A detailed examination of the antibody prevalence and characteristics of anti-ESA antibodies

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Abstract

Background. The antibody characteristics in erythropoiesis-stimulating agent (ESA)-treated patients who develop antibody-mediated pure red cell aplasia (PRCA; amPRCA) can be described as high-affinity, neutralizing anti-ESA antibodies with a mixed immunoglobulin G (IgG) subclass. The characteristics of an early-onset anti-ESA antibody response are not well documented, especially in the months prior to the development of amPRCA. Therefore, a detailed characterization of anti-ESA antibodies was performed in patients in both clinical studies and in a post-market setting. Both baseline and post-dose samples were tested and antibody-positive samples were characterized. Antibody characteristics such as concentration, isotype and specificity were evaluated in subjects with non-neutralizing anti-ESA antibodies and subjects that developed neutralizing anti-ESA antibodies associated with amPRCA.

Methods. Serum samples were analyzed for the presence of anti-ESA antibodies, using a validated surface plasmon resonance (SPR)-based immunoassay or SPRIA.

Results. Among the clinical studies, pre-existing non-neutralizing anti-ESA antibodies were found in 6% of the subjects from clinical studies in nephrology, oncology and congestive heart failure (CHF). After ESA treatment, 2.3% of the subjects developed binding, non-neutralizing antibodies with 0.1% confirmed as having an IgG isotype and were specific to the ESA protein. IgM antibodies were detected at baseline and post-ESA treatment and reported to be specific to the glycosylation of the ESA. No clinical study subjects progressed to amPRCA. In contrast, anti-ESA antibody-positive subjects from the post-market setting with a confirmed IgG subclass were specific to the ESA protein. Subjects that had progressed to amPRCA were noted to have high antibody concentrations with neutralizing activity and a diverse IgG subtype.

Conclusions. A low prevalence of non-neutralizing anti-ESA IgM specific to glycosylation on the ESA and IgG1 antibodies specific to the ESA protein was detected across all clinical patient populations. Patients with amPRCA were noted to have high IgG antibody concentrations, neutralizing antibodies and the presence of anti-ESA IgG4 antibodies.

Keywords: ESA; pre-existing antibodies; PRCA; SPRIA

Introduction

The initial administration of a foreign, non-tolerant antigen will induce a classic primary immunoglobulin M (IgM) antibody response [1, 2]. Subsequent antigen exposure will result in the development of both antigen-specific T helper cells and B cells. This T-dependent B cell response provides the necessary signals to drive B cells to undergo isotype switching (IgM to IgG1 through IgG4) and somatic hypermutation resulting in the affinity maturation of antigen-specific antibody-producing plasma cells [3, 4].

Recombinant human erythropoietin (EPO) has been used to treat anemia for more than two decades. In healthy subjects, the immune system does not respond to circulating recombinant EPO because of self-tolerance. Despite the immunological processes of central and peripheral tolerance, failures of self-tolerance or ‘breaking’, tolerance can occur. Although EPO is generally well tolerated, rare cases of antibody-mediated pure red cell aplasia (amPRCA) have been reported [5, 6].

The development of autoantibody to numerous endogenous self-antigens in healthy individuals has also been reported [7] and cytokine-specific autoantibody has been demonstrated against interleukin (IL)-1α, IL-6, IL-10, interferon (IFN)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). Bendtzen et al. [8] reported autoantibodies to IL-1α in up to 75% of a Caucasian population and <3% for the other cytokines. Although some antibodies to these pleiotropic cytokines were neutralizing, their immunological function remains unclear. Results from a series of studies on autoantibodies suggest that physiological autoimmunity may play a role in regulating the immune system [9].

Erythropoietin is the primary hematopoietic growth factor essential for governing the rate of red cell production. Since regulation of EPO expression is under tight control and is the terminal regulator of erythropoiesis, a biological role for a second level of regulation by autoantibody is questionable. Despite this, erythropoiesis-stimulating agent (ESA) naïve subjects have detectable...
levels of non-neutralizing anti-ESA antibodies [10, 11]. The lack of detection and reporting of these antibodies may be related to limitations in detection methods. The bridging enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific method, but owing to its inherent wash steps [12], detection of low-affinity antibodies poses a challenge. The radioimmunoprecipitation assay (RIPA) is also a sensitive method; however, detection of IgM antibodies would likely be missed since they have limited binding to Protein A or Protein G. The surface plasmon resonance-based immunoassay (SPRIA) [13] is not as sensitive as the ELISA or RIPA methods, but it has demonstrated detection of a broad range of anti-ESA antibodies, including low-affinity and IgM antibodies [14].

The detection of anti-EPO antibodies and its clinical association with anemia have been reported in systemic lupus erythematosus (SLE) subjects [15] as well as in patients with human immunodeficiency virus Type 1-related anemia [16]. In both cases, a fundamental immune disturbance results in the loss of immune tolerance to numerous self-antigens, including erythropoietin. Despite the lack of a reported neutralizing anti-EPO antibody in these studies, a clinical association of anti-EPO autoantibodies with anemia suggests that an etiological role may be possible. In spite of many years of study, it is still uncertain why tolerance to these endogenous self-proteins is lost or broken.

The mechanism(s) that break tolerance to exogenous recombinant proteins such as erythropoietin is also not well understood. In the case of the exogenous administration of recombinant EPO, glycosylation, other post-translational modifications and the aggregation state of the protein are important intrinsic factors that influence immunogenicity. Equally important, extrinsic factors such as route of administration, genetic background and immune status of the patient and use of certain concomitant medications also contribute to this phenomenon [17–20].

Here, we have compiled antibody data from over 6000 patients analyzed for pre-existing and ESA-induced antibodies detected by SPRIA from clinical studies and the post-market setting. The specific aims were: (i) to determine the prevalence and incidence of anti-ESA antibodies across different patient populations, (ii) to evaluate whether subjects with non-neutralizing, binding anti-ESA antibodies affinity mature and isotype switch and (iii) to evaluate the anti-ESA antibody characteristics of isotype, specificity and concentration in antibody-positive subjects with non-PRCA and in patients that develop amPRCA.

Materials and methods

Anti-ESA antibody characterization

Clinical and post-market safety samples were analyzed with a validated semi-quantitative SPR-based immunoassay or SPRIA previously described [21]. Briefly, diluted serum samples were passed over the flow cells of a biosensor chip immobilized with Epoetin alfa or darbepoetin alfa followed by an injection of goat anti-human IgG, IgM and IgA antibodies. The assay was validated to detect at least 80 ng/mL anti-Epoetin alfa antibodies. Antibody-positive samples demonstrating at least 50% signal inhibition in the presence of soluble drug were confirmed to be drug-specific. Relative antibody concentrations were interpolated from a 7-point anti-Epoetin alfa standard curve (10.0–0.16 µg/mL) using an anti-ESA IgG standard. It is important to note that for anti-ESA IgM antibody concentrations, the 5-fold mass difference between IgM and IgG antibodies results in an over estimation of the actual concentration of IgM antibodies. Identification of the antibody isotype of positive samples with antibody concentration >0.25 µg/mL was done by sample injection across the Epoetin alfa or darbepoetin alfa surface followed by an anti-human isotype-specific antibody.

Anti-ESA antibody specificity to aglycosylated and glycosylated EPO

Selected samples were tested for reactivity to Escherichia coli (E.coli) EPO and aglycosylated Aranesp. Preparation of E.coli EPO has been previously described [22]. Aglycosylated Aranesp was prepared by deglycosylation of Aranesp using Sialidase, O-glycanase and N-glycanase under native conditions. Dithiothreitol (DTT) was added into the deglycosylation mix to increase the rate of deglycosylation. Then, the DTT was immediately removed by size exclusion chromatography, thereby oxidizing the material to its native state. Denaturants were not used during N-glycan removal. Tryptic peptide mapping indicated that the disulfide bonds were intact and that the N- and O-linked carbohydrates were removed.

The following EPO molecules were immobilized onto the flow cell of a sensor chip in order of increasing glycosylation: E.coli EPO, aglycosylated Aranesp, Epoetin alfa and darbepoetin alfa were immobilized to flow cells 1, 2, 3 and 4, respectively. Patient serum samples were analyzed by SPRIA. Signal inhibition of at least 50% when treated with 100 µg/mL E.coli EPO confirmed antibodies to be specific to E.coli EPO.

Clinical study samples

Antibody results from serum samples were analyzed in the following study populations: nephrology (=8 studies), oncology (=7 studies) and CHF (n = 5 studies). It is recognized that there were protocol differences between study populations including, but not limited to, the number of subjects, subject type (healthy or disease population), dosing schedule, dose amount and delivery method, length of study and inclusion of placebo group. The aim of this work was to gain perspective of the type and frequency of anti-ESA antibodies detected in clinical studies regardless of the specific conditions of any one study.

Post-market safety samples

Antibody results were compiled from specimens collected from and analyzed in patients treated with Epoetin alfa or darbepoetin alfa who had experienced a suspected drug-related adverse event in a post-market setting. Additional follow-up specimens were requested and analyzed to monitor the patient’s antibody response over time for patients determined to be positive for anti-ESA antibodies. The safety specimens were predominantly from the chronic kidney disease population who had received one or more marketed ESA products via subcutaneous injection. Retrospective analysis of data for safety specimens analyzed between April 2000 and May 2011 included 243 patients tested for anti-Epoetin alfa antibodies and 400 patients tested for anti-darbepoetin alfa antibodies.

Cell-based bioassay method

A cell-based bioassay, previously described [23], was used to assess whether the detected binding antibodies in patient samples neutralize the biological activity of Epoetin alfa or darbepoetin alfa.

Statistical methods

The cutoff concentration between neutralizing and non-neutralizing antibody specimens was estimated using a logistic regression on a log-transformed antibody concentration as an independent variable and weighted on the absolute value of the log-transformed antibody concentration.

Results

Clinical study results

The immunoassay results from clinical studies in nephrology, oncology and CHF were compiled to evaluate the prevalence of pre-existing, developing and confirmed anti-ESA antibody isotype (Table 1). The prevalence of anti-ESA antibodies in the three patient populations was
compared with regard to the antibody incidence rate with a logistic regression model by the method of maximum likelihood. The odds ratio, 95% confidence interval and P-value were calculated (see Supplementary data, Table S1). There were no statistically significant differences in any of the three populations compared with one another at the α = 0.05 significance level. Thus, individual anti-ESA antibody results from the three populations were merged to determine the overall rates of anti-ESA antibody prevalence. The cumulative pre-existing antibody rate was 6.0% (409/6870) and the developing antibody rate was 2.3% (158/6870). All pre-existing and developing anti-ESA antibodies in the clinical study samples were binding and non-neutralizing and none of the subjects exhibited clinical signs or symptoms of amPRCA.

A small number of subjects across all clinical study groups had pre-existing antibodies (baseline positive) that were post-dose antibody negative. These ‘baseline only’ antibody-positive samples were 1.7% in each group: 21/1235 for nephrology, 88/5051 for oncology and 10/584 for CHF. The majority of the antibodies (72.3%) in the ‘baseline only’ antibody-positive subgroup were below the assay quantifiable limit (0.25 µg/mL) and their significance is unknown. The results of antibody concentration and isotype for the nephrology study population illustrate that the IgM isotype is commonly detected in both the pre-existing and developing antibody groups (Figure 1). Examples of the IgG1 isotype were limited to the pre-existing antibody group in nephrology studies, but both pre-existing and low levels of developing IgG antibodies occurred in subjects in the oncology and CHF studies (Table 1). The method of identifying antibody isotype identification was less sensitive than the screening method and was highly dependent on the antibody concentration and affinity. Consequently, the antibody isotype was not confirmed for the majority (80.2%) of positive samples. Antibody concentrations measured in the pre-existing and developing antibody groups were <1 µg/mL in 93.0% of the subjects positive for anti-ESA antibodies (see Figure 1).

Evidence of ESA-induced antibody development in clinical studies

As previously noted, 101 of the 1235 nephrology study subjects (8.2%) were determined to develop anti-ESA antibody after ESA-treatment. The subset of the 101 subjects was analyzed further and subjects with anti-ESA antibody characteristics associated with antibody development and maturation were evaluated. None of the subjects showed any signs of developing amPRCA. Although antibody concentrations increased over time in some subjects, there was no evidence of isotype class switching. Similar observations were made for the oncology and CHF study subjects (data not shown).

Glycosylation and binding antibody specificity

The binding specificity of selected samples from clinical studies and post-market safety subjects were analyzed.

Table 1. Prevalence of pre-existing and developing anti-ESA antibodies in clinical studies was found to be similar across study populations regardless of differences in study protocols

<table>
<thead>
<tr>
<th>Anti-ESA antibody classification</th>
<th>Nephrology (n = 1235)</th>
<th>Oncology (n = 5051)</th>
<th>CHF (n = 584)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-existing</td>
<td>Totala</td>
<td>71 (5.75%)</td>
<td>308 (6.10%)</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>13 (1.05%)</td>
<td>33 (0.65%)</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>5 (0.40%)</td>
<td>16 (0.32%)</td>
</tr>
<tr>
<td>Developing</td>
<td>Totala</td>
<td>30 (2.43%)</td>
<td>113 (2.24%)</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>2 (0.16%)</td>
<td>7 (0.14%)</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>0 (0%)</td>
<td>5 (0.10%)</td>
</tr>
</tbody>
</table>

*The isotype classification could not be confirmed for most detected antibodies because of low concentration.
Post-market safety samples

Patients that received Epoetin alfa or darbepoetin alfa treatment were first divided into two groups on the basis of antibody type, anti-Epoetin alfa or anti-darbepoetin alfa, and then subdivided on the basis of the presence of neutralizing antibodies in a bioassay. Binding antibodies were detected in 43 of 243 patients when analyzed for anti-Epoetin alfa antibodies; 14 of the 43 patients with binding antibodies were determined to have neutralizing antibody activity. Binding anti-ESA antibodies were detected in 40 of 400 patients when analyzed for anti-darbepoetin alfa antibodies; 13 of the 40 patients with binding antibodies were determined to have neutralizing antibodies. Compiled results from all subjects and all time points determined to have anti-ESA antibodies indicate that patients positive for anti-ESA IgG neutralizing antibodies are likely to have higher antibody concentrations (see Figure 3) and anti-EPO IgG4 subtype (see Table 2). In addition, samples from three patients with amPRCA demonstrated specificity to the ESA protein (see Figure 2b).

Statistical analysis on all time points for each patient was carried out to determine the association of the antibody concentration or antibody isotype/IgG subclass with neutralizing antibodies. Neutralizing antibodies were associated with an anti-Epoetin alfa antibody concentration of >2.81 µg/mL and the presence of anti-ESA IgG4 antibodies. Similarly, neutralizing antibodies were associated with an anti-darbepoetin alfa antibody concentration of >6.43 µg/mL and the presence of anti-darbepoetin IgG4...
antibodies (κ coefficient 0.4522). The data can be found in Supplementary data, Table S2.

In the post-market patients, there was no evidence of antibody development or maturation in any patient regardless of the treatment received or neutralizing classification. Within the non-neutralizing antibody population, nearly all anti-ESA antibody concentrations tested either decreased or remained constant (data not shown). Only one subject showed an increase in antibody concentration (2.76 µg/mL); however, the antibody was characterized as non-neutralizing IgM at all time points. Patients with neutralizing antibodies decreased in antibody concentration, lost confirmed isotypes or lost neutralizing activity (data not shown). There were four patients with neutralizing antibodies that increased antibody concentration over time that were unchanged for confirmed antibody isotypes.

**Discussion**

The development of amPRCA is a life-threatening condition that, in rare cases, can result in amPRCA. From 1998 to 2003, an increase in the incidence of amPRCA in Europe occurred with Eprex® that coincided with a manufacturing change. While the precise cause has not been determined, it has been suggested to be related to leachates in the syringe, removal of stabilizers and/or biophysical changes in structure [24–29]. Cases of amPRCA have also been reported due to follow-on ESA biologics [30, 31].

**Table 2.** Post-market study samples with detected anti-ESA antibodies were analyzed for antibody isotype, neutralizing antibodies and relative antibody concentration

<table>
<thead>
<tr>
<th>Antibody detected in sample</th>
<th>Number of samples (n)</th>
<th>Antibody isotype confirmed</th>
<th>Neutralizing antibody incidence (%)</th>
<th>Relative concentrationa median (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Epoetin alfa (n = 93)</td>
<td>14 IgG1, IgG2, IgG3, IgG4</td>
<td>100.0</td>
<td>38.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 IgG1, IgG2, IgG4</td>
<td>90.0</td>
<td>3.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 IgG1, IgG4</td>
<td>33.3</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 IgG1</td>
<td>25.0</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 All othersb</td>
<td>0.0</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Anti-darbepoetin alfa (n = 73)</td>
<td>2 IgG1, IgG3, IgG4</td>
<td>100.0</td>
<td>13.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 IgG1, IgG2, IgG3</td>
<td>100.0</td>
<td>33.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 IgG1, IgG2, IgG3, IgG4</td>
<td>75.0</td>
<td>13.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 IgG1, IgG2, IgG4</td>
<td>64.3</td>
<td>5.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 IgG1, IgG4</td>
<td>60.0</td>
<td>14.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 IgG, subtype not determined</td>
<td>25.0</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 IgG1</td>
<td>13.3</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 IgG4</td>
<td>0.0</td>
<td>1.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27 All othersb</td>
<td>0.0</td>
<td>1.53</td>
<td></td>
</tr>
</tbody>
</table>

Samples were then classified by the type of antibody detected (anti-Epoetin alfa or anti-darbepoetin alfa) and by the antibody isotypes confirmed. All time points for patients were included.

*Antibody concentrations reported were relative to a rabbit polyclonal anti-Epoetin alfa antibody.

bIncluded IgM, other mixed IgG1, IgG2 and IgG3 isotypes, and samples with no isotype confirmed.

Therefore, we have compiled anti-ESA antibody data from three clinical patient studies and from patients in the post-market safety setting. The prevalence and characteristics of anti-ESA antibodies were similar across the three different clinical studies. Pre-existing antibodies were detected in 6% of the subjects with a low concentration of autoantibody to the ESA (91.7% of the subjects <1.0 µg/mL). Despite the lack of a confirmed antibody isotype in most subjects, ~1% were IgM antibodies with specificity to the glycosylation moieties on the ESA, while ~0.3% were confirmed anti-ESA IgG antibody and specific to the ESA protein.

Most of the IgM antibodies in normal serum are derived from CD5+ B-cells (B1 cells) that express polyreactive IgM antibodies in response to carbohydrates on glycoproteins and glycolipids [32, 33]. Although the source of the anti-ESA IgM antibodies has not been

Fig. 3. Results are shown for all post-market anti-ESA antibodies for non-neutralizing antibody (non-Nab) and neutralizing antibody (Nab) groups with median values illustrated. The median anti-darbepoetin alfa (darbepo) antibody concentrations were higher for Nab (n = 21) than for non-Nab (n = 51); P-value <0.0001. Similarly, the median anti-Epoetin alfa (EPO) antibody concentrations were higher for Nab (n = 29) than for non-Nab (n = 59); P-value <0.0001. Specimens with indeterminate concentrations were not included in the calculated median. Antibody concentrations were relative to a rabbit polyclonal anti-Epoetin alfa antibody.

Table 2. Post-market study samples with detected anti-ESA antibodies were analyzed for antibody isotype, neutralizing antibodies and relative antibody concentration
confirmed to be B-1 or the conventional B-2 cell population, it is noteworthy that the IgM-positive subjects in these clinical studies did not demonstrate an augmentation in IgM concentration or demonstrate isotype switching following ESA administration, suggestive of IgM-producing B-1 cells. On the basis of previous reports [34], it is likely that these are poly-reactive, low-affinity antibodies. The physiologic relevance of pre-existing T-cell-independent IgM antibodies to an ESA is likely of little clinical relevance. The Chinese Hamster Ovary (CHO) host cell line used to express EPO and the conditions in which the host cell is grown can impact on post-translational modification, such as glycosylation. Although the mammalian CHO cell line processes recombinant glycoproteins in a manner similar to humans, there can be minor differences in glycosylation with variation in the sialic acid composition within the oligosaccharide groups including N-glycolyneuraminic acid (Neu5Gc) and may contribute to the IgM reactivity observed. But, a low content of Neu5Gc has been reported for EPO (Procrit®) [35], and therefore, it is unlikely to be the target of the IgM reactivity [36].

The development of a primary IgM response to a protein therapeutic has been reported with a corresponding switch to IgG [37]. The sole reason why a primary anti-ESA IgM response is not reported in ESA-treated subjects is that samples are generally not collected during this early onset of an immune response. Similar pre-existing antibody rates have been reported for several other growth factors and cytokines [8]. Along these lines, autoantibody to EPO has been found in SLE [38], a chronic and systemic autoimmune disease. In this case, there is a general loss of tolerance to numerous self-proteins, one of which is to erythropoietin.

The analysis of anti-ESA antibodies in clinical study subjects showed a modest increase in antibody concentration in a small population, low rates of anti-ESA IgM and IgG antibody development and a lack of maturation of antibody isotype. A majority of the subjects (96.3%) from the three clinical studies that developed an anti-ESA antibody response had <1 µg/mL of antibody (see Figure 1). Despite a confirmed human immunoglobulin binding to the ESA in the SPRIA screening assay, identification of the specific isotype was not possible with commercially available isotype-specific reagents when anti-ESA antibody levels were <1 µg/mL. In the remaining subjects with an antibody concentration of >1 µg/mL, the identification of an IgM and IgG1 isotype characteristic of an early immune response was predominant. These subjects did not demonstrate isotype switching, characteristic of a maturing immune response. One explanation for the lack of progression from binding, non-neutralizing anti-ESA antibodies to the development of amPRCA is maintenance of peripheral tolerance to the exogenous ESA.

Post-market safety samples from patients with confirmed amPRCA were not accompanied by a baseline sample nor was there a sample collected prior to a precipitous drop in hemoglobin (Hb). Since sampling prior to ESA administration and prior to a drop in Hb are typically not collected, conclusions about the antibody time course from onset to the development of amPRCA cannot be made. To better understand the differences between non-neutralizing antibodies detected in clinical studies and neutralizing antibodies associated with amPRCA, antibody concentration and isotype data were compiled and analyzed. In patients with neutralizing antibodies, there was a strong statistical association with anti-Epoetin alfa antibody concentrations >2.81 µg/mL and the presence of anti-Epoetin alfa IgG4 antibody. Similar results are shown for darbepoetin alfa (see Supplementary data, Table S2). These results are consistent with a previous report [12]. Subsequent sample analysis from some patients with neutralizing positive antibodies demonstrated declining antibody concentrations, loss of confirmed isotype and loss of neutralizing activity. This is not unexpected since ESA treatment is generally withdrawn and immunosuppressive therapy is considered once amPRCA is confirmed. One study suggested that anti-ESA antibody concentrations above 1000 ng/mL are likely to show neutralizing antibodies [39]. The antibody concentration associated with neutralizing activity as demonstrated by our data was at a higher concentration and dependent on ESA type. Furthermore, there are examples of neutralizing antibodies below that antibody concentration as well as non-neutralizing antibodies above the limit (see Supplementary data, Table S2) which suggests that the 1000 ng/mL concentration can be used only as a guide and cannot replace a cell-based bioassay to confirm the presence of neutralizing antibodies.

The development of binding, non-neutralizing IgG detected in non-PRCA subjects did not demonstrate augmentation post ESA treatment but did have specificity for the ESA protein, not the carbohydrate. A study of the first cases of amPRCA in 2000 by Casadevall et al. [40] demonstrated that anti-EPO antibodies bound equally well to the glycosylated and deglycosylated epoetin.

In summary, the immunogenicity data from the three clinical patient populations and the commercial safety samples compiled here reveal: (i) an immature anti-ESA antibody signature in subjects having low concentrations (<1 µg/mL) of binding, non-neutralizing IgG1 and IgM antibodies with no adverse clinical effects and (ii) a mature, high concentration of binding and neutralizing anti-ESA antibody of mixed IgG subclass containing IgG4 associated with amPRCA. Since amPRCA is a rare event, more frequent sampling may not be warranted. To further our understanding of the etiology from early onset to the development of amPRCA, more novel analytical approaches are needed. We have demonstrated an association between IgG4 detection in serum from subjects with neutralizing antibodies and amPRCA. We have only detected anti-ESA IgM and IgG1 antibodies pre-existing, and in a small proportion of patients that developed antibodies after ESA treatment. The presence of an anti-ESA IgG4 antibody is not likely a cause but a consequence of a maturing antibody response. We have previously published the detection of a mixed IgG subclass response that includes IgG4 in amPRCA patients [14], so an accurate measure of an anti-ESA IgG4 antibody in the presence of other, more prevalent IgG subclasses is challenging by any of the current immunoassay platforms [41]. The association between amPRCA and the presence of anti-ESA IgG4 antibodies is interesting. We suggest that the development of a sensitive immunoassay to detect anti-
ESA IgG4 antibodies may provide a much needed biomarker for the detection of early-onset amPRCA.

Supplementary data

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

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Macrophage impairment causes LPG


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Macrophage impairment produced by Fc receptor gamma deficiency plays a principal role in the development of lipoprotein glomerulopathy in concert with apoE abnormalities

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Abstract

**Background.** To obtain a clear understanding of the pathogenesis of lipoprotein glomerulopathy (LPG), we studied the role of the deficiency of Fc receptor gamma chain (FcRγ) for the development of LPG in concert with apolipoprotein E (apoE) abnormalities.

**Methods.** We generated apoE and FcRγ double-knockout (FcRγ/apoE-KO) mice, and subsequently introduced several kinds of human recombinant apoE genes. At 21 days after infection, the mice were sacrificed and histologically examined. Peritoneal macrophages were evaluated for their response to modified lipids.

**Results.** In the FcRγ/apoE-KO mice, the human apoE3-injected mice showed the most drastic LPG-like changes, as well as prominent hypertriglyceridemia. Meanwhile, relative to the human apoE3-injected mice, the FcRγ/apoE-KO mice showed greater lipoprotein deposition and less macrophage infiltration into the mesangial area. Moreover, the peritoneal macrophages in the apoE/FcRγ-KO mice were impaired in lipid uptake and secretion of the cytokines monocyte chemotactic protein-1 and regulated upon activation, normal T-cell expressed and secreted, after the uptake of oxidized low-density lipoprotein.

**Conclusions.** These results suggest that the impairment of macrophage function resulting from FcRγ deficiency plays a principal role in the development of LPG in the presence of apoE abnormalities.

**Keywords:** apolipoprotein E; Fc receptor gamma chain; lipoprotein glomerulopathy; macrophage

Introduction

Lipoprotein glomerulopathy (LPG) is a recently identified renal disease [1] that was originally described in 1989 by Saito et al. and mainly affects people of Japanese and Chinese origin. LPG patients commonly show severe proteinuria and progression to renal failure. A histological characteristic of LPG is the deposition of a thrombus-like substance in markedly dilated glomerular capillaries; these substances positively stain for Sudan IV and oil red O. Moreover, patients with LPG have elevated concentrations of intermediate-density lipoprotein (IDL) and plasma apolipoprotein E (apoE) levels similar to those seen in type III dyslipidemia [2].

In 1997, a novel apoE variant, apoE-Sendai (Arg145Pro), was identified in Japanese patients with LPG [3]. Since the identification of apoE-Sendai, various other apoE mutations have subsequently been discovered [4–12]. In murine studies, Ishigaki et al. [13] and our group [14] reported that the apoE-knockout (KO) mice that received virus-mediated transduction of apoE-Sendai developed the murine counterpart of LPG. Therefore, the dyslipidemia that resulted from the apoE variants was