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The Role of Complement in Cryoglobulin-Induced Immune Complex Glomerulonephritis

Marten Trendelenburg,* Liliane Fossati-Jimack,2* Josefina Cortes-Hernandez,2* Daniel Turnberg,* Margarita Lewis,† Shozo Izui,‡ H. Terence Cook,† and Marina Botto3*

Many forms of glomerulonephritis are triggered by Ab localization in the glomerulus, but the mechanisms by which this induces glomerular inflammation are not fully understood. In this study we investigated the role of complement in a mouse model of cryoglobulin-induced immune complex glomerulonephritis. Several complement-deficient mice on a C57BL/6 and BALB/c genetic background were used and compared with strain-matched, wild-type controls. Cryoglobulinemia was induced by i.p. injection of 6-19 hybridoma cells producing an IgG3 cryoglobulin with rheumatoid factor activity against IgG2a of allotype a present in BALB/c, but not C57BL/6, mice. Thus, the cryoprecipitate in C57BL/6 mice consisted of the IgG3 cryoglobulin only (type I cryoglobulinemia) compared with IgG3-IgG2a complexes in BALB/c (type II cryoglobulinemia). The survival of mice was not affected by complement deficiency. Glomerular influx of neutrophils was significantly less in C3-, factor B-, and C5-deficient mice compared with wild-type and C1q-deficient mice. It did not correlate with C3 deposition, but did correlate with the amount of C6 deposited. Deficiency of CD59a, the membrane inhibitor of the membrane attack complex, did not induce an increase in neutrophil infiltration, suggesting that the generation of C5a accounts for the effects observed. There was no apparent difference between cryoglobulinemia types I and II regarding the role of complement. Our results suggest that in this model of cryoglobulin-induced glomerulonephritis the neutrophil influx was mediated by C5 activation with the alternative pathway playing a prominent role in its cleavage. Thus, blocking C5 is a potential therapeutic strategy for preventing renal injury in cryoglobulinemia. The Journal of Immunology, 2005, 175: 6909–6914.

Immune complex glomerulonephritis is an important feature of a number of human diseases, including cryoglobulinemia, systemic lupus erythematosus, serum sickness, and bacterial endocarditis. It is characterized by the formation and/or deposition of Ag-Ab complexes in the glomerulus, followed by an influx of inflammatory leukocytes. The mechanisms inducing the glomerular inflammation and the subsequent tubulointerstitial injury are not well understood.

Complement is well known to mediate the processing of immune complexes (1). However, the relative importance of complement activation by immune complexes in the induction of immune complex-mediated disease manifestations remains unclear. Although the role of the complement system has been investigated in a number of experimental models of immune complex glomerulonephritis using various strategies, the findings are still conflicting. Since the early studies performed by Unanue and Dixon (2), complement has been considered to mainly play a proinflammatory role in the glomerulus. However, recent studies have shown that mice lacking C1q, the first component of the classical pathway of complement activation, develop a more severe glomerular inflammation and thrombosis than their wild-type controls in the accelerated nephrotic nephritis model, suggesting that the role of complement may be protective (3).

Cryoglobulinemia is caused by Igs that precipitate in the cold. Depending on the clonality of the precipitating Igs, three types of cryoglobulinemia can be distinguished (4). Although type I cryoglobulinemia consists of a single monoclonal cryoprecipitable Ig, type II and III cryoglobulins are mixed, i.e., composed of monoclonal (type II) or polyclonal (type III) Abs with rheumatoid factor activity against other Igs. In 1987, Gyotoku et al. (5) described a mouse model of cryoglobulinemia using a cryoprecipitating IgG3 6-19 mAb derived from autoimmune MRL/MpJ-lpr/lpr mice. This Ab had additional rheumatoid factor activity against IgG2a of allotype a. Therefore, depending on the IgG2a allotype, injected mice developed type I or type II cryoglobulinemia. Both types of cryoglobulinemia led to severe glomerular injury with predominant infiltration of polymorphonuclear neutrophils (6), as may sometimes be seen in human disease (7). Additional studies of this model of cryoglobulinemia demonstrated that the glomerular inflammation was dependent on cryoprecipitation of the monoclonal component, but not on its rheumatoid factor activity (8). The infiltrating polymorphonuclear neutrophils played an active role in the development of the wire-loop glomerular lesions observed in this model (9), and the inflammation was not mediated by FcγRs (10) or C3 (11).

In the present study we analyzed the role of complement in the pathogenesis of the initial glomerular inflammation induced by 6-19 IgG3 monoclonal cryoglobulins. We used two different strains of mice developing either type I (C57BL/6 mice) or type II (BALB/c mice) cryoglobulinemia. Our results suggest that C5 had a predominant role in neutrophil recruitment. Furthermore, C5
cleavage occurred only in mice with a fully functional alternative pathway. Interestingly, the glomerular inflammation was independent of glomerular C3 deposition, because C3 deposition occurred via the classical and the alternative pathway to a similar extent.

Materials and Methods

Animals

Complement-deficient (C1q<sup>-/-</sup>, C3<sup>-/-</sup>, Bf<sup>-/-</sup>, CD59<sup>-/-</sup>, BfC2<sup>-/-</sup>, C4<sup>-/-</sup>) mice were generated as described previously (12–14). C5-deficient mice were generated by backcrossing the mutated C5 gene present in DBA/2 mice into C57BL/6 mice for 10 generations. Age-, strain-, and sex-matched, wild-type mice were used in all experiments. Complement-deficient mice studied on the C57BL/6 genetic background were backcrossed to that strain for 10 generations. On the BALB/c genetic background, only C1q<sup>-/-</sup>, C3<sup>-/-</sup>, and CD59<sup>-/-</sup>-deficient mice were available. Mice lacking other complement components, specifically factor B, C2, and C4, were not investigated on the BALB/c genetic background, because these complement genes are part of the H2 region. Therefore, even after extensive backcrossing there would be an MHC mismatch between the backcrossed mice carrying the H<sub>2</sub><sup>a</sup> haplotype of the original 129 embryonic stem cells instead of the H<sub>2</sub><sup>b</sup> haplotype of wild-type BALB/c controls. All animal procedures were performed in accordance with institutional guidelines.

Cryoglobulinemia

Cryoglobulinemia was induced using a protocol modified from that described previously (5). Briefly, 10<sup>7</sup> hybridoma cells producing a cryoprecipitating murine IgG Ab 6-19 were injected i.p. without pretreatment with pristane. To avoid rejection of the hybridoma cells, immunosuppression was achieved by a simultaneous injection of a mixture of anti-mouse IgG3 6-19 induced proliferative glomerulonephritis (ICN) Abs were used for direct immunofluorescence studies. All incubations were performed for 1 h at room temperature, and all Abs were appropriately diluted in PBS. Sections were mounted in Permafluor. In quantitative immunofluorescence studies, to exclude artifacts due to variable decay of the fluorochrome, all sections from one experiment were stained and analyzed at the same time. Sections were examined at ×40 magnification using a BX4 fluorescence microscope (Olympus Optical). A Color Coolview digital camera (Photicon Science) was attached to the microscope, and using Image-Pro Plus software (Media Cybernetics), images were captured for analysis. For each section, 20 glomeruli were examined, and the mean fluorescence intensity was recorded, with results expressed as arbitrary fluorescence units (AFU).<sup>4</sup>

C6 staining was conducted by incubation of the sections with a rabbit anti-mouse C6 Ab (18) (provided by Dr. A. Tenner, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA) diluted 1/400 in TBS/0.1% BSA for 60 min. Ag-bound Abs were detected using a secondary goat anti-rabbit IgG HRP-labeled Ab (DakoCytomation). The sections were then washed with 3,3<sup>-</sup>-diaminobenzidine (Sigma-Aldrich), counterstained with Mayer’s hematoxylin solution (Sigma-Aldrich), and then dehydrated through graded alcohols and xylene. C6-deficient mice (provided by Dr. P. Morgan, Department of Biochemistry, University of Wales College of Medicine, Cardiff, U.K.) were used as negative controls. For a quantitative assessment of glomerular C6 deposition, the observer was blinded to sample identity, and the intensity of glomerular staining was ranked with 0 indicating the lowest staining intensity.

For the quantification of glomerular macrophages, kidneys were fixed in paraformaldehyde-hyline-periodate for 4 h, then transferred to 7% sucrose overnight before snap-freezing in isopentane and storage at −70°C. For the staining, a primary monoclonal rat anti-mouse CD68 Ab (Serotec) was used. Sections were then dehydrated with a 1% solution of hydrogen peroxide in 50% methanol. A mouse anti-rat secondary Ab and a rat peroxidase anti-peroxidase tertiary Ab (both purchased from Jackson ImmunoResearch Laboratories) were then applied. The sections were developed with 3,3<sup>-</sup>-diaminobenzidine, counterstained with Mayer’s hematoxylin solution, and then dehydrated through graded alcohols.

Statistical analysis

All values described in the text and figures are expressed as the median and range. Statistical analysis was conducted using PRISM 3.2 (GraphPad). Nonparametric tests were applied throughout, with differences considered significant at \( p < 0.05 \).

Results

The i.p. implantation of hybridoma cells secreting the cryoprecipitating murine IgG3 6-19 induced proliferative glomerulonephritis with prominent endocapillary hypercellularity and neutrophil infiltration (Fig. 1A). This was followed by systemic disease manifestations. The mice usually developed severe signs of disease between 8 and 12 days after injection. As expected, complement-deficient mice had no survival advantage over wild-type controls (data not shown). Renal disease was usually best seen on days 7–8 after the hybridoma injection and before the development of other clinical signs. Therefore, this time point was chosen for all additional analyses. Mice that rejected the hybridoma cells were excluded from further analysis.

<sup>4</sup>Abbreviation used in this paper: AFI, arbitrary fluorescence intensity.
globulinemia. As shown in Figs. 1 and 2, C3- and factor B-
A
C57BL/6 genetic background. These mice developed type I cryo-
The model of cryoglobulinemia was first analyzed in mice with a
Type I cryoglobulinemia
The model of cryoglobulinemia was first analyzed in mice with a
C57BL/6 genetic background. These mice developed type I cryo-
A
merular tuft and prominent infiltration of neutrophils. C1q-deficient mice showed a similar appearance (B). In mice lacking factor B (C) or C3 (D), there was less glomerular hypercellularity and fewer neutrophils in the glomeruli (periodic acid-Schiff stain; magnification, ×40).

FIGURE 1. Light microscopy of glomeruli 7 days af-
ter peritoneal implantation of hybridoma cells in mice on
a C57BL/6 background. Wild-type mice (A) showed pro-
iferative glomerulonephritis with enlargement of the glo-
mericulin-induced immune complex glomerulonephritis.

Type II cryoglobulinemia
In a second step, the model of cryoglobulinemia was applied to
mice on a BALB/c genetic background. Because BALB/c mice
have an IgG2a of allotype α, the cryoprecipitating IgG3 had ad-
ditional rheumatoid factor activity leading to a coprecipitation of
monoclonal IgG3 and polyclonal IgG2a. Therefore, in these mice
the model resembled type II cryoglobulinemia.

As seen in C57BL/6 mice, C3-deficient mice on a BALB/c ge-
genetic background were also protected from severe glomerular neu-
A
phil infiltration, whereas C1q-deficient mice were similar to the
wild-type controls (Fig. 5A), indicating that the alternative path-
way played a predominant role even in this model of disease. The
differences observed could not be attributed to variation in glo-
mericulin-induced immune complex glomerulonephritis.

Taken together, these results indicated that there was no direct
pathogenic role for C3 and that neutrophil recruitment was depend-
ent on activation of the late complement components predomi-
nantly via the alternative pathway. To provide additional support
for this observation, glomerular neutrophil infiltration in diseased
mice was compared between wild-type and C5-deficient mice. As
seen for C3- and factor B-deficient mice, C5 deficiency led to
significantly reduced glomerular neutrophil infiltration, confirming
the suggested role for the late complement components in neutro-
phil recruitment (Fig. 4). This could be mediated by the membrane
attack complex (C5b-9) or by the chemotactic properties of
cleaved C5. To address this question, we analyzed mice deficient
in CD59a, the cell surface inhibitor of the membrane attack com-
plex. Compared with wild-type controls, similar numbers of glo-
mericulin-induced immune complex glomerulonephritis.

To analyze whether the reduced neutrophil influx in alternative
pathway-deficient mice (i.e., factor B- and C3-deficient mice) was
due to reduced complement deposition, kidney sections were
stained for C3 and C6. Although there was a trend toward less glu-
merular C3 deposition in C1q- and factor B-deficient mice, there
was no overall difference compared with wild-type mice
(Fig. 3A). Furthermore, C5 deposition in C1q- and factor B-defi-
cient mice was very similar, suggesting that both pathways of com-
plement activation were activated by the deposited cryoglobulins.
However, in contrast to the C3 deposition, staining for glomerular
C6 reflected the differences in glomerular neutrophil influx, with
significantly less C6 deposition in the C3- and factor B-deficient
mice compared with wild-type controls (Fig. 3B). The presence
of very low level of C6 deposits in C3-deficient mice may represent
local C6 synthesis.
I cryoglobulinemia, C6 deposition strongly reflected the number of infiltrated neutrophils, with almost undetectable levels in C3-deficient mice (Fig. 5B). Therefore, although it was not possible to test factor B-deficient mice on a BALB/c genetic background, the same prominent role of the alternative pathway of complement as that seen in mice with type I cryoglobulinemia (C57BL/6) was likely. In addition, we were able to address the question of whether neutrophil influx is due to an effect of the membrane attack complex (C5b-9) or to the chemotactic properties of cleaved C5 by analyzing mice deficient in CD59a. Similar to the results obtained in C57BL/6 mice, the comparison of CD59a-deficient and wild-type mice did not reveal a significant difference in the number of glomerular neutrophils. The median number of neutrophils was 6.2 (range, 0.5–8.2) in wild-type mice and 5.2 (range, 3.2–7.1) in CD59a-deficient mice, again supporting a major role for C5a in neutrophil recruitment in this model of cryoglobulin-induced immune complex glomerulonephritis.

**Discussion**

In this study we demonstrated that complement plays a predominant role in neutrophil recruitment in a model of cryoglobulin-induced immune complex glomerulonephritis. Neutrophils have been shown to be the major inflammatory cells, which are initially observed at the sites of tissue injury induced by 6-19 monoclonal cryoglobulins including the kidney (6). Although neutrophils

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total serum IgG3 (mg/ml)</th>
<th>Cryoprecipitating IgG3 (mg/ml)</th>
<th>Glomerular IgG3 deposition (AFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.26 (0.64–1.59)</td>
<td>0.59 (0.2–5.8)</td>
<td>27.8 (23.7–32.0)</td>
</tr>
<tr>
<td>C1qa−/−</td>
<td>1.0 (0.84–1.84)</td>
<td>0.48 (0.4–1.1)</td>
<td>31.5 (23.5–46.1)</td>
</tr>
<tr>
<td>C3−/−</td>
<td>0.84 (0.52–1.84)</td>
<td>0.69 (0.3–2.9)</td>
<td>31.6 (22.3–43.6)</td>
</tr>
<tr>
<td>Bf−/−</td>
<td>0.91 (0.68–1.89)</td>
<td>1.75 (0.7–2.9)</td>
<td>31.3 (23.0–37.1)</td>
</tr>
</tbody>
</table>

Data are expressed as the median, with the range of values in parentheses. There was no significant difference between the groups of mice.
played an active role in the development of wire-loop glomerular lesions (9), how the glomerular deposits of 6-19 cryoglobulins led to neutrophil infiltration remained unclear (11). In the previous study it was shown, using cobra venom factor to deplete murine C3, that complement deposition did not play a major role in the development of the wire-loop lesions, but glomerular neutrophil infiltration was not quantitated in the C3-depleted animals. Our study using complement-deficient mice, demonstrated that there was an important role for complement in the recruitment of neutrophils to the glomeruli. There are, however, some differences between the studies. In our study we did not pretreat the animals with pristane, because this might cause an additional and independent effect on complement activation. Consequently the cryoglobulin concentrations achieved in our model were markedly lower than those reported previously (6, 8) and were not sufficiently high to induce skin vasculitis in BALB/c mice. Furthermore, the predominant glomerular lesions we found were neutrophil infiltration, with no evidence of wire-loop lesions, indicating that the level of circulating cryoglobulins was not sufficient to lead to visible deposits in glomerular capillary lumens or beneath the endothelium.

The role of complement in our study seemed to be independent of the type of cryoglobulinemia, because C57BL/6 mice (type I cryoglobulinemia) and BALB/c mice (type II cryoglobulinemia) developed similar glomerular lesions, and the results in complement-deficient mice of both strains were similar. The neutrophil influx observed in this study was likely to be C5 dependent, because C5-deficient mice had the lowest numbers of glomerular neutrophils detected. This would be consistent with the fact that C5a, the major cleavage product of C5, is well known to be an important chemoattractant for neutrophils. Although not excluded, it is unlikely that the membrane attack complex played a major role in this model of cryoglobulinemia, because CD59a-deficient mice had similar disease expression to wild-type controls.

FIGURE 4. Neutrophil recruitment in wild-type and C5-deficient C57BL/6 mice developing type I cryoglobulinemia 7 days after injection of hybridoma cells. The number of neutrophils was significantly reduced in C5-deficient mice. The horizontal bar represents the median of each group.

FIGURE 5. Type II cryoglobulinemia in wild-type and C1q- and C3-deficient BALB/c mice 8 days after injection of 6-19 hybridoma cells. A. Mean number of neutrophils per glomerulus. Although C1q-deficient mice showed similar neutrophil influx compared with wild-type controls, the glomerular neutrophils were significantly reduced in C3-deficient mice. B. Ranking of glomerular C6 deposition revealed a similar staining intensity between C1q-deficient and wild-type mice. C3-deficient mice had almost undetectable C6 deposition compared with controls. The horizontal bar represents the median of each group.
References