Membrane complement regulators protect against fibrin exudation increases in a severe peritoneal inflammation model in rats

Masashi Mizuno,1,2 Yasuhiro Ito,1,2 Tomohiro Mizuno,3 Claire L. Harris,4 Yasuhiro Suzuki,1,2 Noriko Okada,5 Seiichi Matsuo,2 and B. Paul Morgan4

1Renal Replacement Therapy, 2Division of Nephrology, 3Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; 4Complement Biology Group, Infection, Immunology and Biochemistry, School of Medicine, Cardiff University, Cardiff, UK; and 5Immunology, Nagoya City University Graduate School of Medicine, Nagoya, Japan

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Mizuno M, Ito Y, Mizuno T, Harris CL, Suzuki Y, Okada N, Matsuo S, Morgan BP. Membrane complement regulators protect against fibrin exudation increases in a severe peritoneal inflammation model in rats. Am J Physiol Renal Physiol 302: F1245–F1251, 2012. First published February 15, 2012; doi:10.1152/ajprenal.00652.2011.—Peritonitis and the rare sequela of encapsulating peritoneal sclerosis (EPS) are serious problems in patients on peritoneal dialysis therapy. Chronic and persistent peritoneal injuries may be a risk factor of EPS. We previously reported that a chronic, proliferative peritonitis developed when zymosan was administered intraperitoneally following scraping injury of rat peritoneum (Mizuno M, Ito Y, Hepburn N, Mizuno T, Noda Y, Yuzawa Y, Harris CL, Morgan BP, Matsuo S. J Immunol 183: 1403–1412, 2009). Peritoneal membrane complement regulators (CRegs), especially Crry and CD59, protected from injury by inhibiting local complement activation, suggesting that CRegs play important roles in maintaining homeostasis in rat peritoneum. Here, we investigated roles of complement in the development of EPS by neutralizing CReg function with monoclonal antibodies (MAbs). Proliferative peritonitis was induced by scraping the peritoneum, followed by daily intraperitoneal administration of zymosan. When either Crry or CD59 alone was neutralized by MAb, the tissue injuries were not significantly changed compared with rats without neutralizing MAb. When both Crry and CD59 were neutralized in this model, severe fibrin exudation was observed on the peritoneal surface on day 5, accompanied by inflammatory cell infiltration, resembling the early stages of development of EPS. Dense peritoneal deposition of C3 fragments and membrane attack complex were observed, along with the fibrin exudates. Intravenous administration of cobra venom factor, which profoundly activates complement, further enhanced these pathological changes. Our results show that complement activation in injured peritoneum drives peritoneal inflammation, and that enhancement of complement activation by inhibiting CReg and/or enhancing systemic activation contributes to the initiation of EPS; therefore, anti-complement agents might be of therapeutic value in humans for the treatment of EPS.

peritonitis; peritoneal dialysis; complement regulation; zymosan

PERITONITIS IS A SERIOUS PROBLEM for patients on peritoneal dialysis (PD) therapy and often prevents adherence to long-term PD therapy in Japan and around the world (13, 28). Peritonitis is a risk factor for the development of encapsulating peritoneal sclerosis (EPS), a potentially lethal complication of PD. Exudation of fibrinogen and the development of fibrin layers along the peritoneal surface are early pathological changes seen in EPS. In the advanced stages, changes in fibrous encapsulation involve the intestine and progress to paralytic ileus, which has a high mortality and poor prognosis. Prognosis of fungal or yeast peritonitis is particularly poor, because these infections can induce EPS, even after a single episode (2, 6). Our laboratory previously reported a severe proliferative peritonitis that developed when zymosan was administered following mechanical scrape injury of rat peritoneum (Zy/scrape peritonitis) (14). Zymosan is a cell membrane component of yeast and activates complement (C) through the alternative pathway (30). When scraping was used to mimic the physical stress placed on the peritoneum in PD therapy, local expression of membrane C regulators (CRegs) along the peritoneum was reduced, demonstrating uncontrolled C activation and stressing the importance of C regulation in the peritoneum (26). Notably, Crry and CD59 collaborated to protect against autologous C activation in rat peritoneum, demonstrating their importance in maintaining peritoneal homeostasis (20) and further implicating the C activation system in peritoneal dysfunction.

C plays key roles, not only in the maintenance of host homeostasis by eliminating infectious microorganisms and abnormal cells as a part of innate immunity, but also in the regulation of cellular immunity for both T and B cells, and in controlling fertilization (11, 31). In contrast, uncontrolled C activation can induce inflammation in various tissues (15) and augment inflammatory conditions, such as some collagen diseases (16). There are numerous reports showing that CRegs are important in preventing excessive C activation in host tissues (15). Because the development of EPS involves chronic inflammation and fibrosis, C activation might also be related to the induction and/or development of EPS.

In regard to the progression to EPS pathology, a two-hit theory has been proposed by Kawanishi et al. (6, 7). The first hit to the peritoneum and initial peritoneal injuries is caused by a combination of long-term usage of nonphysiological peritoneal dialysates, particularly with low pH and high osmolarity, and repeated mechanical stress caused by the PD catheter. The second hit, for example, is provided by repeated peritoneal infections and is an obligatory step for the development of EPS. In the present study, we investigated whether the further enhancement of C activation by functional blocking of two CRegs, Crry and CD59, with or without cobra venom factor (CVF) as an enhancer of systemic activation, could provide a second hit in the Zy/scrape model and cause the development
of EPS-like peritoneal changes that mimic the course of fungal peritonitis in PD patients.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing ~250 g (Japan SLC, Hamamatsu, Japan) were used for in vivo experiments in the present study. Animals were maintained under conventional laboratory conditions and given free access to water and food. Rats were operated on, intraperitoneally (ip) injected, and/or killed under ether anesthesia. All animal experiments described herein were approved by the Nagoya University Animal Committee and were carried out according to the Regulations on Animal Experiments in Nagoya University.

Reagents and antibodies. Zymosan A was purchased from Sigma-Aldrich (St. Louis, MO). "Dianead PD-4 4.25%" was used as a 4.25% PD fluid (pH of 5.5; Baxter, Tokyo, Japan). Anti-rat Cry [monoclonal antibody (MAb) S12] and anti-rat CD59 (MAb 6D1) were characterized as previously described (4, 17). Both MAbs are mouse MAbs of isotype IgG1; that block the function of their respective CRegs. Fluorescein isothiocyanate (FITC)-labeled rabbit antirat mouse IgG (Cappel) was purchased from Cappel Laboratories (Westchester, PA). To observe C3b and C5b-9 [membrane attack complex (MAC)] deposition, we used FITC-rabbit anti-rat C3 (Cappel, Solon, OH) and our in-house polyclonal rabbit anti-rat C9, respectively (14, 12, 18, 19), followed by incubation with FITC-goat anti-rabbit IgG (Cappel). For the detection of inflammatory cells, we used FITC-labeled mouse monoclonal anti-rat leuocyte common antigen (LCA) (clone OX1; Chemicon International, Temecula, CA) and FITC-labeled mouse anti-rat monocyte (clone ED1; Serotec, Indianapolis, IL). To visualize fibrinogen deposition, we used FITC-labeled rabbit anti-human fibrinogen cross-reactive against rat fibrinogen (DakoCytomation, Glostrup, Denmark).

Experimental protocol. In the present study, we used a zymosan-induced peritonitis model induced by five daily ip injections of 5 mg zymosan mixed with 4.25% PD fluid (2 ml for day 0, 5 ml for day 1, and 10 ml for the following days) in rats prepared by mechanical scraping of the right side of the parietal peritoneum (Zy/scrape peritonitis), as described previously (14).

First, the roles of two CRegs, Cry and CD59, were tested by blocking with 0.5 mg of MAbs S12 or 6D1, respectively, either individually or in combination, on day 5.

Second, to investigate the requirement of C activation in the acceleration of peritoneal injuries following blockade of Cry and CD59, systemic C activation was achieved by intravenous administration of 25 units of CVF in 0.5 ml of isotonic saline or vehicle for 10 min after neutralization of CRegs.

All rats were killed on day 6 for histological and immunohistochemical analysis. The detailed experimental protocol is shown in Table 1.

Histological analysis. The parietal peritoneum was dissected and cut into strips of ~5 × 20 mm, and a total of four strips from the right-side parietal peritoneum with mechanical scraping were obtained. Two strips were fixed in 20% buffered formalin, embedded in paraffin, cut into 4.5-μm-thick sections, and stained with hematoxylin and eosin for histological analysis. The other two strips were used for immunohistochemical analysis. To estimate peritoneal thickness as a measure of tissue damage in the parietal peritoneum, 20 random fields for each rat were observed at ×50 and ×100 magnifications under light microscopy (LM), and the average peritoneal thickness was recorded for each rat.

Immunohistochemical analysis. The peritoneal strips were snap-frozen, cut into 4.5-μm-thick sections on a cryostat, and fixed in acetone, according to our laboratory’s previous report (14). For the detection of infiltrated cells, frozen sections were incubated with FITC-labeled anti-rat LCA or with FITC-labeled ED1. The number of LCA-positive cells or ED1-positive cells was calculated by the following formula: number of LCA or ED1-positive cells = (total number of LCA- or ED1-positive cells in 20 sequential peritoneal areas under ×200 magnification)/20.

To investigate C3b deposition, FITC-labeled rabbit anti-rat C3 was incubated with frozen sections. For the detection of C5b-9, frozen sections were incubated with rabbit anti-rat C9, followed by incubation with FITC-labeled goat anti-rabbit IgG absorbed with untreated rat serum (1:1 vol/vol). For C3 and C9 staining, normal rabbit serum was used as a negative control for antibody-treated samples, and the peritoneum of untreated rats was provided as an additional untreated negative control. To score the deposition of C3b or C5b-9, a semiquantitative scale was used, as follows: 0, minimum to trace amounts along the peritoneal surface; 1, deposition of up to one-half of the area within the thin layer; 2, deposition of more than one-half of the area within the thin layer; 3, deposition extending into the deep layer in addition to 2; and 4, deposition of >80% of the area of a visual field. For the semiquantitative evaluations of fibrin deposition in the peritoneum, frozen sections were stained with FITC-labeled rabbit anti-human fibrinogen cross-reactive against rat fibrinogen and fibrin. The degree of fibrin deposition was scored as follows: 0, minimum to trace amounts along the peritoneal surface; 1, deposition of up to one-half of the area of a visual field; 2, deposition of more than one-half of the area; and 3, deposition extending into the deep layer in addition to 2. The C3b, C5b-9, or fibrin deposition index was calculated as the average of the total score for 20 fields under ×200 magnification.

Statistical analysis. All values are expressed as means ± SE. Statistical analysis was performed by one-factor ANOVA. When significant differences were present, data were further analyzed using the Wilcoxon test between two groups. P values of <0.05 (5%) were considered to be statistically significant.

RESULTS

Macroscopic findings after blocking of CRegs, Cry and CD59, individually or in combination, in zymosan-induced peritonitis. After 5 days of daily administration with zymosan in the mechanical scraping rat model (Zy/scrape peritonitis), white color changes were observed on the surface of the parietal peritoneum, and surface vessels were obscured, similar to the findings in our laboratory’s previous report (Fig. 1A) (14). When single blocking MAbs were injected 24 h before death, no obvious macroscopic changes were observed in the peritoneum between those with and without CReg neutralization (Fig. 1, A–C). In contrast, simultaneous blocking of Cry and CD59 resulted in increased edematous and fibrinous changes with adhesions on the liver and intestine compared with rats without blocking of CRegs (Fig. 1D). Untreated peritoneum was smooth, and vessels running under it were clearly visible (Fig. 1E).

Table 1. In vivo experimental protocol

<table>
<thead>
<tr>
<th>Group No.</th>
<th>n</th>
<th>Zymosan</th>
<th>MAb</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>5 mg × 5 days</td>
<td>Vehicle</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5 mg × 5 days</td>
<td>S12</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5 mg × 5 days</td>
<td>6D1</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>5 mg × 5 days</td>
<td>S12 + 6D1</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>5 mg × 5 days</td>
<td>S12 + 6D1</td>
<td>Vehicle</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>5 mg × 5 days</td>
<td>S12 + 6D1</td>
<td>CVF</td>
</tr>
</tbody>
</table>

n, No. of rats. Zymosan was injected intraperitoneally once a day for 5 consecutive days after the mechanical scraping of the parietal peritoneum. Monoclonal antibody (MAb) administered on day 5 was 1 mg of each MAb per rat, or 0.5 mg of each S12 and 6D1 together per rat. Posttreatment. 25 units per 0.5 ml of cobra venom factor (CVF) in isotonic saline per rat, or 0.5 ml of isotonic saline per rat, were intravenously injected 10 min after intraperitoneal injection of MAbs S12 + 6D1.
rats at significantly higher numbers than in the other groups (Fig. 4). On the other hand, deposition of C3b and C5b-9 along the peritoneum was clearly and significantly increased when both Crry and CD59 were blocked in this model (group 4).

**DISCUSSION**

Our laboratory previously showed that both acute and chronic peritoneal inflammation in Zy/scrape peritonitis were partly dependent on C activation (14). In the present study, we investigated whether unregulated activation of the C system could trigger further peritoneal damage, including fibrin exudation on the surface of the injured peritoneum, a hallmark of the early stages of EPS. When the CRegs, Crry and CD59, were functionally blocked with MAbs in this Zy/scrape peritonitis model, fibrin exudation and fibrinogen deposition along the peritoneal surface, and the accumulation of inflammatory cells and deposition of C activation products in the peritoneum, were all increased. Importantly, these events required the simultaneous blockade of Crry and CD59; blocking of either alone had no discernable effect, demonstrating that dysregulation at both the C3 convertase and MAC assembly stages of C activation was necessary to induce EPS and strongly implicating C5a and MAC as triggers. Tissue deposits of C3b and MAC were detected only when both CRegs were blocked, confirming their involvement in amplifying tissue damage (14). When acute C activation was generated by a bolus intravenous injection of CVF, deposition of fibrinogen, accumulation of inflammatory cells, and thickening of the peritoneum were further enhanced compared with the injuries seen in the Zy/scrape peritonitis model with blocking of both Crry and CD59. These results...
suggest that exudation of fibrinogen along the peritoneum is induced by unregulated C activation in the peritoneum, a result of impaired CReg function, and is likely associated with an increase in vascular permeability of the peritoneal surface driven by C activation products such as C3a, C5a, and MAC, which are known chemotactic, anaphylactic, and cytotoxic factors (10, 16).

A two-hit theory has been proposed for the progression to EPS pathology (6). Peritoneal fibrosis has been considered as a pathological hallmark of the pre-EPS state (22). Indeed,

![Image of peritoneal thickness and severity of fibrin deposition along peritoneal surface.](image1)

![Image of fibrin exudation and deposition of C3b and C5b-9 in the peritoneum.](image2)
fibrosis in EPS was associated with the production of transforming growth factor (TGF-β), which was reported to be an important mediator of EPS development in PD patients (1, 9), and in animal models, where suppression of TGF-β production decreased peritoneal fibrosis (27). Our human study also showed the importance of TGF-β1 and connective tissue growth factor in the progression of peritoneal impairment in PD patients with ultrafiltration failure (21). Although peritoneal fibrosis is common in patients on long-term PD, EPS does not always occur in the peritoneum.

Fig. 4. Peritoneal thickness, deposition of fibrin, leukocyte common antigen (LCA)- and ED1-positive cells, and C3 and C5b-9 depositions in the peritoneum. Peritoneal thickness (A), fibrin deposition (B), accumulation of LCA- (C) and ED1-positive cells (D), C3b deposition (E), and C5b-9 deposition (F) were measured in the Zy/scrape peritonitis model, with or without monoclonal antibody blocking of CRegs. When both CRegs (Cry and CD59) were blocked (group 4), peritoneal thickness, deposition of fibrin, numbers of LCA-positive cells and ED1-positive cells, and deposition of C3b and C5b-9 were significantly increased in the peritoneum compared with all other groups.

Fig. 5. Enhancement of C activation by cobra venom factor (CVF) administration in rats with blocked CRegs, Cry and CD59. Compared with vehicle alone (group 5; A), administration of CVF augmented macroscopic peritoneal injuries with enhanced fibrin exudation (group 6; B). Peritoneal thickness (C and D), fibrin layer formation (arrows), fibrin exudation (E and F), deposition of C3b (G and H), and deposition of C5b-9 (I and J) were all increased following CVF treatment in the peritonitis model. *The peritoneal surface. Original magnification is ×100 for C and D, and ×200 for E–J. Scale bars are in the top left corner of C and E. LM, light microscopy.
Other factors influencing disease progression include the level of IL-6, which is strongly implicated in the progression to chronicity of other inflammatory diseases (5). In recent reports, mineralocorticoids, macrophages, and angiogenesis have all been implicated in the progression of fibrosis (22, 25, 26, 29). Although many factors have been linked to fibrosis, the precise mechanism of progression to EPS remains unresolved.

Fungal or yeast peritonitis is associated with a poor prognosis in PD patients. Although the development of EPS usually requires repeated peritoneal infections, it can sometimes be induced by a single episode of peritonitis caused by a fungal or yeast infection (6, 24). Zymosan, a yeast cell wall component, induced C-dependent proliferative peritonitis in the presence of a physical peritoneal injury induced by mechanical scraping (14), suggesting that C activation could be an initiating factor for acute and chronic peritoneal inflammation (14). Although this severe inflammation continued for over 2 mo, it did not directly proceed to an EPS state. It was considered that some additional activators and/or enhancers might be required for the development of EPS in chronic peritoneal fibrosis; these may represent the other second hits, as defined by the two-hit theory for EPS (3). Here, we show that local dysregulation of C by blocking CRegs on the peritoneum induced fibrin exudation in the Zy/scraping peritonitis model, resulting in the formation of a thick fibrin layer and associated adhesions along the injured peritoneum, closely resembling the initial phase of EPS. The data suggest that the C activation system plays a role, not only in the first hit, but also in the second hit for the progression of EPS pathology. These injuries were further enhanced by acutely activating systemic C to amplify the second hit.

In summary, we showed that local blockade of two CRegs, Crry and CD59, in the Zy/scrape rat peritonitis model augments peritoneal injuries and causes an increase in fibrin exudation along the peritoneum. These early EPS-like findings were induced by C activation and exacerbated by acute systemic C activation. These results suggest that dysregulated C activation in the peritoneum is a factor that leads to EPS in human PD patients. EPS is a lethal complication of PD therapy with very limited treatment options. Surgical therapy, steroids, and immune-suppressive agents, such as tamoxifen, have all been used to treat EPS in humans (7, 8, 23), but none has much effect on prognosis. Our results suggest that anti-C therapy, targeting C5a and/or MAC formation, might be a useful strategy for preventing or inhibiting the development of EPS in PD patients. Eculizumab, an anti-C5 MAb that blocks C5 cleavage, preventing formation of C5a and MAC, is already in the clinic and is an attractive candidate for prevention and/or treatment of EPS.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.M. conception and design of research; M.M., T.M., Y.S., and N.O. performed experiments; M.M. and Y.I. analyzed data; M.M., Y.I., C.L.H.,...
and S.M. interpreted results of experiments; M.M. prepared figures; M.M., N.O., and B.P.M. drafted manuscript; M.M. and B.P.M. edited and revised manuscript; M.M. approved final version of manuscript.

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