γ-Globulins prepared from sera of multiparous women bind anti-HLA antibodies and inhibit an established in vivo human alloimmune response

John W. Semple, Michael Kim, Alan H. Lazarus, and John Freedman

It has previously been shown that sera from multiparous women have increased levels of anti-idiotypic antibodies specific for anti-HLA molecules. γ-Globulins prepared from these sera may be superior to commercial preparations of intravenous γ-globulin (IVIg) for inhibiting HLA alloimmunization. To test this, F(ab′)2 fragments prepared from either commercial IVlg or from the sera of men or multiparous women were coupled to CNBr-Sepharose and tested for their ability to bind F(ab′)2 fragments derived from polyspecific anti-HLA sera. As determined by flow cytometry, compared with columns coated with F(ab′)2 derived from commercial IVlg or sera from men, columns coated with F(ab′)2 prepared from the sera of multiparous women bound significantly more anti-HLA. In addition, intact IgG molecules prepared from the sera of multiparous women significantly neutralized the reactivity of the anti-HLA F(ab′)2 fragments. To determine whether the intact IgG molecules or their corresponding F(ab′)2 fragments could affect in vivo alloimmunity, they were tested for their ability to inhibit an established IgG human alloimmune response in humanized severe combined immunodeficient (SCID) mice. Compared with commercial IVlg, when intact IgG or F(ab′)2 fragments derived from multiparous women were administered to SCID mice making human anti-HLA antibodies, a significant reduction in anti-HLA reactivity was observed. The findings suggest that IgG molecules prepared from the sera of multiparous women have increased anti-idiotypic reactivity against anti-HLA antibodies, which can significantly inhibit an established human IgG alloimmune response in an Fc-independent manner. (Blood. 2002;100:1055-1059)

© 2002 by The American Society of Hematology

Introduction

Intravenous γ-globulin (IVlg) is widely used to treat patients with immunoregulatory disorders, particularly chronic autoimmune thrombocytopenic purpura (AITP).1-4 Although IVlg therapy has shown to have a benefit in raising platelet counts in autoimmune platelet disorders, it appears to be not efficacious in patients with platelet-induced HLA alloimmunization.5-8 The reasons for this are unclear but may relate to the nature of the immune response and the mechanisms of action of IVlg, that is, Fc receptor blockade/inhibition9,10 or anti-idiotypic regulation.10-12

One of the mechanisms by which the peripheral antibody repertoire is regulated is via the production of antibodies reactive with the variable regions of other antibodies, that is, anti-idiotypes.13-15 It has also been shown that the production and reactivity of anti-HLA antibodies are under the regulation of anti-idiotypic antibodies.16-18 Perhaps the most striking example of natural in vivo anti-idiotypic regulation of alloimmunization to HLA is that associated with pregnancy, both at the level of the fetus and of the mother. Phelan et al19 elegantly demonstrated that normal individuals contain anti-idiotypic antibodies to HLA molecules exquisitely specific for the HLA antigens encoded from the noninherited maternal allele (NIMA), but not paternal HLA alleles. Additionally, anti-HLA reactivity may be transient in patients with malignancy,20 and it has been shown that in these patients, as in pregnant women, anti-idiotypic antibodies may actively down-regulate anti-HLA antibodies.21-24

We speculated that pooling sera from multiparous women might increase the content of anti–HLA-specific anti-idiotypes to produce a more effective IVlg product for alloimmunized patients. We tested this by using affinity chromatography techniques combined with a humanized severe combined immunodeficiency (SCID) mouse model of alloimmunization. Our results indicate that, compared with commercial IVlg, IgG prepared from sera of multiparous women has higher anti-idiotypic binding capacity for anti-HLA and is superior to commercial IVlg or IgG prepared from men in inhibiting a secondary human alloimmune response.

Materials and methods

Sources and preparation of IgG

Three sources of IgG preparations were examined for anti–HLA-specific anti-idiotypic antibodies: (1) a commercial IVlg preparation (Immune Globulin Intravenous, 5%, Bayer, Etobicoke, ON, Canada), (2) the pooled sera of 34 never-transfused male volunteers (age range, 25-58 years), or (3) pooled sera from 47 multiparous women (age range, 27-47 years) who had their last pregnancy at least 1 year before blood sampling. Before pooling, anti-HLA reactivity in each serum was tested in a microlymphocytotoxicity (LCT) assay using a 30-cell panel of HLA typing cells (Canadian Blood Services, Toronto Center, Toronto, ON, Canada) and all were negative for anti-HLA antibodies. Equal amounts of serum from each...
individual were pooled. As a source of IgG anti-HLA antibodies, high-
titered polyclonal anti-HLA human sera were obtained from the Canadian
Blood Services (Dr B. Hannach, Toronto Center). For all 3 sources, IgG
molecules were prepared by precipitation with 50% saturated ammonium
sulfate followed by dialysis against 50 mM Tris-saline, pH 8.0. The IgG
molecules were further purified by adsorption on QAE-Sephadex A-50
(Pharmaclia, Mississauga, ON) to remove contaminating albumin.25

$F(ab')_2$ preparation

The $F(ab')_2$ fragments of the IgG molecules were prepared by standard
methods to differentiate between idiotypic andFc-mediated effects.25 Briefly, the IgG molecules (1%–3% wt/vol) were dialyzed against 0.2 M
sodium acetate, pH 4.5, and digested with 2% (wt/wt) pepsin (Sigma, St
Louis, MO) for 24 hours at 37°C. The $F(ab')_2$ fragments were then purified
by Sephadex G150 (Pharmacia) gel filtration and protein G-Sepharose
(Pharmacia) adsorption. Purity of the $F(ab')_2$ fragments was determined by
high-performance liquid chromatography (HPLC) analysis using a Beck-
man Gold HPLC with an Altex TSK-3000 size exclusion column (1.5 × 30
cm) equilibrated in 0.05 M NaPO4/0.1 M NaSO4, pH 6.75. The final $F(ab')_2$
purity was typically more than 96%.

Anti-HLA neutralization assay

The 10-fold serial dilutions (from $10^{-5}$ M) of intact IgG molecules from (1)
commercial IVIg, (2) men, or (3) multiparous women were mixed with an
equal volume (100 μL) of anti-HLA $F(ab')_2$ fragments ($10^{-5}$ M final
concentration) and incubated at 4°C for 18 hours. The mixtures were gently
resuspended and 100 μL of protein A–conjugated Sepharose beads
(Pharmacia) added for 45 minutes at 20°C to remove intact IgG. The beads
were then centrifuged for 2 minutes at 800g, proteins in the supernatants
quantified, and residual anti-HLA reactivity determined by flow cytometry.

Affinity chromatography

To study anti-idiotypic interactions, $F(ab')_2$ fragments derived from either
commercial IVIg, the sera of men, or multiparous women were covalently
coupled to CNBr Sepharose beads (Pharmacia) and 3.0-mL beads contain-
ing 60 mg coupled protein was poured into 1 × 8-cm glass chromatography
columns (Pharmacia). Each column was extensively characterized with
respect to baseline protein interactions. None of the columns could retain
reactivity of a 50-μg load of a goat antihuman Fc-specific polyclonal
antibody but did retain the reactivity from a 50-μg load of goat antihuman
H+L chain–specific antibody. Additionally, none of the columns retained a
1-mg load of human albumin. For anti-idiotypic binding studies, all the
columns were subjected to the same chromatographic protocol. One
milligram of anti-HLA $F(ab')_2$ fragments (in 1 mL) was loaded onto the
columns and allowed to continuously circulate at 0.5 mL/min for 18 hours
at 4°C using a pump-driven closed loop system.26 Columns were then
washed with 20 column volumes of running buffer and the unbound protein
collected. The columns were subsequently washed with 20 column volumes
of 0.2 M glycine, pH 2.8, to elute bound proteins, which were then
neutralized by adding 2 M Tris base (final concentration, 2.4% vol/vol). The
eluted $F(ab')_2$ fragments were concentrated by membrane filtration.
Both loaded and eluted proteins were made to the same concentration and
anti-HLA reactivity was determined by flow cytometry.

SCID mouse model of human alloimmunization

Female CB.17 SCID mice (6-8 weeks of age) were obtained from Jackson
Laboratories (Bar Harbor, ME) and human alloimmunization was induced
as previously described.26 Briefly, human peripheral blood mononuclear
cells (PBMCs) were obtained by 1.077 g/mL Percoll fractionation from a
female blood donor with a history of prior pregnancy and low levels of
circulating anti-HLA class I alloantibodies; she was blood group B+, HLA
A24/A34, B51/B62, Cw4, Bw4, Bw6 and had low but stable levels of
circulating anti-HLA-B7 alloantibodies. The SCID mice were first en-
graffed with $1 \times 10^7$ of the donor’s PBMCs and then challenged twice
weekly for 4 weeks with $10^7$ irradiated (2500 Gy) HLA A2/A2, Cw7+
PBMCs from healthy laboratory volunteers. Anti-HLA antibody develop-
ment was monitored weekly by flow cytometry. At the fourth week, if mice
developed anti-HLA, they were randomized to receive 1 g/kg intraperitone-
ally of either intact IgG or $F(ab')_2$ fragments derived from commercial IVIg
or from sera from men or from multiparous women, twice weekly for 4
weeks. These IgG dosages are similar to those used for human patients with
AITP.3 Serum anti-HLA reactivity after administration was compared with
preadministration anti-HLA levels (week 4 of challenge).

Flow cytometry

For detection and characterization of anti-HLA, serial dilutions of the
indicated test samples were incubated with 100 PBMCs from HLA-typed
individuals for 45 minutes at 20°C and washed once. Fluorescein isothiocya-
nate (FITC)-conjugated goat antihuman IgG (H+L chain– or Fc–specific,
Cedarlane Laboratories, Hornby, ON) was then added to the cells for 30
minutes at 20°C in the dark. Cells were analyzed by flow cytometry as
previously described23 using a FACSort flow cytometer (Becton Dickinson,
San Jose, CA) equipped with an argon laser operating at 15 mW; 10 000
events were acquired through an electronic cellular gate set on lymphocytes
based on forward and side scatter and were analyzed using LYSYS II
software (Becton Dickinson).

Statistical analysis

Significance between means ± SD of the flow cytometric data was
determined by Student unpaired t test for analysis of means.

Results

IgG derived from sera of multiparous women contains
increased anti–HLA-specific anti-idiotypes

The intact IgG preparations were tested for their ability to
neutralize the binding of anti-HLA antibodies. When titrations of
intact IgG molecules derived from (1) commercial IVIg or from sera
from (2) men or (3) multiparous women were incubated with
$F(ab')_2$ fragments of anti-HLA, the IgG from multiparous women
demonstrated significantly greater inhibition ($P < .01$) than was
seen with IgG from either commercial IVIg or from men (Figure 1).
To measure anti-HLA idiotypic binding, affinity columns coated
with $F(ab')_2$ fragments derived from commercial IVIg, men, or
multiparous women, were loaded with $F(ab')_2$ fragments made
from anti-HLA sera and, after elution, the column-bound proteins
were examined by flow cytometry. Compared with the anti-HLA

\[ \text{anti-HLA F(ab')2 molar ratio} = \frac{\text{anti-idiotypic binding}}{\text{intact IgG}} \]

\[ \text{MedChan} = \text{Mean ± SD} \]

\[ n = 6 \]

\[ P < .05 \]

\[ P < .01 \]

\[ \text{Figure 1. Inhibition of anti-HLA F(ab')2 fragments. Ability of intact IgG derived from} \]

\[ \text{commercial IVIg (C), sera from men (●), or sera from multiparous women (○)} \]

\[ \text{to inhibit reactivity of anti-HLA F(ab')2 fragments. Anti-HLA reactivity is expressed as} \]

\[ \text{median channel fluorescence (mean ± SD, n = 6) at the indicated intact IgG/anti-HLA} \]

\[ \text{F(ab')2 molar ratios. As controls, the anti-HLA reactivity of the 3 intact IgG} \]

\[ \text{preparations (x-axis = C) and of anti-HLA F(ab')2 fragments (x-axis = 0, ○} \]

\[ \text{incubated only with phosphate-buffered saline is shown. The stars indicate significance} \]

\[ (\star: P < .05, \star\star: P < .01) \text{ between data points for intact IgG from multiparous} \]

\[ \text{women versus intact IgG derived from commercial IVIg.} \]

From www.bloodjournal.org by guest on October 28, 2017. For personal use only.
IgGs of Multiparous Women Inhibit Alloimmunization

Figure 2. Flow cytometric histograms. Representative examples of flow cytometric histograms of the anti-HLA reactivities of 1 µg of (A) anti-HLA F(ab')2 fragments loaded onto affinity columns, (B) protein eluates from the affinity columns coated with F(ab')2 fragments from commercial IVIg (---) or from sera of men (-----), and (C) protein eluates from the affinity columns coated with F(ab')2 fragments derived from the sera of multiparous women.

Figure 3. Anti-HLA reactivity. Anti-HLA reactivity (mean ± SD) of the median channel fluorescence values (n = 5) of the anti-HLA F(ab')2 fragments loaded onto the affinity columns (·), and of bound proteins eluted from the affinity columns coated with F(ab')2 derived from (1) commercial IVIg (○), (2) sera from males (□), or (3) sera from multiparous women (●). The median channel fluorescence of phosphate-buffered saline only is shown (x-axis = 0 [●]). The stars indicate significance (*: P < .01) between data points comparing proteins eluted from the affinity columns coated with F(ab')2 derived from commercial IVIg versus from sera of multiparous women.

Figure 4. Anti-HLA alloimmune response in SCID mice. Development of human anti-HLA alloimmune response in SCID mice (n = 93) engrafted with PBMCs from an alloimmunized donor and challenged twice weekly for 4 weeks with allogeneic PBMCs. Data are expressed as the median channel fluorescence (mean ± SD) of SCID mouse sera at a 1:100 dilution.

Discussion

Therapy with IVIg is effective in treating immunodeficiency states, bacterial/viral infections, and immunoregulatory disorders, particularly immunohematologic disorders such as autoimmune thrombocytopenia, autoimmune neutropenia, and autoimmune hemolytic anemia.1-3,7 Although the mechanisms of action of IVIg in immune regulation are complex and not yet fully elucidated, several theories have been postulated. In autoimmune thrombocytopenic disorders, for example, several experimentally supported theories of the mechanism of action of IVIg have been proposed. These include reticuloendothelial Fc receptor blockade,4 down-regulation of FcγRIIIa via FcγRIIb,5 anti-idiotypic regulation,6,10,12 and cytokine alterations.5 In contrast to the recognized efficacy of IVIg therapy in autoimmune disorders, there is controversy regarding its benefit in transfusion-induced HLA alloimmunization.5,9 Although several investigators have demonstrated that commercial IVIg preparations can inhibit anti-HLA in vitro,28-30 the inhibition has been incomplete and may be the result of absence of the necessary...
anti–HLA-specific anti-idiotypes in commercial IVIg.36,37 Our results indicate that, compared with commercial IVIg or IgG prepared from the sera of men, the IgG derived from multiparous women has higher anti-idiotypic binding capacity for anti-HLA and can significantly inhibit an established human anti-HLA immune response in humanized SCID mice. Overall, these results support the hypothesis that IgG molecules prepared from multiparous women may be an effective γ-globulin product for the treatment of alloimmune platelet disorders.

Compared with the commercial IVIg or IgG derived from men, intact IgG molecules derived from multiparous women bound and neutralized significantly more anti-HLA F(ab')2 fragments (Figure 1). Furthermore, compared with F(ab')2-coupled affinity columns derived from either commercial IVIg or the sera from men, affinity columns coated with F(ab')2 fragments from the sera of multiparous women bound significantly more anti-HLA in an idiotypic-dependent fashion (Figures 2 and 3). It is, however, important to note that IgG prepared from commercial IVIg or male donors was prepared in an identical fashion and used at the same concentration as the IgG derived from multiparous women; nonetheless, bound anti-HLA reactivity was different. The fact that IgG from a relatively small pool of male donors (n = 34) behaved similarly to the commercial IVIg (pooled from thousands of donors) suggests that pool size is not likely the reason for the lower anti-HLA binding capabilities (eg, due to dilution of anti-idiotypes on large-scale pooling). However, whether increasing the pool size of sera from multiparous women will change their anti-idiotypic binding patterns toward anti-HLA has not yet been established; we are currently studying this.

The probable explanation for higher anti-HLA idiotype reactivity in the IgG preparations from multiparous women is likely because of the women’s prior multiple exposures to paternal HLA antigens during pregnancy, resulting in anti-HLA alloimmunization and subsequent development of cross-reactive anti-HLA idiotypes. This contention is supported, in part at least, by the observation that despite exposures to paternal HLA antigens, none of the sera collected after at least 1 year from the last pregnancy contained anti-HLA reactivity. Similarly, 94% of 109 multiparous women (different from those used in this study) screened by LCT had no detectable anti-HLA reactivity (B. Hannach, Canadian Blood Services, Toronto Center, personal communication). The need to screen the sera of multiparous women for anti-HLA reactivity before being used as a source of IgG production may be important because the presence of these antibodies may, when transfused, lead to the development of severe side effects such as transfusion-related acute lung injury (TRALI).38 For example, it was recently demonstrated that administration of plasma from multiparous women to patients in intensive care units had a higher incidence of TRALI reactions compared to patients receiving control plasma units,39 although donor plasma anti-HLA reactivity was not specifically determined.

To determine whether the IgG purified from the sera of multiparous women could inhibit an established human alloimmune response, we used a SCID mouse model of human alloimmunization.26 When the F(ab')2 preparations were administered to SCID mice already anti-HLA alloimmunized, F(ab')2 preparations derived from the sera of multiparous women significantly inhibited SCID mouse serum anti-HLA reactivity. This supports the conclusions based on the affinity chromatography experiments that sera from multiparous women contain more anti–HLA-specific anti-idiotypes and suggests that these anti-idiotypes can more effectively inhibit a human anti-HLA response than does commercial IVIg. The time kinetics of in vivo HLA alloimmunization within the first week after the initial F(ab')2 administration have not been determined; however, the data are in agreement with those reported in several transplant studies showing that anti–HLA-specific anti-idiotypes are correlated with lower alloimmunization rates.16–18

Intact IgG preparations from multiparous women or commercial IVIg caused a greater inhibition of anti-HLA reactivity in the SCID mice than did their corresponding F(ab')2 fragments (Figure 5). Although this may suggest that the presence of the Fc region has an additive effect to anti-idiotypic regulation in reducing HLA alloimmunization, it could not be ruled out that the differences may be also due to different in vivo half-lives of intact IgG and F(ab')2 fragments in this mouse model.

Studies in several animal models have recently shown that the mechanism of IVIg in the reversal of either autoimmune- or xenoimmune-mediated thrombocytopenia is primarily due to Fc-dependent inhibition of the reticuloendothelial system.9,40,41 Our results suggest that, with respect to HLA alloimmunization, an IgG product can be produced from the sera of multiparous women that mediates its effects via anti-idiotypic interactions. These apparent discrepant results underscore that γ-globulins may have multiple mechanisms of action, which may be reflected by differing methodologies or type of immune phenomenon studied.
In summary, sera from multiparous women have an increased content of anti-idiotypic antibodies specific for anti-HLA alloantibodies. These purified anti-idiotypic antibodies can significantly inhibit an established IgG anti-HLA immune response in a humanized SCID mouse model. The results suggest a new and relatively simple approach to producing a superior γ-globulin product for the treatment of platelet alloimmunization.

Acknowledgment

The authors would like to thank Mr Andrew R. Crow (Senior Research Assistant, Canadian Blood Service) for his excellent technical assistance.

References

31. Tyan DB, Li VA, Czer L, Trento A, Jordan SC. In- travenous immunoglobulin G (IVIg) for high- titer anti-HLA alloantibody in highly sensitized transplant candidates and transplant with a histocompatible or- gan. Transplantation. 1994;57:553-559.
37. Mahoney RJ, Breggia AE. Inhibition of HLA anti- body cytotoxicity by intravenous immunoglobulin G Fab (a) dimers, monomers, and monovalent Fab (ab). Hum Immunol. 1999:60:492-498.
γ-Globulins prepared from sera of multiparous women bind anti-HLA antibodies and inhibit an established in vivo human alloimmune response

John W. Semple, Michael Kim, Alan H. Lazarus and John Freedman