In vivo Administration of Intravenous Immunoglobulin (IVIg) can Lead to Enhanced Erythrocyte Sequestration

H. Kessary-Shoham¹, Y. Levy², Y. Shoenfeld², M. Lorber³ and H. Gershon¹

¹Department of Immunology, Rappaport Faculty of Medicine, Technion, Haifa, Israel
²Department of Medicine B, Research Unit of Autoimmune Disease, Sackler Faculty of Medicine, Tel Aviv University, Sheba Medical Center, Tel Hashomer, Tel Aviv, Israel
³Institute for Immunology, Allergy and AIDS, Rambam Hospital, Haifa, Israel

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Enhanced erythrocyte sequestration is one of the very few major adverse effects of intravenous immunoglobulin (IVIg). IVIg contains high molecular weight IgG complexes (~300 kDa) which, in the presence of serum, mimic immune complexes by activating complement, binding to CR1 of red blood cells (RBC) (CD35) and mediating erythrophagocytosis. Four of seven patients undergoing IVIg therapy showed significant drops in haematocrit and haemoglobin that were not due to isoantibodies in the IVIg. Prior to treatment, patients' RBC carried IgG and complement (C₃d) that were not bound as immune complexes via CR1 (CD35). The patients whose RBC bound immune complex-like moieties and showed drops in haematocrit and haemoglobin subsequent to IVIg were young adults (22–35 years); older patients (50–69 years) showed no ill effects. In the presence of complement, RBC of young patients bound IVIg complexes in vitro while those of older patients did not. It is not the absolute levels of erythrocyte-associated IgG or C₃ fragments, neither pre- nor post-therapy, which are predictive of IVIg associated decreases in haematocrit and haemoglobin levels. Patient age and RBC inability to bind the IVIg immune complex-like moieties in vitro both appear to be predictors of resistance to sequestration after in vivo treatment with IVIg.

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Introduction

We have recently determined that immunoglobulin for intravenous use (IVIg) contains high molecular weight moieties that can activate complement and bind to erythrocytes via their complement receptor, CR1 (CD35) [1]. This binding to erythrocytes can mediate their sequestration in vitro [1]. These high molecular weight moieties thus behave like complement-bearing immune complexes which can attach to red blood cells (RBC) and mediate complement dependent erythrophagocytosis (‘innocent bystander’ sequestration) [2, 3]. Erythrocyte CR1 (CD35), with a high affinity for immune complex-bound complement (C)₃b, is the major carrier of complement-bearing immune complexes in the circulation. CR1 also functions as a cofactor for the protease factor I which cleaves C₃b to C₃bi and its subsequent degradation products, C₃c and C₃dg [4]. The lower affinity of CR1 for C₃bi results in the release of the complex from the RBC [5]. Under dynamic conditions in the circulation, erythrophagocytosis due to ‘innocent bystander’ sequestration will be dependent upon the levels of RBC-bound opsonins (IgG, C₃b, C₃bi, C₃dg) at the time of exposure of the erythrocyte to splenic and hepatic macrophages.

Immunoglobulin for intravenous use (IVIg) is widely used as a safe and effective treatment for immunodeficiencies as well as autoimmune and other immunologically mediated diseases [6]. There have, however, been reports of enhanced [7], albeit subclinical, erythrocyte sequestration in healthy individuals [8] and augmented RBC turnover in idiopathic thrombocytopenic purpura (ITP) [8] and anaemic patients [9], and in rare cases, evidence has been presented for the onset of hemolytic anemia subsequent to IVIg treatment [9–12].

This study was undertaken to investigate the effect of IVIg on erythrocyte sequestration and to determine whether the immune complex-like moieties found in IVIg bind to erythrocytes in vivo as they do in vitro [1]. For this purpose, we examined the erythrocytes of patients undergoing treatment with IVIg for the binding of IgG and C₃ fragments and correlated this binding with enhanced sequestration of erythrocytes subsequent to the treatment.
Materials and Methods

Blood donors

Erythrocytes from seven patients undergoing treatment with IVIg and from 10 healthy controls were obtained from venous blood (2 ml) taken in heparin under sterile conditions and used immediately. Healthy controls were of both sexes and either 22–40 years old (young controls) or 70–75 years old (elderly controls). Patient ‘P1’ was Coombs- negative with pemphigus vulgaris. Patient ‘P2’ was treated for scleroderma. Patient ‘P3’ was Coombs’-positive with systemic lupus erythematosus (SLE), ITP and autoimmune haemolytic anaemia after splenectomy. Patient ‘P4’ was treated for acute exacerbation of SLE, Coombs’-positive anaemia and glomerulonephritis. Patient ‘P5’ was Coombs’-positive with SLE, anti-phospholipid antibodies, autoimmune haemolysis and arthritis. Patient ‘P6’ was treated for SLE, anti-phospholipid antibodies, Coombs’-positive anaemia, fever and skin involvement. Patient ‘P7’ was treated for rheumatoid arthritis. Blood type, age and sex of patients can be found in Table 1. Haematocrit and hemoglobin levels of each patient were determined prior to administration of IVIg and within 2 h of the final treatment. The additional volume of fluid administered with the IVIg in the final treatment was taken into consideration when determining the expected haematocrit and haemoglobin values subsequent to treatment.

Exp=expected results; obs=observed results. Bold data demonstrate patients with observed haematocrit and/or haemoglobin levels after IVIg which were lower than expected.

### Table 1. Haematocrit and haemoglobin levels prior to initiation of IVIg therapy and subsequent to final IVIg treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex (F/M)</th>
<th>Blood group</th>
<th>Haematocrit Prior to IVIg</th>
<th>Post-IVIg (exp)</th>
<th>Post-IVIg (obs)</th>
<th>Haemoglobin Prior to IVIg</th>
<th>Post-IVIg (exp)</th>
<th>Post-IVIg (obs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>35</td>
<td>M</td>
<td>B+</td>
<td>49</td>
<td>46.6</td>
<td>44</td>
<td>15.6</td>
<td>14.8</td>
<td>14.4</td>
</tr>
<tr>
<td>P2</td>
<td>50</td>
<td>F</td>
<td>AB+</td>
<td>41</td>
<td>39</td>
<td>40</td>
<td>12.5</td>
<td>11.9</td>
<td>12.3</td>
</tr>
<tr>
<td>P3</td>
<td>65</td>
<td>F</td>
<td>A+</td>
<td>31</td>
<td>30</td>
<td>31</td>
<td>10</td>
<td>9.5</td>
<td>10.1</td>
</tr>
<tr>
<td>P4</td>
<td>22</td>
<td>F</td>
<td>O+</td>
<td>30.3</td>
<td>28.8</td>
<td>27</td>
<td>10.2</td>
<td>9.7</td>
<td>8.7</td>
</tr>
<tr>
<td>P5</td>
<td>30</td>
<td>F</td>
<td>B+</td>
<td>34.6</td>
<td>32.9</td>
<td>28.8</td>
<td>11.7</td>
<td>11.1</td>
<td>10.1</td>
</tr>
<tr>
<td>P6</td>
<td>27</td>
<td>F</td>
<td>A+</td>
<td>31</td>
<td>29.5</td>
<td>25</td>
<td>9</td>
<td>8.6</td>
<td>7</td>
</tr>
<tr>
<td>P7</td>
<td>67</td>
<td>F</td>
<td>A+</td>
<td>33.5</td>
<td>32.3</td>
<td>31.3</td>
<td>10.7</td>
<td>10.3</td>
<td>10.4</td>
</tr>
</tbody>
</table>

Erythrocytes (RBC)

Leukocyte and platelet-free RBC [13] were prepared as previously described [2].

Intravenous Immunoglobulin (IVIg)

Isiven VI. (Instituto Sierovaccinogeno Italiano, S. Antimo, Italy) was used for all treatments and experimentation. Patients received a total of 2 gm IVIg/kg body weight divided equally between 2 to 5 consecutive daily treatments. IVIg alleviated clinical symptoms of all patients but P4.

Complement (C)

AB serum pooled from at least six donors was stored in aliquots at −70°C and used as a source of complement.

Immune complexes (IC)

Soluble tetanus–anti-tetanus immune complexes were prepared as previously described from human antiserum consisting primarily of IgG antibody to tetanus toxoid [2]. Tetanus toxoid was a generous gift from L. Grundman, Rafa Laboratories, Jerusalem, Israel.

Sephacryl S-200 HR

IVIg was separated according to molecular weight on Sephacryl S-200 HR columns (Pharmacia, Uppsala, Sweden) in 0.05 M Tris-HCl, pH 7 as previously described [1]. Fractions were collected and assayed for protein concentration by the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). Molecular weight markers (Kit MW-GF-1000, Sigma Chemical Company, St. Louis, MO, USA) were bovine serum albumin (MW 66,000), yeast alcohol dehydrogenase (MW 150,000), sweet potato β-amylase (MW 200,000) and horse spleen apoferritin (MW 443,000).

Factor I activity

Pooled AB serum was diluted in Veronal buffer, pH 7.2, to a final EDTA concentration of 10 mM and used as a source of factor I activity as previously described [1, 3].
Treatment of erythrocytes with IVlg or immune complexes with or without complement

Erythrocytes were incubated at 37°C for 20 min in veronal buffered saline (VBS) in the presence or absence of AB serum as a source of complement with various concentrations of IVlg, tetanus-anti-tetanus immune complexes, or IVlg fractions derived from a Sephacryl S-200 HR column. Aliquots of IVlg or immune complex and complement-treated erythrocytes were exposed to factor I activity by washing with VBS containing 10 mM EDTA, resuspension in pooled AB serum diluted to a final EDTA concentration of 10 mM and incubation for 90 min at 37°C. The erythrocytes were then washed with cold PBS and analysed by flow cytometry for the binding and release of IgG, C3c and C3d. Aliquots of these RBC were also examined in a phagocytosis assay.

Flow cytometry

RBC were prepared for flow cytometry as previously described [2]. In brief, IgG on RBC was detected by rabbit anti-human IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) followed by biotinylated goat anti-rabbit IgG (Sigma) and then ExtrAvidin FITC (Sigma). C3c and C3d were detected by rat IgG2a monoclonal antibodies (mAb) (clone 4 and clone 3 respectively) (generous gifts from PJ. Lachmann, Cambridge, England), followed by FITC-labelled mouse anti-rat IgG (Jackson ImmunoResearch Laboratories) [14]. Control staining was performed with species and isotype matched non-specific antibodies. Subsequent to staining, erythrocytes were rewarshed, suspended in PBS and examined by FACScan (Becton Dickinson, Mountain View, CA, USA). Light scatter was used to differentiate erythrocytes from particulate debris. Fluorescence was measured by an argon laser using a 530 nm filter. Net mean fluorescence (NMF) in experimental samples was determined by subtraction of the fluorescence of cells stained with second antibody alone from the mean fluorescence of specifically stained cells. The staining of cells with second antibody alone did not differ from that obtained with species and isotype matched non-specific antibodies.

Phagocytosis assay

This assay was performed as published by Sheiban and Gershon [15]. In brief, human peripheral blood monocytes were incubated for 48 h in a lymphokine-rich supernatant obtained from Con-A-stimulated human non-adherent peripheral blood mononuclear cells. Monocytes were then washed and distributed to round bottom microtiter wells in RPMI-1640, 1% glutamine, 1% non-essential amino-acids, 1% Na-pyruvate, penicillin and streptomycin plus 10% heat inactivated fetal calf serum containing 3.5 mg/ml glucose. Cultures were incubated for 5–6 h at 37°C, centrifuged at 800×g, the supernatant removed, and the cells fixed with 0.025% glutaraldehyde in PBS. The residual number of RBC in wells with and without phagocytosis was counted in a haemocytometer. Percent phagocytosis was calculated as follows:

\[
\frac{\text{residual RBC (no monocytes) - residual RBC (incubated with monocytes)}}{\text{residual RBC (no monocytes)}} \times 100
\]

Table 2. Erythrocytes (O+) exposed to IVlg or immune complexes in the presence of complement bind IgG, C3c and C3d and become susceptible to erythrophagocytosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net mean fluorescence (RBC)</th>
<th>Phagocytosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVlg</td>
<td>16±4</td>
<td>0±1</td>
</tr>
<tr>
<td>C'</td>
<td>3±3</td>
<td>1±1</td>
</tr>
<tr>
<td>3±5</td>
<td>2±3</td>
<td>2±1</td>
</tr>
<tr>
<td>IVlg+C'</td>
<td>10±2</td>
<td>9±2</td>
</tr>
<tr>
<td>IC+C'</td>
<td>56±17</td>
<td>10±2</td>
</tr>
</tbody>
</table>

IVlg = intravenous immunoglobulin; C' = pooled AB serum as a source of complement; IC = tetanus-anti-tetanus immune complexes.
Results are the average±SEM of five donors.

Results

In vitro exposure of O+ erythrocytes in the presence of complement to IVlg at concentrations (10 mg/ml) reached in the serum of patients after treatment with high doses of IVlg (≥ 500 mg/kg) leads to the binding of IgG, C3c and C3d with concomitant susceptibility to phagocytosis (Table 2). Binding of immunoglobulin and complement and susceptibility to phagocytosis are similar to that observed when these same erythrocytes are exposed to complement-bearing immune complexes (IC+C') but not to complement (C') or immune complexes (IC) alone. These experiments suggest that within the IVlg there are immune complex-like forms which can, in the presence of complement, bind to erythrocyte CR1 and serve as opsonins for the mediation of phagocytosis.

We have previously shown that sandoglobulin IVlg contains both monomeric and higher molecular weight (~dimers) IgG [1]. In the present study, we used isiven IVlg for the in vivo treatment of patients. Sephacryl S-200 HR fractionation of isiven IVlg confirms that this IVlg source consists of 83% of the protein in the form of IgG monomers (fraction II) of molecular weight (MW) 150 kDa and 9% of the IgG (fraction I) with the approximate MW of an IgG dimer (~300 kDa) (Figure 1). In the presence of complement, it is the ‘dimeric’ fraction I that mimics the ability of immune complexes to bind opsonins (IgG, C3b [C3c, C3dg]) to erythrocytes (Table 3).

The question still remained as to whether in vivo exposure to IVlg could lead to the binding of immune complex-like moieties to erythrocyte CR1 (CD35) and thus to the binding of the opsonins IgG and C3b and...
enhanced sequestration. To address this point, we examined a series of patients undergoing IVIg therapy in order to evaluate the possible effects of IVIg on erythrocyte sequestration. Haematocrit and haemoglobin levels of these patients were examined prior to the initiation of the first IVIg treatment and immediately after the termination of the last treatment (Table 3). Expected values were computed with the presumption that none of the diluent volume of the IVIg had yet appeared in the urine. Since the actual volume of urine made in this interval is unknown, it is probable that the true expected values were even higher and closer to the initial pretreatment values.

Four of the seven patients examined (P1, P4, P5 and P6) showed marked RBC sequestration subsequent to IVIg treatment while patients P2, P3 and P7 showed little or no effect of IVIg on erythrocyte sequestration as determined by haematocrit and haemoglobin. Although we have been able to detect significant levels of IgG anti-A and B blood group antibodies in various preparations of IVIg [16], they do not appear to play a role in the in vivo sequestration of erythrocytes in these patients, since patients P2, P3 and P7 with blood groups AB and A did not demonstrate reduced haematocrit or haemoglobin levels after treatment.

To determine whether in vivo exposure to IVIg indeed leads to the binding of immune complex-like moieties to erythrocyte CR1, we examined erythrocytes from our patients undergoing IVIg therapy for bound IgG and C3d prior to the initiation of the first IVIg treatment and subsequent to the termination of the last treatment. RBC samples from patients undergoing IVIg therapy were either prepared directly for analysis of bound IgG and C3d or exposed to pooled AB serum containing 10 mM EDTA, as a source of complement (C). RBC were prepared for the flow cytometric detection of membrane bound IgG, C3c or C3d. Specific activity was calculated by dividing the net mean fluorescence (NMF) of each sample of erythrocytes by the amount of protein to which they were exposed in the appropriate Sephacryl fraction. Results are from a representative experiment of four performed.

<table>
<thead>
<tr>
<th>Erythrocytes exposed to:</th>
<th>Net mean fluorescence/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Fraction I+C′</td>
<td>763.0</td>
</tr>
<tr>
<td>Fraction II+C′</td>
<td>78.1</td>
</tr>
</tbody>
</table>

O′ RBC were treated with the IVIg Sephacryl fractions I (MW ≥300 kDa) or II (MW ~150 kDa) in the presence of pooled AB serum as a source of complement (C). RBC were prepared for the flow cytometric detection of membrane bound IgG, C3c or C3d. Specific activity was calculated by dividing the net mean fluorescence (NMF) of each sample of erythrocytes by the amount of protein to which they were exposed in the appropriate Sephacryl fraction. Results are from a representative experiment of four performed.

Figure 1. ISIVEN IVIg contains 150 kDa and ≥300 kDa forms of IgG. ISIVEN IVIg was separated on Sephacryl S-200 HR. Molecular weight markers ranged from 66 to 443 kDa. Fractions were collected and assayed for protein concentration by the Bio-Rad protein assay. Protein concentration is expressed in arbitrary units. The fractions under peaks marked I and II were pooled for subsequent analysis. The results are the average of two separations performed.

Table 3. The immune complex-like forms in ISIVEN IVIg that activate complement and bind to erythrocytes are found in the higher molecular weight (≥300 kDa) Sephacryl S-200 HR fraction of IVIg (fraction I).

Figure 2. Immune complex-like moieties bind in vivo to RBC of patients treated with IVIg (ISIVEN). Erythrocytes of patients were obtained prior to initial administration of IVIg [pretreatment I(□)] and within 2 h of the last treatment [post-treatment III(■)]. Aliquots of all RBC were exposed to pooled AB serum containing 10 mM EDTA as a source of factor I [pretreatment I/EDTA (■)], post-treatment III/EDTA (■)]. All cells were stained for IgG and C3d and analysed by flow cytometer. Results are expressed as net mean fluorescence.
factor I, to allow for the cleavage of C'3b and the release of C'3c immune complexes, provided such were bound to CR1 (CD35), or the release of C'3c alone, leaving C'3dg bound to RBC (provided the binding was mediated by RBC specific immunoglobulin) [3]. Prior to IVIg treatment, most patients had well detected levels of IgG and C'3 breakdown products (C'3d) bound to their RBC (Figure 2). These opsonins were not bound to the erythrocyte as immune complexes via CR1 (CD35) as demonstrated by their resistance to removal with EDTA/serum [3, 5]. Subsequent to IVIg treatment, complex-like moieties were found bound to the erythrocytes of patients P1, P4, P5 and P6, while the erythrocytes of patients P2, P3 and P7 bound low levels of immune complexes, if at all. This was demonstrated by the minimal removal of C'3d in the presence of EDTA/serum. Erythrocytes, taken before IVIg therapy, of those patients who demonstrated enhanced binding of immune complex-like moieties during IVIg therapy and enhanced sequestration also bound significant levels of IgG +C'3 fragments when exposed to IVIg and complement in vitro (Figure 3). Subsequent exposure of these erythrocytes to factor I activity led to the

Figure 3. RBC from patients susceptible to IVIg-mediated haemolysis bind IVIg immune complexes in vitro while RBC from patients resistant to haemolysis do not bind IVIg immune complexes. Erythrocytes from patients were incubated in vitro with 10 mg/ml IVIg (preadsorbed on A+ or B+ RBC to remove isoantibodies) in the presence or absence of pooled AB serum as a source of complement. Aliquots of these erythrocytes and those not exposed to IVIg were then exposed to pooled AB serum containing 10 mM EDTA as a source of factor I. All erythrocytes were then stained for IgG or C'3d and analysed by a flow cytometer. Results are presented as net mean fluorescence. Typical results of patients susceptible to IVIg-mediated haemolysis are represented by patient P5. Typical results of patients resistant to IVIg-mediated haemolysis are represented by patient P7. Results show background (—), and levels of IgG and C'3d as detected in situ (· · ·), IVIg +C' (---), IVIg +C' +EDTA (-- --).
shocking of IgG from these erythrocytes. Upon exposure to IVIg in the presence of complement, erythrocytes of those patients who did not demonstrate enhanced binding of immune complex-like moieties during IVIg therapy and did not suffer from enhanced sequestration, bound minimal amounts of IgG in the form of immune complexes. This minor amount of complexes was released from the erythrocytes upon exposure to factor I activity. It should be noted that the patients that showed this adverse effect of IVIg (patients P1, P4, P5, P6) were all under 35 years of age, while those patients 50 years of age or older (P2, P3, P7) did not show this ill effect.

**Discussion**

IVIg is considered a relatively safe and effective reagent for the treatment of various immunologically mediated diseases [6, 7]. IVIg has not been found equally effectual, however, in the treatment of all autoimmune diseases. Autoimmune haemolytic anaemia is a conspicuous example of a disorder in which major difficulty in treatment with IVIg has been encountered. There have reports of successful treatment [17], others of little or no efficacy [8, 18] and many of detrimental effects [9–12; 19–21]. It has even been reported that haemolytic anaemia can develop subsequent to IVIg treatment [9, 12]. IVIg treatment has also been reported to lead to enhanced erythrocyte sequestration in patients [8] and in healthy individuals [8].

In investigating the possible relationship between IVIg therapy and enhanced erythrocyte sequestration, we have demonstrated the presence of IgG of molecular weight of a dimer or greater (≥ 300 kDa) in IVIg from various manufacturers (Figure 1) [1], which in the presence of serum can mimic immune complexes and bind to erythrocytes via CR1 [1]. In previous studies, we have shown that complement-bearing immune complexes bound to erythrocyte CR1 can mediate erythrocyte sequestration in vitro [2, 3]. Similar ‘innocent bystander’ sequestration was shown to occur when erythrocytes are exposed to IVIg and complement in vitro [1].

In the present report, we studied patients undergoing IVIg therapy to determine whether the immune complex-like moieties in IVIg indeed bind to erythrocytes in vitro and serve as oposins in the mediation of erythrocyte sequestration. Enhanced sequestration was observed in four of seven patients, as demonstrated by significant drops in haematocrit and haemoglobin levels. Those patients who suffered from enhanced erythrocyte sequestration after in vivo treatment were all young adults (22–35 years of age) whose erythrocytes demonstrated the ability to bind IVIg immune complex-like moieties in vitro (Figure 2) and in vitro (Figure 3). Those patients who did not demonstrate enhanced erythrocyte sequestration after in vivo treatment were the three oldest patients in our study (50–69 years). These findings correlate with previous reports of ours which demonstrate that erythrocytes of elderly individuals are less competent to bind immune complexes via CR1 (CD35) than are those of young individuals [2, 3].

Our findings suggest that neither pre- nor post-therapy levels of erythrocyte-associated IgG or C3 fragments are predictive of hemolysis subsequent to IVIg treatment. They imply that the presence of immune complex-like moieties bound to erythrocyte CR1 (CD35) after IVIg treatment is correlated with in vivo haemolysis. The ability to bind immune complex-like moieties is more pronounced on erythrocytes of young individuals than on those of older individuals. Therefore, patient age and inability to bind the immune complex-like moieties in IVIg in vitro both appear to be predictors of resistance to haemolysis after in vivo treatment with IVIg.

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