice from Taconic, Denmark, were
housed at the research vivarium of Active Biotech AB in
Lund. All animal experiments were performed in agree-
ment with the ethical approval by Malmö/Lund animal
ethics committee (permit M72-13).

Sera from HLA-sensitized patients with CKD, included
in an earlier reported phase 2 dose-finding study\textsuperscript{13}
(EudrACT no. 2013-005417-13 and ClinicalTrials.gov
Identifier NCT02224820) was used. The study protocol
was approved by the regional ethics committee in Uppsala,
Sweden (approval no. 2014/131), and all study subjects
provided written informed consent.

Preparation of HLA-, Erythrocyte-, Thrombocyte-, and
CD20-reactive scIgG and F(ab\textsuperscript{'})\textsubscript{2}

Human HLA-reactive patient serum, human control
serum, rabbit antihuman erythrocyte (Rockland; 209-
4139), rabbit antimuscle erythrocyte antibody (Rockland;
210-4139), protein G-purified rabbit antimuscle thrombo-
cyte serum (Cedarlane, CLA31440), and rituximab (human
monoclonal anti-CD20 IgG1, MabThera/Paranova
Läkemedel AB, Sweden) were incubated with different
concentrations of imlifidase at 37°C for 1 h, followed by
heating (56°C, 30 min). Samples with desired amounts of
scIgG and F(ab\textsuperscript{'})\textsubscript{2} were identified by SDSPAGE/Western
blot and were tested for activity in different assays, with
mock-treated intact IgG as positive control.

Single-antigen Bead Assays

Sera from the HLA-sensitized imlifidase-treated patients
and fractions of intact IgG, scIgG, or F(ab\textsuperscript{'})\textsubscript{2} generated
from patient pretreatment serum were analyzed with the
LABScreen and C1qScreen assays using a Luminex 200
instrument and HLA Fusion 2.0 software (One Lambda,
ThermoFisher Scientific). All samples were heat-inacti-
vated (56°C, 30 min) before the analysis.

CDC-PRA: Complement-dependent Killing by
Panel-reactive Antibody

The CDC-PRA analysis was performed at the
Department of Clinical Immunology and Transfusion
Medicine, Lund, Sweden. Intact IgG, scIgG, and F(ab\textsuperscript{'})\textsubscript{2}
fractions, generated in vitro from HLA-sensitized patient
serum, were tested against a mini panel of purified T and B
cells from 5 representative donors, with cytotoxic fractions
retested after serial dilution.

Complement-fixing Capacity of Single-cleaved HLA-
reactive IgG

HLA-sensitized patient serum and nonsensitized healthy
control serum were heat-inactivated (56°C, 30 min) and
screened for IgG binding to Raji cells by flow cytometry
using biotin-conjugated polyclonal donkey antihuman
IgG (H+L) in combination with phycoerythrin-conjugated
streptavidin.

To investigate the complement-fixating capacity of pre-
treatment patient and control IgG, scIgG, and F(ab\textsuperscript{'})\textsubscript{2},
Raji cells were incubated with the different IgG fractions
using noncytotoxic human serum (10%) as complement
source. After incubation, the cells were divided into differ-
ent plates and stained for C1q and C4d before analysis by
flow cytometry.

For detailed information, see Supplemental Digital
Content (SDC), http://links.lww.com/TP/C327.
Complement-dependent Cytotoxicity Activity of Single-cleaved Rituximab

To evaluate the potential cytotoxic capacity of scIgG, Daudi cells were incubated with different concentrations of rituximab preparations (Rtx, scRtx, or F(ab′)2 Rtx; 10 or 30 µg/mL depending on read-out), followed by addition of noncytotoxic human serum (8%–10%) as complement source. Plates were incubated at 37°C, and cells were analyzed with respect to cell viability (colorimetry) and cell death (7AAD), C1q and C4d (flow cytometry).

For detailed information, see SDC, http://links.lww.com/TP/C327.

Crossmatching

The complement-dependent cytotoxicity crossmatch (CDCXM) and flow cytometric crossmatch (FCXM) were performed according to standard procedures, as previously described.15 The donor cells for the FCXM test were not treated with pronase.

FCγ Receptor-mediated Interactions of Single-cleaved Antierythrocyte IgG (Binding, ADCC and ADCP)

For FCγR binding and ADCC experiments, THP-1 monocytes were preactivated with lipopolysaccharide (LPS) and IFN-γ, after which erythrocytes opsonized with antierythrocyte preparations (intact IgG, scIgG, and F(ab′)2) or PBS were added. After overnight incubation, the degree of erythrocyte retention to adherent THP-1 cells was evaluated. Supernatants were collected for analysis of hemoglobin content. For ADCP, erythrocytes were labeled with CellTrace Far Red dye, washed, and incubated with different concentrations of rabbit antihuman erythrocyte preparations. Opsonized erythrocytes were added to PMA- and LPS-activated THP-1 cells. After 5 h incubation at 37°C, nonphagocytized erythrocytes were lysed, and the percentage of THP-1 cells that had engulfed labeled erythrocytes was analyzed by flow cytometry. For detailed information, see SDC, http://links.lww.com/TP/C327.

Experimental Autoimmune Hemolytic Anemia

Eleven-week-old female BALB/c mice (Taconic) were weighed and injected intraperitoneally with 1 mg preparations of intact IgG, scIgG, or F(ab′)2, generated from rabbit antimouse erythrocyte antibody. Blood was collected into Microvette CB 300 µL, lithium heparin tubes (Sarstedt, 16.443). Hematocrit and erythrocyte number (by DAF assay; see SDC, http://links.lww.com/TP/C327) were assessed daily. For hematocrit, 30 µL blood was taken up in LightCycler Capillaries (Roche, 1909339) and centrifuged in capillary adapters (Roche, 1909312) for 10 min at 510 g, after which the ratio of packed erythrocytes to total blood volume was measured. Spleen weights were registered at termination (day 3).

Experimental Immune Thrombocytopenic Purpura

Nine-week-old female BALB/c mice were injected intraperitoneally with 0.25 mg preparations of intact IgG, scIgG, or F(ab′)2 generated from rabbit antimouse thrombocyte serum. Blood plasma was collected into Microvette CB 300 µL, K2 EDTA tubes (Sarstedt, 16.444) 1 d after

FIGURE 1. Analysis of serum samples obtained before and after treatment of an HLA-sensitized patient with 0.12 mg/kg imlifidase. In (A), SDS-PAGE analysis of IgG cleavage in consecutive serum samples obtained before (pre) and at various time-points after treatment, with an additional 0.12 mg/kg dose given after 30 h. In (B) and (C), anti-F(ab) and anti-Fc Western blots corresponding to the SDS-PAGE analysis in (A). In (D) and (E), LABScreen and C1qScreen SAB analyses of samples collected before (predose) and 1 h after imlifidase treatment, with each bar representing the signal obtained for a specific HLA class I-presenting bead. IgG, immunoglobulin G; MFI, mean fluorescence intensity; SAB, single-antigen beads; scIgG, single-cleaved IgG; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.
induction, and platelets were counted on a VetScan HM5 (Abaxis).

RESULTS
ScIgG Produces a Similar Signal as Intact IgG in the LABScreen Assay But Is Undetectable by C1qScreen
To investigate the potential influence of scIgG on SAB assays, consecutive serum samples from an HLA-sensitized patient treated with a suboptimal dose (0.12 mg/kg; $C_{\text{max}}$ 2.1 µg/mL) of imlifidase were analyzed by SDS-PAGE and Western blot. All of the intact IgG in the pretreatment serum sample was cleaved to scIgG 1 h after the first dose and was further converted to F(ab$'$)2 and Fc fragments at 6 h postdose (Figure 1A–C). Pretreatment serum generated strong signals in both LABScreen and C1qScreen assays, with median MFI values >20 000. Despite the total elimination of intact IgG 1 h after imlifidase treatment (Figure 1B and C), the LABScreen signals were only slightly reduced in comparison with predose signals (Figure 1D). This indicated that HLA-binding scIgG is well-recognized by the Fc-specific detection antibody used in LABScreen. Importantly, even the very low levels of scIgG still present 2 d after treatment initiation and only very weakly detected by Fc-specific Western blot (Figure 1C; lane 2 d) gave a median MFI signal in the 2000–3000 range, with 4 individual beads being still above 6000 (data on file). In contrast, no signal was generated in the C1qScreen at any posttreatment time-point between 1 h (Figure 1E) and 2 d (data on file).

To confirm this observation, pretreatment serum was used to generate preparations with different proportions of

![FIGURE 2](image-url)

**FIGURE 2.** The impact of different imlifidase-generated anti-HLA fractions (intact IgG, scIgG, and F(ab$'$)$_2$) on SAB analyses. Different IgG fractions were generated by in vitro treatment of serum from an HLA-sensitized patient with PBS (sample a) or increasing concentrations of imlifidase (samples b–d). In (A), SDS-PAGE analysis, in (B) and (C), anti-F(ab$'$)- and anti-Fc-specific Western blots, respectively. In (D) and (F), the samples containing intact IgG (sample a) and mainly scIgG (sample b) were analyzed with LABScreen and C1qScreen, with each bar representing the signal obtained for a specific HLA class I-presenting bead. In (E) and (G), the LABScreen and C1qScreen data for all 4 samples are shown, with each box summarizing the signal obtained from all beads (median, interquartile range, and 10–90 percentile). IgG, immunoglobulin G; MFI, mean fluorescence intensity; SAB, single-antigen beads; scIgG, single-cleaved IgG; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.
intact IgG, scIgG, and F(ab′)2 (referred to as samples a–d in accordance with Figure 2A–C). When analyzing the fraction in which all intact IgG had been cleaved to scIgG, but with only a small proportion of the scIgG having been further converted to F(ab′)2 and Fc (sample b in Figure 2A–C) in the LABScreen assay, no reduction in signal was observed when compared with sample a (intact IgG) (Figure 2D and E). Although the MFI signal decreased somewhat for sample c, which contained mainly F(ab′)2 fragments with only a small amount of residual scIgG, the reduction was significant only for sample d, which contained only trace amounts of scIgG (Figure 2E). Although sample d contained only trace amounts of scIgG (Figure 2A–C), the signals from many SAB were still considerably higher than background (median MFI [interquartile range]: 1977 [72–4202]; Figure S1, SDC, http://links.lww.com/TP/C327). In contrast, sample b, which contained mainly scIgG (Figure 2A), was entirely negative in the C1qScreen SAB assay (Figure 2F–G). This result was consistent with the C1qScreen analysis of the clinical trial serum sample collected 1 h after imlifidase treatment (Figure 1E).

**ScIgG Retains a Certain Degree of Activity in the CDC-PRA Test**

As previously reported by Lorant et al, treatment serum from the HLA-sensitized patient was positive against 90% of T cells and 100% of B cells in a CDC-PRA panel (n = 30). This patient was treated with an imlifidase dose of 0.12 mg/kg, which was subsequently judged suboptimal based on the insufficient Cmax achieved in plasma (2.1 µg/mL to be compared with 5.8 µg/mL, which is the mean Cmax achieved at the optimal dose level of 0.25 mg/kg). Although significantly reduced, 14% and 38% T- and B-cell positivity still remained 24 h after dosing. At this time-point, intact IgG was no longer present, and scIgG was only very weakly detected by Western blot using anti-Fc detection (Figure 1C). To follow-up on these earlier findings and further investigate the clinical effect of scIgG, the samples generated from the pretreatment serum of the patient were tested against a mini panel of blood donor cells (n = 5). As shown in Figure 3, the serum fractions containing mainly F(ab′)2 fragments with either small (sample c) or trace (sample d) amounts of scIgG (Figure 2A–C), were negative against all donor T and B cells in the mini panel. In contrast, mock-treated serum containing intact IgG (sample a) gave maximum reactivity in all donors (Figure 3). The serum preparation containing a substantial amount of scIgG but no intact IgG (sample b in Figure 2A–C) remained positive against 4/5 and 5/5 of T and B cells, respectively, however, with CDC reactivity scores being reduced in 2/5 (T cell) and 4/5 (B cell) donors. Follow-up analysis of serially diluted preparations of intact IgG (sample a) and scIgG (sample b) revealed a clear difference in their intrinsic capacity to trigger complement-dependent cytotoxicity, with 2048- and 256-fold dilutions being required to turn the intact IgG and the scIgG preparations completely inactive in all donor T- and the B-cell tests. It should here be emphasized that the extremely high level of scIgG in sample b is artificial and unlikely to be obtained at imlifidase Cmax levels achieved at the selected clinical dose (0.25 mg/kg).

**C1q Binding Is Impaired But Not Abrogated for scIgG**

The CDC-PRA results indicated that the cytotoxic capacity of scIgG was clearly reduced though not eliminated. To further evaluate this, different preparations of rituximab (Figure S2, SDC, http://links.lww.com/TP/C327) were evaluated in a system with CD20-expressing Daudi cells using human serum as complement source.
In contrast to intact rituximab, single-cleaved and fully cleaved rituximab were both without detectable cytotoxic effect at 10 µg/mL (Figure 4A). When tested at 30 µg/mL, single-cleaved rituximab retained a certain capacity to bind C1q (Figure 4B), but even at this concentration, the binding was nonproductive and did not result in downstream complement activation (C4d deposition; Figure 4C) and cytotoxicity (7AAD; Figure 4D).

Because activation of complement by monoclonal antibodies depends on binding to a single antigen epitope, structural constraints related to epitope density, and geometry might influence the efficacy.16,17 For this reason, serum from an HLA-sensitized patient with known CDC activity toward HLA-expressing Raji cells was used as a source of polyclonal IgG in an additional system (Figure S3, SDC, http://links.lww.com/TP/C327). HLA-sensitized patient serum showed strong reactivity with Raji cells (Figure 5A) and intact HLA-reactive IgG from the same patient was a strong inducer of C1q-binding (Figure 5B), in contrast to control IgG. Although HLA-reactive scIgG had clearly reduced capacity to induce C1q deposition, a certain degree of residual activity was still present when using the polyclonal antibody-based detection of C1q (Figure 5B). In contrast, a comparative assessment using the C1qScreen kit reagent indicated that only intact HLA antibodies were able to fix C1q (Figure 5C). Regardless of this ambiguity, single-chain cleavage of HLA-reactive IgG abrogated its capacity to induce downstream C4d deposition (Figure 5D).

**ScIgG Retains a Certain Degree of FcγR-dependent Effector Function**

To investigate whether complement-independent effector functions were maintained in scIgG, different FcγR-dependent in vitro models were used. FcγR-binding activity of intact IgG and scIgG was shown by the retention of opsonized erythrocytes on a confluent layer of...
FIGURE 5. Complement activation by HLA-reactive IgG, scIgG, and F(ab’)2 in Raji cells. In (A), IgG-binding activity of HLA-reactive (patient) and nonsensitized serum (control) at different dilutions. In (B) and (C), C1q binding to Raji cells induced by different imlifidase-generated IgG fractions (Figure S3A, SDC, http://links.lww.com/TP/C327), detected by polyclonal anti-C1q F(ab’), antibody (B) and C1qScreen kit detection reagent (C). In (D), C4d deposition induced by the different imlifidase-generated IgG fractions. All analyses were performed on duplicate samples (n = 2). IgG, immunoglobulin G; MFI, mean fluorescence intensity; scIgG, single-cleaved IgG.

FIGURE 6. Complement-independent effector functions of imlifidase-generated antierythrocyte fractions. In (A), FcγR-dependent retention of erythrocytes to a confluent layer of THP-1 cells after opsonization with antierythrocyte IgG, scIgG, or F(ab’), in the absence of complement (n = 2). In (B), ADCC activity of THP-1 cells after opsonization of erythrocytes with the different antierythrocyte IgG fractions (n = 2–3). In (C), ADCP activity of THP-1 cells after opsonization with the different antierythrocyte IgG fractions. For more information on the IgG fractions used, see Figure S4, SDC, http://links.lww.com/TP/C327. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; IgG, immunoglobulin G; MFI, mean fluorescence intensity; scIgG, single-cleaved IgG.
THP-1 cells (Figure 6A), with intact IgG being effective already at 0.6 µg/mL, whereas 5 µg/mL scIgG was required for retention to occur. Regarding ADCC, antierythrocyte scIgG retained about 40% of the ADCC lysing activity at 5 µg/mL (Figure 6B). In line with this, 5 µg/mL antierythrocyte scIgG retained about 40% of the opsonizing activity of the intact IgG preparation leading to phagocytic uptake in the ADCP assay (Figure 6C).

The function of scIgG was also evaluated in 2 different antibody-mediated disease models in mice, experimental autoimmune hemolytic anemia (eAIHA), and experimental immune thrombocytopenic purpura (eITP), both considered largely FcγR-dependent.18 At the used concentrations, scIgG had no effect on erythrocyte number and spleen weight in eAIHA (Figure 7A–C) but retained a limited capacity to reduce platelet count in eITP (Figure 7D).

The Flow Cytometric Crossmatch Test Does Not Discriminate Between Intact IgG and scIgG

Clinical serum samples from a second patient treated with a suboptimal dose of imlifidase (0.12 mg/kg) were analyzed by SDS-PAGE, visualizing the stepwise cleavage of IgG into F(ab′)2, via the scIgG intermediate. Comparison of clinical assay data with the SDS-PAGE pattern showed how the different assays are affected by remaining scIgG (Figure 8). Importantly, the data demonstrated a clear influence of remaining scIgG on assays using anti-Fc detection, including the FCXM. Although the nonamplified CDC turned negative 6 h posttreatment, the FCXM remained positive even after 24 h. The patient was given a second dose (0.12 mg/kg) of imlifidase in accordance with the trial protocol (NCT02224820). This resulted in FCXM conversion after another 6 h, which enabled successful transplantation of the patient.

DISCUSSION

The development of crossmatch testing before transplantation has significantly impacted graft survival by allowing the detection of preformed antibodies, which would result in hyperacute or antibody-mediated rejection.19 These tests rely heavily on the determination of a donor-specific HLA antibody’s ability to elicit cytotoxic responses.20 In the setting of evaluating the success of a given desensitization regimen to enable an HLA-incompatible transplant, it
is imperative to have strong understanding of the clinical assays involved in determining a crossmatch conversion from positive to negative. The mechanism of action of imlifidase includes the generation of a sc IgG intermediate that may be indistinguishable from intact IgG when evaluated with assays using anti-Fc detection methods. This study concluded that when imlifidase is used in clinical practice, sc IgG is unlikely to be pathologically important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.21 In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.22,23 In our study, sc IgG demonstrated a detectable capacity to initiate Fc-dependent processes. Sc IgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, sc IgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (234LLGGP238 in IgG1) have, together with residues in the CH2 domain, been shown to be important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.21 In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.22,23 In our study, sc IgG demonstrated a detectable capacity to initiate Fc-dependent processes. Sc IgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, sc IgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (234LLGGP238 in IgG1) have, together with residues in the CH2 domain, been shown to be important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.21 In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.22,23 In our study, sc IgG demonstrated a detectable capacity to initiate Fc-dependent processes. Sc IgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, sc IgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (234LLGGP238 in IgG1) have, together with residues in the CH2 domain, been shown to be important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.21 In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.22,23 In our study, sc IgG demonstrated a detectable capacity to initiate Fc-dependent processes. Sc IgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, sc IgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (234LLGGP238 in IgG1) have, together with residues in the CH2 domain, been shown to be important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.21 In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.22,23 In our study, sc IgG demonstrated a detectable capacity to initiate Fc-dependent processes. Sc IgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, sc IgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (234LLGGP238 in IgG1) have, together with residues in the CH2 domain, been shown to be important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific sc IgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of sc IgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.
process of complement activation. Thus, a clinical decision to proceed to transplantation generally requires a negative crossmatch test involving donor cells.

The herein demonstrated impact of scIgG on the LABScreen assay has important implications for the most used crossmatch tests, that is, the antihuman globulin (AHG)-amplified CDCXM and the FCXM. These tests rely on anti-IgG (AHG) and anti-Fc antibodies (FCXM) in their respective detection steps. Consequently, the FCXM is unable to discriminate between intact IgG and scIgG (Figure 8), and the AHG-amplified CDCXM reacts to intact IgG and scIgG as well as F(ab′)2. It should be emphasized that in the AHG-amplified CDCXM, the amplifying antibody is likely to be the main contributor to complement activation. This means that even in cases of complete imlifidase-mediated conversion of anti-HLA IgG into F(ab′)2 and Fc fragments, there is a considerable risk that cell-bound anti-HLA F(ab′)2 may cause a false positive result. Therefore, the AHG-amplified CDCXM test needs to be interpreted with caution in patients treated with imlifidase.

As suggested by this study, postimlifidase transplant eligibility is best evaluated in a system considering the complement-activating capacity of scIgG without giving scIgG (or F(ab′)2) a disproportionate impact on assay outcome.

![FIGURE 9. Schematic illustration of the detection of intact anti-HLA IgG, anti-HLA scIgG, and anti-HLA F(ab′)2 fragments by an Fc-specific detection antibody (left panel) and by AHG (anti-IgG; middle panel). Explanation in right panel. AHG, anti-human globulin; IgG, immunoglobulin G; scIgG, single-cleaved IgG.](image)

| TABLE 1. | Influence of imlifidase-generated HLA-reactive scIgG and F(ab′)2 on different crossmatch tests |
|----------|-----------------------------------------------------------------------------------------------|
| **Assay for assessment of transplantation eligibility** | **Is the detection reagent compromised by the presence of HLA-reactive scIgG?** | **Is the detection reagent compromised by the presence of HLA-reactive F(ab′)2?** |
| CDCXM (nonamplified)a | No | No |
| AHG-amplified CDCXM | Yes | Yesb |
| FCXM | Yes | No |
| Anti-HLA SAB | Yes | No |
| C1q SAB | Noe | No |

*aIncludes no amplification step and thus provides a direct measure of the CDC activity of any IgG-derived component.  
bAHG with Fab′, reactivity will detect HLA-binding Fab′, without intrinsic capacity for complement activation.  
cWith reservation that the residual complement-activating effect of scIgG in vivo might be underestimated.

AHG, anti-human globulin; CDCXM, complement-dependent cytotoxicity crossmatch; FCXM, flow cytometric crossmatch; IgG, immunoglobulin G; SAB, single-antigen bead; scIgG, single-cleaved IgG.
CDCXM is considered to best match these requirements. Although it is the least sensitive of the currently used crossmatch tests, CDCXM provides a physiologically relevant assessment of transplant eligibility by measuring lytic activity on live donor cells in which several different HLAs can be simultaneously assessed at relevant levels and in which potential clustering/capping of antigen is allowed.

Additional information on posttreatment donor-specific antibodies levels might be obtained by the highly sensitive SAB assays. However, our data demonstrate that the LABscreen assay is likely to underestimate the clinical efficacy of imlifidase, because even minute amounts of scIgG cause a signal of similar strength as intact IgG. On the other hand, the C1qScreen may overestimate the clinical efficacy of imlifidase, because scIgG is entirely undetected.

In clinical practice, a combination of assays may be used for clinical decision-making and the pros and cons of each should be assessed.

Although AHG-amplified CDCXM is less compatible with transplant eligibility assessment after imlifidase treatment due to its recognition of both scIgG and F(ab’)2, results from other common methods that are based on anti-Fc detection, including FCXM and anti-HLA IgG SAB (eg, LABScreen), should also be interpreted with their limitations in mind. Although a positive posttreatment result in any of these assays is most often explained by low levels of residual scIgG, any signal above the predefined threshold for positivity will still require precautions, especially because the performance of FCXM tests is not standardized across different HLA laboratories and patient groups, including the use of prame, which was not investigated in this study.

In conclusion, advances in new therapies often require a close evaluation of these medications on current assays, laboratory tests, and personalized medicine in decision-making. In the context of crossmatch conversion in desensitization therapies, imlifidase-generated scIgG impacts the clinical assays used to assess transplant eligibility, and interpretation of these tests should be made with careful consideration of the timing of the imlifidase dose in relation to the crossmatch testing. Knowledge of the pharmacokinetics/pharmacodynamics of imlifidase in evaluating the process of intact IgG through scIgG and ultimately to fully cleaved IgG as well as the clinical impact of scIgG, potentially maintaining residual Fc-mediated effector functions, should be considered to facilitate clinical decision-making following desensitization with imlifidase.

REFERENCES

1. Jackson KR, Covarrubias K, Holscher CM, et al. The national landscape of deceased donor kidney transplantation for the highly sensitized: transplant rates, waitlist mortality, and posttransplant survival under KAS. Am J Transplant. 2019;19:1129–1138.
2. Stewart DE, Wilk AR, Klassen DK. KAS turns four: the state of deceased renal transplant candidates in the era of kidney paired donation and the new kidney allocation system: is there still a role for desensitization? Cln Transplant. 2019;33:e13751.
3. Vindelbro R, Spierry C, von Pawel-Rammingen U. Rapid IgG heavy chain cleavage by the streptococcal IgG endopeptidase IdeS is mediated by IdeS monomers and is not due to enzyme dimerization. Febs Lett. 2013;587:1818–1822.
4. von Pawel-Rammingen U, Johansson BP, Björck L. IdeS, a novel streptococcal cysteine protease with unique specificity for immunoglobulin G. EMBO J. 2002;21:1607–1615.
5. Hansa Biopharma AB. Annex I: summary of product characteristics. Available at https://www.ema.europa.eu/en/documents/product-information/idefix-epar-product-information_en.pdf. Accessed May 7, 2021.
6. Jordan SC, Lorant T, Choi J, et al. IgG endopeptidase in highly sensitized patients undergoing transplantation. N Engl J Med. 2017;377:442–453.
7. Lorant T, Bengtsson M, Eich T, et al. Safety, immunogenicity, pharmacokinetics, and efficacy of degradation of anti-HLA antibodies by IdeS (imlifidase) in chronic kidney disease patients. Am J Transplant. 2018;18:2752–2762.
8. Reutelingsperger CP, Petrone D, Nemeth JF, et al. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol. 2008;45:1837–1846.
9. Wahlberg J, Bengtsson M, Bergström C, et al. Impact of flow cytometry cross-matching results on the outcome of cadaveric kidney transplantation. Transplant Proc. 1994;26:1752–1753.
10. Bockermann et al. 2018;18:2752–2762.
11. Kushihata F, Watanabe J, Mulder A, et al. Human leukocyte antigen and human complement activation: role of IgG subclass, specificity, and cytotoxic potential. Transplantation. 2004;78:995–1001.
12. Sylvestre D, Clynes R, Ma M, et al. Immunoglobulin G-mediated inflammatory responses develop normally in complement-deficient mice. J Exp Med. 1996;184:2385–2392.
13. Tait BD, Süssal C, Gebel HM, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013;95:19–47.
14. Ge S, Chu M, Choi J, et al. Imlifidase inhibits HLA antibody-mediated NK cell activation and antibody-dependent cell-mediated cytotoxicity (ADCC) in vitro. Transplantation. 2020;104:1574–1579.
15. Boccalia et al. 2018;45:1837–1846.
16. Bockermann et al. 2019;33:e13751.
17. Bockermann et al. 2019;33:e13751.
18. Boccalia et al. 2019;33:e13751.
27. Guddat LW, Herron JN, Edmundson AB. Three-dimensional structure of a human immunoglobulin with a hinge deletion. *Proc Natl Acad Sci USA*. 1993;90:4271–4275.

28. Hezareh M, Hessell AJ, Jensen RC, et al. Effector function activities of a panel of mutants of a broadly neutralizing antibody against human immunodeficiency virus type 1. *J Virol*. 2001;75:12161–12168.

29. Radaev S, Motyka S, Fridman WH, et al. The structure of a human type III Fc gamma receptor in complex with Fc. *J Biol Chem*. 2001;276:16469–16477.

30. Radaev S, Sun PD. Recognition of IgG by Fc gamma receptor. The role of Fc glycosylation and the binding of peptide inhibitors. *J Biol Chem*. 2001;276:16478–16483.

31. Rayner LE, Hui GK, Gor J, et al. The solution structures of two human IgG1 antibodies show conformational stability and accommodate their C1q and FcR ligands. *J Biol Chem*. 2015;290:8420–8438.

32. Sondermann P, Huber R, Oosthuizen V, et al. The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIIa complex. *Nature*. 2000;406:267–273.

33. Wines BD, Powell MS, Parren PW, et al. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gammaRI and Fc gammaRIIA bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. *J Immunol*. 2000;164:5313–5318.

34. Dall’Acqua W, Simon AL, Mulkerrin MG, et al. Contribution of domain interface residues to the stability of antibody CH3 domain homodimers. *Biochemistry*. 1998;37:9266–9273.

35. Rispens T, Davies AM, Ooijevaar-de Heer P, et al. Dynamics of inter-heavy chain interactions in human immunoglobulin G (IgG) subclasses studied by kinetic Fab arm exchange. *J Biol Chem*. 2014;289:6098–6109.