Effect of neutralizing antibodies to IL-10 and C5 on the renal damage caused by a pathogenic human anti-dsDNA antibody*

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Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized by the production of a number of autoantibodies. Amongst these, anti-double-stranded deoxyribonucleic acid (anti-dsDNA) antibodies are involved in the pathogenesis of lupus nephritis. It was shown previously using severe combined immune deficient (SCID) mice that when the hybridomas secreting human immunoglobulin G (IgG) anti-dsDNA antibodies, RH14 and DIL-6, were implanted intraperitoneal the antibodies produced by RH14, but not DIL-6, deposited in the kidneys, caused pathological changes in the renal tissues and induced proteinuria. In this study we have further analysed the effect of activated terminal complement proteins and interleukin-10 (IL-10) in the pathogenesis of glomerulonephritis caused by the RH-14.

Methods. SCID mice implanted with RH-14 or DIL-6 cell lines were treated with neutralizing antibodies to IL-10 (mAb B-S10) or anti-complement factor 5 (anti-C5) (mAb BB5.1) intraperitoneally. Control groups received either an isotype control antibody (135.8) or phosphate-buffered saline (PBS). Serum human IgG levels and proteinuria were estimated and the extent of renal involvement was examined by histopathological and electron microscopic techniques.

Results. While there was a tendency to reduce proteinuria in the anti-IL-10 injected group the anti-C5 injected group showed a significant reduction in proteinuria (P < 0.01) compared with the groups injected with either the control mAb or PBS. There was a considerable reduction in the serum human IgG levels in the anti-IL-10 but not in the anti-C5 treated animals. Both anti-IL-10 and anti-C5 treated groups showed significantly reduced renal impairment as revealed by histopathological examination and proteinuria assessment.

Conclusion. The findings, while confirming the role of IL-10 and activated terminal complement component in the production of antibody at the cellular level and at the site of glomerular immune deposition in this model, respectively, also suggest the beneficial effect of a combined therapy using both anti-IL-10 and anti-C5 mAb to prevent or reduce the effect of the humoral immune response in lupus disease.

Key words: Systemic lupus erythematosus, Anti-DNA antibodies, Nephritis, IL-10, C5.
Thus the immunological imbalance of SLE may be related to an abnormally high production of IL-10 or hypersensitivity of immune cells to this cytokine. Moreover peripheral blood mononuclear cells (PBMC) from SLE patients spontaneously release large amounts of IL-10 [9, 11]. Treatment of NZB/WF1 mice with anti-IL-10 mAb delayed the onset of autoimmune manifestations and the production of autoantibodies [12]. Further, it has been shown that IgG production by the B-lymphocytes from SLE patients is largely IL-10 dependent [13]. Our own studies have shown that IL-10 has profound effects on anti-single-stranded DNA (anti-ssDNA) and anti-dsDNA production when co-cultured with PBMC from patients with SLE [14]. Furthermore, the experimental data from human SLE patients also suggest that specific IL-10 antagonists may have beneficial effects in patients with SLE [15].

Although various studies have explored the factors responsible for the onset of autoimmunity in mice [16], little is known regarding the pathogenic mechanisms of renal disease following deposition of immune complex. An important step in immune complex-initiated inflammation is the activation of the complement cascade through both the classical and alternative pathways [17]. During complement activation the C5 component is cleaved to form products C5a and C5b-9, with multiple proinflammatory effects. Cellular activation by C5a induces the release of multiple additional inflammatory mediators [18, 19]. In addition C5b-9 can also stimulate the release of multiple proinflammatory molecules [20–22] and may well play an important role in inflammation. Thus blocking C5a as well as C5b generation may be required for optimal inhibition of the inflammatory response. The role of the activated terminal complement component in the progression of the renal disease in NZB/WF1 mice has been studied, using a mAb specific for murine C5 [23]. Injection of monoclonal anti-C5 antibody resulted in significant amelioration of the course of glomerulonephritis and increased survival [24].

In this study we have examined the role of IL-10 and activated terminal complement in the pathogenesis of the glomerulonephritis caused by the human anti-DNA monoclonal antibodies in SCID mice [7].

Materials and methods

Antibody treatments

The anti-mouse C5 monoclonal antibody (BB5.1, IgG1) and the control murine anti-human C8 monoclonal antibody (135.8, IgG1) have been described previously [23, 24]. The anti-IL-10 monoclonal antibody (B-S10, IgG1, directed against human IL-10) [12] was provided by Diaclone, France.

Experimental design

Four-week-old SCID mice were treated with 0.2 ml sterile pristane 10 days prior to intraperitoneal (i.p.) implantation of hybridoma cells. Hybridoma cells producing human IgG mAbs (RH-14 or DIL-6) were harvested from midlog phase cultures and resuspended in 0.5 ml RPMI so that the total number of cells was 10^6. The SCID mice (Harlan, UK), implanted with RH-14 or DIL-6 cell lines, were treated with anti-IL-10 (B-S10), anti-C5 (BB5.1) and isotype control antibody (135.8). After the intraperitoneal cell transfer, 10 SCID mice in each group were injected intraperitoneally either with 100 μg anti-IL-10 or 600 μg anti-C5 twice weekly until the end of the experiment. The same number of controls were injected either with 600 μg control mAb 135.8 or phosphate-buffered saline (PBS). The mice were killed either when their body weight increased by 20% due to ascites, or if they appeared unwell. Blood samples were collected and the protein excretion in the urine was measured by impregnating Albustix (Bayer Diagnostics) with urine, reading the colour change visually against a standard and graded from 0 to 4+. A full autopsy was performed and kidneys were sampled. Part of the tissue was snap frozen, fixed in 5% buffered formalin or fixed in 2% glutaraldehyde in PBS.

The study did not require ethical approval.

IgG ELISA

The serum human immunoglobulin was analysed using solid phase ELISA. Maxisorp plates (Nunc, Denmark) were coated with goat anti-human IgG (Sigma) at 10 μg/ml and 100 μg/ml. After incubation for 1 h at 37°C the wells were washed twice with PBS. The plates were blocked with 200 μl/well of 2% bovine serum albumin (BSA) for 1 h at 37°C, then rinsed twice with PBS plus 0.1% Tween 20 (PBS-T). Serum samples diluted 1/50 in PBS-T were added and incubated for 1 h at 37°C. After washing the wells three times with PBS-T, 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was added for another 1 h incubation. The plates were then washed four times with PBS-T and developed with substrate 0.1% p-nitrophenyl phosphate (Sigma, St Louis, MO) for 1 h at 37°C. After washing the wells three times with PBS-T, 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was added for another 1 h incubation. The plates were then washed four times with PBS-T and developed with substrate 0.1% p-nitrophenyl phosphate (Sigma, St Louis, MO) for 1 h at 37°C. The plates were then washed twice with PBS-T and incubated with substrate 0.1% p-nitrophenyl phosphate (Sigma, St Louis, MO) for 1 h at 37°C.

Histopathological analysis of renal sections

Sections of renal sections from frozen kidneys obtained from the animals treated with monoclonal antibodies BB5.1, B.S10 and 135–8 were stained for human IgG using fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (1:50; Southern Biotechnology, Birmingham, AL). The slides were washed in PBS and viewed under ultraviolet (UV) light (filter set at 530 nm) using a MRC 600 scanning system connected to image analysis software (Bio-Rad, GB) or using a Nikon microscope attached to a camera. The intensity of immunoglobulin deposition was graded from 0 to 4+. Kidney sections were also coded and screened blind for the evidence of glomerular pathology, vasculitis and lymphoid infiltration.

Electron microscopy

The glutaraldehyde-fixed renal tissues were embedded in Araldite. Thin sections of renal glomeruli were contrasted with uranyl acetate and lead citrate and viewed in a Jeol (JEM-1200EX) electron microscope. Ultrathin sections also were immunolabelled following a method adapted by Cooper et al. [25]. Briefly, sections were blocked with 5% BSA in PBS and reacted with rabbit anti-human IgG (DAKO) at a dilution of 1:4000 in 5% BSA for 120 min. After washing in PBS, 20 nm Protein-A gold (Biocell) at a dilution of 1:1000 in 5% BSA was added and incubated for 60 min. Sections were contrasted with uranyl acetate and examined as described above.

Haemolytic assay

Mouse serum samples were diluted to 10% (vol/vol) with gelatine veronal buffer 2+ (Sigma) and added (50 μl per well) to 96-well optiread microtitre plates containing 50 μg of human C5-deficient serum per well (Quidel, San Diego, CA). The plates were incubated for 30 min at room temperature. Erythrocyte preparation and haemolytic assays were then performed as described in [26].

Statistical analysis

Student’s t-tests assuming two samples with unequal variance were performed with the Microsoft Excel 2000 data analysis program.
Results

Anti-C5 treatment

To explore the role of activated terminal complement component in the development of glomerulonephritis in the pathogenic human IgG anti-dsDNA-implanted SCID mice, 8-week-old SCID mice implanted with RH-14 and DIL-6 hybridoma were begun on biweekly treatment with either anti-C5 or an isotype-matched control mAb. *In vivo* inhibition of complement by anti-C5 mAb was ascertained by the measurement of complement-dependent serum haemolytic activity (Fig. 1). The mAb treatments continued until the body weight of the mice increased by 25% due to ascites or if they appeared unwell. The animals were killed within 29 ± 4 days after the initial implantation of the hybridoma.

Anti-C5 mAb administration was able to sustain complement inhibition *in vivo* for the entire experimental period. The serum haemolytic activity of the control mAb and PBS treated mice declined to less than 65% of normal levels at the end of experiment, presumably secondary to systemic consumption of complement after widespread tissue deposition of immune complexes (Fig. 1). The decline in the haemolytic activity in the sera of control animals correlated with elevated titres of human IgG measured at the end of the experiment (Table 1). Serum and ascites from both anti-C5 treated and control animals showed anti-dsDNA reactivity (data not shown). In addition, quantitative immunofluorescence demonstrated equivalent levels of immune complex (Fig. 2) and C3 (data not shown) deposition in the glomeruli of anti-C5 treated and control RH-14 mice. Consistent with our previous findings [7] crescent formation and tubular cast were not found in this model (data not shown).

The influence of mAb-mediated C5 inhibition on the course of human anti-dsDNA antibody immune complex nephritis was examined both clinically and histopathologically. A marked decrease in the proteinuria, defined as equal to or less than 300 mg/ml (<3+) (*P* < 0.01), was achieved in anti-C5 mAb-treated RH-14 mice relative to control mAb treated animals (Table 1); all the control mAb and PBS treated RH-14 animals developed a high level of proteinuria (>3+). As expected DIL-6 mice did not develop proteinuria.

Electron micrographs of renal sections of RH-14 mice treated with control mAb and PBS showed mesangial cell hypertrophy as well as an increase in the amount of matrix in which deposits were found (Fig. 3a). In the capillary loops there was interposition of mesangial cells under the endothelium and prominent subendothelial deposits. In addition there was generalized thickening of glomerular basement membranes and patchy effacement of the epithelial foot processes. Immunolabelling showed the presence of gold particles over what appeared to be dense deposit in both central and paramesangial areas of matrix (Fig. 3c). Pronounced gold labelling also occurred over the plasma within capillary lumens. No immunolabelling was observed along the glomerular basement membrane. In contrast the anti-C5 treated animals were less affected in terms of their renal pathology. These animals showed minimal increase in the amount of matrix in the mesangial region and little effacement of epithelial foot processes (Fig. 3b).

Anti-IL-10 treatment

Human IgG levels in both RH-14 and DIL-6 mice treated with anti-IL-10 were significantly reduced (*P* < 0.01) compared with the control groups treated with PBS (Table 1). Quantitative immunofluorescence of kidney sections demonstrated significantly low levels (*P* < 0.001) of immune complex in the anti-IL-10 treated RH-14 mice (Fig. 2). There was only a slight tendency to reduce proteinuria in the anti-IL-10 injected RH-14 mice (Table 1). The electron micrograph of renal sections of anti-IL-10 treated RH-14 mice showed minimal increase in the amount of matrix in the mesangial region and showed little effacement of epithelial foot processes (data not shown).

Discussion

The progression of glomerulonephritis in RH-14 hybridoma-implanted SCID mice is associated with a rising titre of human anti-dsDNA antibodies and deposition of immune complexes in the glomeruli. Here we have shown that inhibition of the complement cascade with anti-C5-specific mAb markedly ameliorates the course of nephritis, clearly implicating the products of terminal complement activation in the inflammatory process leading to renal failure.

![Fig. 1. The haemolytic activity of mouse sera from the RH-14 and DIL-6 anti-DNA hybridoma-implanted SCID mice treated with anti-C5, anti-IL-10, control monoclonal antibody or PBS was measured using haemolytic assay and expressed as % haemolytic activity (mean ± s.d.). The treatments continued until the body weight of the mice increased by 25% due to ascites and the haemolytic activity of the sera from the terminal blood samples was measured. Anti-C5 administration was able to sustain complement inhibition *in vivo* for the entire experimental period. The serum haemolytic activity of the control mAb and PBS treated mice declined to less than 65% of normal levels at the end of experiment, presumably secondary to systemic consumption of complement after widespread tissue deposition of immune complexes.](image-url)
Table 1. B-S10, BB5.1 and 135.8 are anti-IL-10, anti-C5 and control monoclonal antibodies respectively. Proteinuria was measured by impregnating Albustix with urine, reading the colour change visually against a standard and grading from 0 to 4+ (shown as mean ± s.d.). Serum human IgG was measured by ELISA and expressed as optical density (OD) values (mean ± s.d.). Renal sections were stained for human IgG using FITC-conjugated anti-human IgG and viewed under UV light using a MRC 600 scanning system connected to image analysis software. The intensity of immunoglobulin deposition was graded from 0 to 4+. Groups receiving anti-IL-10 and anti-C5 were compared with the group receiving control monoclonal antibody. Student’s t-test was used to analyse the difference between the groups.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Cell lines</th>
<th>Proteinuria RH-14 (n = 10)</th>
<th>Serum human IgG RH-14 (n = 10)</th>
<th>Intensity of IgG in renal glomeruli RH-14 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B-S10 (anti-IL-10)</td>
<td>BB5.1 (anti-C5)</td>
<td>135.8 (control)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>RH-14</td>
<td>2.3 ± 0.7</td>
<td>1.3 ± 0.5*</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>DIL-6</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.4</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Serum human IgG</td>
<td>RH-14</td>
<td>162 ± 42*</td>
<td>310 ± 112</td>
<td>312 ± 76</td>
</tr>
<tr>
<td></td>
<td>DIL-6</td>
<td>54 ± 28*</td>
<td>136 ± 74</td>
<td>148 ± 58</td>
</tr>
<tr>
<td>Intensity of IgG</td>
<td>RH-14</td>
<td>1.2 ± 0.3**</td>
<td>3.3 ± 0.5</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>DIL-6</td>
<td>0</td>
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*P < 0.01; **P < 0.001; n = number of animals in each group.

Fig. 2. Immunofluorescence staining of the renal glomerulus from RH-14-implanted SCID mouse treated with (a) control monoclonal antibody (135.8), (b) anti-C5 (BB5.1) and (c) anti-IL-10 (B-S10). There is granular deposition of human IgG in the mesangial areas of SCID mouse treated with control monoclonal antibody and anti-C5. There is a significant reduction (P < 0.001) in the immune complex deposition in the anti-IL-10 treated group. (Magnification ×330.)

Fig. 3. Electron micrographs of renal glomeruli from RH-14-implanted SCID mice treated with (a) control monoclonal antibody (135.8) and (b) anti-C5 (BB5.1). While glomeruli from the control group showed mesangial cell hypertrophy, increase in the amounts of matrix with deposits and interposition of mesangial cells under the endothelium, anti-C5 treated animals showed minimal increase in the amount of matrix in the mesangial region and showed little effacement of epithelial foot processes. (c) Immunolabelling of renal glomeruli showed the presence of gold particles (black dots as indicated by the arrows) over the dense deposit in both central and paramesangial areas of matrix in RH-14-implanted, control monoclonal antibody injected mouse. Pronounced gold labelling found in the plasma within capillary lumens showed the presence of IgG in the plasma. (Magnification ×10 000.)
It has been shown that the cleavage products of C5, C5a and C5b-9 have pleiotropic cell-activating properties, as they have been shown to amplify the release of downstream inflammatory factors such as hydrolytic enzyme, reactive oxygen species, arachidonic acid metabolites, as well as various cytokines [12, 27–30]. In the anti-C5 treated mice the renal pathology was markedly less severe than the control groups treated with control mAb or PBS. This is presumably related to the absence of proinflammatory effects mediated by C5a and C5b-9. Our results suggest that complement activation following tissue deposition of autoantibody-containing immune complexes plays a prominent role in initiating the renal inflammatory response in this SCID model. It further confirms the earlier finding that anti-C5 treatment has dramatically reduced the kidney damage and improved the survival rate of C5-sufficient NZB/W F1 animals [24]. Here we were able to show, using a human anti-dsDNA antibody, that the same mechanism is involved in the glomerulonephritis caused by a pathogenic human autoantibody.

While preventing the generation of the proinflammatory terminal complement components, mAb-mediated complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic micro-organisms as well as for solubilization and clearance of immune complex [31]. Retaining the capacity to generate C3b would appear to be a particularly important aspect of a therapeutic approach to complement inhibition in an inflammatory disease like SLE, where both increased susceptibility to infection and impaired clearance of immune complex are pre-existing clinical features of the disease [32].

Several other immunotherapeutic approaches to the treatment of autoimmune disease, such as blockade of T-cell co-stimulation through the B7 pathway [27] and antagonism of the activity of cytokines such as IL-10 [12], have been associated with suppression of autoantibody production, and thus also support a role for humoral immunity in disease pathogenesis. The reduced serum human IgG levels in the SCID mice treated with anti-IL-10 may be the direct effect of anti-IL-10 on the cells secreting antibodies, where it decreased the production of IgG anti-DNA antibodies, RH-14 and DIL-6 in vivo, possibly by neutralizing the effect of IL-10. The mechanisms responsible for this observation remain completely unclear. The significant reduction in the intensity of immunoglobulin deposition in anti-IL-10 treated RH-14 animals is the reflection of low levels of circulating anti-DNA antibodies in those mice. The electron microscopic studies show that this level of antibody is sufficient to produce deposition of immune complex and cause renal damage. However, the pathological changes in the renal sections are minimal compared with the control groups. These animals also exhibited a low degree of proteinuria compared with controls. This is the first report showing the effect of anti-IL-10 treatment on the nephritis caused by a single pathogenic human anti-dsDNA antibody in a SCID model.

Our conclusions are also supported by an earlier study using an in vivo SCID model. It has been shown that anti-IL-10 mAb had a larger effect on the production of anti-dsDNA antibody than that of total human IgG by peripheral blood mononuclear cells [8]. Further, it also has been observed that increased production of IL-10 could be restricted to B lymphocytes with autoreactive properties [9, 11, 33]. In addition to monocytes and B lymphocytes, some extra immunological sources of IL-10 also influence the immunological imbalance of SLE. In mice, placenta [34] and UV-irradiated keratinocytes [35] produce large amounts of IL-10. In humans these kinds of extra sources may contribute to the exacerbation of SLE by pregnancy and exposure to the sun [36]. All these observations indicate that neutralizing the spontaneous production of IL-10 in SLE patients may represent a new way to interfere with the development of autoimmune process.

This study suggests that in autoimmune disease like SLE where there is a continuous production of autoantibody by the B cells and deposition of immune complex in the tissues, a combined therapy using both anti-IL-10 and anti-C5 mAb may be beneficial to prevent or reduce the effect of humoral immune response while preserving immune complex solubilization and clearance.

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References


