Specific collaboration between rat membrane complement regulators Crry and CD59 protects peritoneum from damage by autologous complement activation

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Abstract

Background. The peritoneal cavity is isolated from the outside and is usually a sterile environment. Patients on peritoneal dialysis (PD) have PD fluid (PDF) infused into the peritoneal cavity. We previously showed that unregulated complement activation could contribute to the development of peritoneal inflammation in yeast peritonitis in PD therapy. In that situation, suppression of local complement activation is essential to protect the host from further injury. The membrane complement regulators (CRegs), Crry, CD55 and CD59, are expressed in the rat peritoneum, especially along the mesothelial cell layer.

Methods. We investigated CRegs’ functional roles in the peritoneal cavity using blocking mAb against each CReg and complement activation in different PDFs.

Results. Blockade of any single CReg did not cause spontaneous peritoneal injury in rat. Combined blockade of Crry and CD59 induced focal peritoneal tissue injury and heavy accumulation of inflammatory cells with peritoneal edema at 24 h. Deposits of C3 and C5b-9 were found on the peritoneal surface after combined blocking of Crry and CD59. Systemic complement depletion by cobra venom factor abrogated these inflammatory changes. When combined blockade of Crry and CD59 was performed with PDF of different pH and glucose concentration in rats, the peritoneal injuries were enhanced with lower pH and higher glucose concentration. These results were confirmed by in vitro experiments using primary rat mesothelial cell culture.

Conclusions. Rat CRegs, Crry and CD59, specifically collaborate to control complement activation in rat peritoneum. During PD, impairment of CReg might contribute to the development of severe peritoneal inflammation.

Keywords: complement; immunology; peritoneal dialysis; peritoneal membrane

Introduction

Complement (C) activation play important roles in innate immunity, protecting the host from invading microorganisms and tumor cells. However, uncontrolled activation of C is harmful for the host. C regulators (CRegs) play important roles to control C activation in the host in both the fluid phase in plasma and solid phase on the cell membrane [1]. Membrane CRegs exist on the membranes to protect self tissues. In animal experiments, including our reports, neutralization of CRegs acting at the C3 convertase level exacerbated various pathologies [2–4]. In most tissues, single neutralization of CD59, a membrane attack complex (MAC; C5b-9) regulator, did not cause significant pathologic changes. An exception was the joint, where neutralization of CD59 induced synovial inflammation [5]. We have shown that inflammation was enhanced in joint and kidney when rat CRegs Crry and CD59 were simultaneously blocked at these sites [6,7].

It is known that some physiological states, such as acidosis, induce C activation [8]. Exposure of the peritoneum to peritoneal dialysis (PD) fluid (PDF) commonly causes physical stresses, including hypertonic and acidic stress [9,10]. In pathogenic conditions such as peritonitis in PD patients, increased C activation products were detected in serum, while C activation products were observed in the peritoneal cavity in chronic PD patients [11–14]. In vitro, exposure of human peritoneal mesothelial cells to PDF caused up-regulation in production of C3 and C4, suggesting that local C production in peritoneum might be asso-
associated with host defense and inflammation [12]. It was also reported that mannose-binding lectin, the key component of the lectin pathway, was lower in sera of chronic PD plasma, perhaps causing increased infection susceptibility [15]. However, there is still very little information regarding peritoneal C activation in PD patients exposed to PDF.

Information on peritoneal expression of CReg is limited. CD59, a CReg inhibiting at MAC level, was expressed on mesothelium in human peritoneum [16]. In rats, we previously described expression of Crry, CD55 and CD59, but not CD46 [17], and we showed that decreased CReg expression contributed to the development of peritonitis [18]. However, there are no reports describing the functional roles of CRegs in normal peritoneum. Here, we investigated roles of CRegs using combinations of mAbs to neutralize function of single or multiple CReg in rat peritoneum, particularly in the context of exposure to PDF.

### Materials and methods

#### Animals

Male Sprague-Dawley (SD) rats weighing ~250 g (Chubu Kagaku Shizai; Nagoya, Japan) were used. All animal experiments were carried out accord-

### Table 1. In vivo experimental protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats (n)</th>
<th>Pretreatment</th>
<th>Injected mAbs (mg/each rat)</th>
<th>Timing of the observation (hours)</th>
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<td>1</td>
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<td>$\beta$1, $\beta$DIII, 6D1</td>
<td>6, 24, 72</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>None</td>
<td>0.5</td>
<td>0</td>
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<tr>
<td>3</td>
<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
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<td>0.5</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>8</td>
<td>6</td>
<td>CVF</td>
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</tr>
<tr>
<td>9</td>
<td>6</td>
<td>Vehicle</td>
<td>0.5</td>
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</table>

#### Fig. 1. Binding of anti-CRegs in peritoneum after 24 h. Typical patterns of binding of anti-Cry (Group 2; frame B), anti-CD55 (Group 3; frame C) or anti-CD59 (Group 4; frame D) at 24 h post-injection. Combination of two anti-CRegs, selected from anti-Cry, anti-CD55 and anti-CD59, was also administered; the binding of mAbs is shown in frames E (Cry/CD55; Group 5), F (CD55/CD59; Group 6) and G (Cry/CD59; Group 7). Frame A is a negative control injected with vehicle only. The peritoneal surface was labeled as ‘*’. Arrowheads show the IgG binding. Original magnification is x200. The scale of specimens is shown in the left-upper corner of frame A.
According to the Animal Experimentation Guide of Nagoya University School of Medicine.

Reagents and antibodies

Mouse mAbs against rat CRegs Crpy (mAb 512), CD46 (MM.1), CD55 (RDIII-7) and CD59 (6D1) were characterized as described [17,19–21]. All mAbs were mouse mAbs of isotype IgG1; all except MM.1 were known to block the function of the respective CReg. FITC-labeled rabbit anti-mouse IgG was purchased from Cappel Labs (Westchester, PA) and was absorbed with normal rat serum (RS). Cobra venom factor (CVF) was purified from lyophilized cobra venom *Naja naja* (Sigma-Aldrich, St. Louis, MO) and was administered as described [5]. Systemic C depletion was confirmed by measuring C hemolytic activity [5].

Experimental protocol

Firstly, rats were intraperitoneally (i.p.) injected with 0.5 mg of mAb, either singly or in combinations according to Table 1. These mAbs were diluted in 10 mL of 4.25% PDF (Dianeal PD-4 4.25%TM; pH ~5; Baxter, Tokyo, Japan; PD4 4.25%). After 24 h, rats were sacrificed and parietal peritoneum collected for examination. To confirm the requirement for C activation to develop peritonitis following blockade of Crpy and CD59, systemic C inhibition was achieved using 25 units CVF in 0.5 mL isotonic saline intravenously 24 h before neutralization of CRegs.

Effects of neutralizing Crpy and CD59 under different osmotic and pH conditions

In a separate experiment, we simultaneously neutralized two CRegs, Crpy and CD59, using mAbs diluted in sterile PBS (isotonic, pH7.4), 1.5% (pH ~6) PDF (Dianeal-N PD-4 1.5%TM; pH ~6; Baxter; NPD4 1.5%), 1.5% (pH ~5) PDF (Dianeal PD-4 1.5%TM; pH ~5.0; Baxter; PD4 1.5%) or 4.25% (pH ~5) PDF (PD4 4.25%). After 24 h, rats were sacrificed and parietal peritoneum collected for examination.

Histological analysis

The parietal peritoneum was dissected in strips (four strips, ~5 × 30 mm, from each side of parietal peritoneum). Two strips from each side of the parietal peritoneum were randomly selected, fixed in 20% buffered formalin and embedded in paraffin. Sections of 4.5 μm thick were stained with hematoxylin and eosin for histological analysis. The other strips were used for immunohistochemical analysis. To estimate tissue damage in parietal peritoneum, 20 fields were randomly observed and scored at ×100 magni-
fication under light microscopy (LM). The severity of peritoneal damage in each rat was scored according to the following formula:

Severity of peritoneal damage (%) = \left( \frac{\text{total number of fields with peritoneal damage}}{20} \right) \times 100

Degree of peritoneal damages in each rat was estimated as follows:
0, 0% peritoneum damaged; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%.

Immunohistochemical analysis

Peritoneal strips obtained as above were snap-frozen, sections of 4.5 μm thick were prepared with a cryostat and fixed in acetone according to our previous report [18]. For the detection of infiltrating cells, frozen sections were incubated with FITC-labeled mouse anti-rat leukocyte common antigen (LCA) mAb (clone OX1; Chemicon International, Temecula, CA) or with FITC-labeled mouse anti-rat monocyte mAb (clone ED1; Serotec, Indianapolis, IL). To investigate C3 deposition, FITC-labeled polyclonal goat anti-rat C3 (Cappel, Solon, OH) was incubated on frozen sections. For the detection of MAC, frozen sections were incubated with rabbit anti-rat C9 followed by incubation with FITC-labeled goat anti-rabbit IgG (Cappel) absorbed with normal RS (1:1/v:v). For C3 and C9/MAC staining, normal rabbit serum was used as negative control for Ab and peritoneum of untreated rats was used as negative control tissue. To score deposition of C3 or C9/MAC, a semi-quantitative scale was used: (−) negative, (+)trace, (+) positive staining <10% of total surface area and (++) positive staining area >10%. The number of LCA-positive cells or ED-1 positive cells per field was calculated by counting positive cells in 20 sequential fields and taking the average.

Neutralization of CRegs in primary cell cultures of rat mesothelial cells

Primary cultures of rat mesothelial cells were obtained from SD rat omentum using a modification of a previous report [22,23] (Supplementary 1). Cells were plated at 2.0 × 10^5 cells/well in M199 medium with 10% FBS and incubated for 12 h. The cells were then incubated in M199 medium with RS or with heat-inactivated RS (incubated at 56°C for 30 min) for 1 h. Firstly, with the aim of studying the effect of neutralizing rat CRegs, preliminary experiments were performed to identify the concentration of RS required to induce 50% positive deposition of C3 in rat mesothelial cells. The concentration of RS was adjusted from 0 to 10%, and a dose of 2% RS or heat-inactivated RS was chosen for the following in vitro experiments. For in vitro neutralization of CRegs, 0.05 mg/mL of mAb 5I2, RDIII7 or 6D1, or every combination of two of them, with RS or heat-inactivated RS, was added to the medium and the mesothelial cells were incubated for 1 h at 37°C. To study the effects of pH or glucose concentration on the neutralizing of CRegs Crry and CD59 on rat mesothelium, we incubated mesothelial cells as described above but with 2% RS or heat-inactivated RS in pH 5.0, 5.4, 6.4 or 7.4 M199 medium adjusted by addition of HCl or in 0, 1.50, 2.5 or 4.25% glucose in M199 medium. After the incubation, the cells were washed with PBS three times and C3 deposition on the cells was detected with FITC-labeled anti-rat C3. Cells were double-stained by DAPI (Sigma–Aldrich) to facilitate cell counting. Total and C3-positive cells were counted in 20 fields randomly, and the C3 deposition was calculated by the following formula;

Degree of C3 deposition (%) = \frac{\text{number of C3 positive cells}}{\text{total number of cells}} \times 100.

The above experiments were repeated five times.

Fig. 3. Light microscopy changes in parietal peritoneum at 24 h after neutralization of CRegs. The mAb treatment administered is displayed to the left of each frame. Original magnification is ×200. Frames A, B, C, D, E, F, G, H show typical images from Groups 1 (vehicle), 2 (anti-Crry), 3 (anti-CD55), 4 (anti-CD59), 5 (anti-Crry + anti-CD55), 6 (anti-CD55 + anti-CD59) and 7 (anti-Crry + anti-CD59), respectively. Frame H shows peritoneum of untreated rat. The bar in the left-upper corner in frame A is shown as scale of 100 μm.
Data displayed are the mean ± SE. Statistical analysis was performed by Kruskal-Wallis test and unpaired t-test for cell counts. Tissue damage was analyzed by the non-parametric Mann-Whitney U-test. P-values < 0.05 (5%) between two groups were considered significant.

**Results**

**Macroscopic and LM findings after suppression of CRegs**

Administered anti-CReg mAbs bound in rat peritoneum (Supplementary 2; Figure 1). Macroscopic findings in rats 24 h after administration of any single mAb or most pairs of mAbs were unremarkable with no significant macroscopic changes (Figure 2A). In contrast, in rats given a combination of mAb against Crry and CD59 (Group 7), the parietal peritoneum was edematous with white stripe-like changes, the mesentery and the visceral peritoneum were dotted with white granules and adhesion of omentum and sclerotic changes were found in the retroperitoneum of the posterior abdominal wall (Figure 2B). Additionally, rats in Group 7 had obvious peritoneal effusion at 24 h after functional suppression of Crry and CD59, while there was zero or trace peritoneal effusion after CReg functional suppression in other groups (1 to 6; Table 2).

In parietal peritoneum under LM, only Group 7 rats showed significant changes. There was accumulation of inflammatory cells, irregularity of peritoneal surface, necrotic changes in peritoneal fatty tissues and muscle destruction in sub-peritoneum in the segmental parietal peritoneum (Figure 3G). In the other groups, there were no significant pathological changes or trace cellular accumulation under the parietal peritoneum (Figure 3A–F). Peritoneal damage under LM was semi-quantitatively estimated in all groups (Figure 4A). Of note, in Group 7 rats, neither pathological changes under LM (Figures 3A–D) nor significant IgG binding (Figures 5I–L) was seen in heart, lung, liver and kidney, although parietal injury and IgG deposition were observed in this group (Figure 1G).

To observe the time course of peritoneal injury in Group 7 rats, we also examined parietal peritoneum at 6 and 72 h after neutralization of CRegs. At 6 h, there were no obvious macroscopic or microscopic changes in the parietal membrane. At 72 h, the peritoneal injuries were largely resolved with only minor residual injuries remaining (data not shown).

**C deposition and tissue damage after the functional suppression of CRegs**

Trace deposition of C3, but no MAC deposition, was found along the mesothelial cell layer in untreated rat peritoneum (Figures 6L and P). At 24 h, neutralization of any single CReg and any combination of two CRegs, except for the combination of Crry and CD59 (Group 7), did not induce significant deposition of C3 or MAC compared with untreated rats (Figures 6A–J, M and N). At 24 h in Group 7, abundant deposits of C3 and MAC were found in damaged tissues, including subperitoneal muscle and peritoneal surface (Figures 6K and O and Table 2).

Although no obvious macroscopic and microscopic pathological changes were found at 6 h in Group 7, abundant C3 and MAC deposits were observed on the peritoneal surface and in the sub-mesothelial layer at this time (data not shown). In other groups, only trace C3 deposition along the mesothelial layer was found at 6 h, similar to untreated rats. In Group 7, C3 and MAC deposition was reduced at 72 h, correlating with the reduced tissue damage in peritoneum in Group 7 at 72 h (data not shown).

**Accumulation of inflammatory cells in peritoneum**

Accumulation of inflammatory cells was compared in Groups 1 to 7 at 24 h. Figures 4B and C shows that numbers of LCA-positive and ED-1 positive cells were counted (B and C). The data are shown as mean ± SE. *P < 0.05, **P < 0.005 and ***P < 0.0001.

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Effects of systemic C depletion by pretreatment with CVF

At 24 h after neutralization of Crry and CD59, no macroscopic changes were observed in CVF-pretreated rats (Group 8) while marked macroscopic changes and abundant peritoneal effusion were found in the control group (Group 9) (Figure 7). Under LM, similar pathological changes to those described in Group 7 were observed in control Group 9 (Figure 7D), while no significant peritoneal injuries were found following systemic C depletion (Figure 7A). No detectable pathological changes were observed in heart (A and E), lung (B and F), liver (C and G) and kidney (D and H) in experimental or control rats. No binding of anti-mouse IgG was found in heart (I and M), lung (J and N) and liver (K and O), while kidney showed trace staining in a glomerular distribution. Original magnification is ×400.

C3 deposition after suppression of CRegs on rat mesothelial cells

Treatment of cells with single mAbs or most pairs of mAbs together with RS caused no increase in C3 deposition compared with RS only (Figure 8C). In contrast, RS combined with mAb against Crry and CD59 induced remarkable C3 deposition on mesothelial cells (Figure 8B and E). Heat-inactivated RS, with or without mAbs, did not cause C3 deposition on cells (Figure 8D).

Effect of different pH and osmotic conditions on C3 deposition in rat mesothelial cells following neutralization of Crry and CD59

Peritoneal injuries and accumulation of infiltrated cells in rat peritoneum at 24 h post-neutralization of Crry and CD59 were assessed at different levels of pH and glucose concentration. Macroscopic changes in the peritoneum of rats treated with neutralizing mAbs in PD2 4.25% (pH ~5) were the most widespread and severe among the four PD solutions tested, while mAbs in PBS caused the least injury (Figure 9A–C). To confirm the influence of acidic pH or glucose as an osmotic stress, we assessed C3 deposition on rat mesothelial cells treated with RS in pH 5.0, 5.4, 6.4 or 7.4 M199 medium after suppression of Crry and CD59 or in 0, 1.5, 2.5 or 4.25% glucose contained in M199 medium. C3 deposition was lowest at pH 7.4 and increased at lower pH (Figure 9D). C3 deposition on cells was significantly greater in 2.5% and 4.25% glucose compared to 0% glucose concentration in the medium (Figure 9E).
Discussion

In our recent report [18], we showed that the CRegs Crry, CD55 and CD59 were expressed in rat peritoneum, but CD46 was not expressed. Therefore, we here investigated functional roles of CRegs, Crry, CD55 and CD59, by blocking with specific mAb in the presence of PDF. Neutralization of individual CReg, Crry, CD55 or CD59, in rat peritoneum did not cause significant tissue damage in the peritoneum. These findings were different from those reported in other tissues [1–3,5,18,24]. When we blocked a specific combination of two CRegs, Crry and CD59, in the peritoneum, acute and transient peritonitis was induced. Although anti-CRegs might in themselves cause some C activation, this is unlikely to be significant because neither single nor any other combination of two anti-CRegs caused any significant inflammation in the peritoneum. These findings were different from those reported in other tissues [1–3,5,18,24]. When we blocked a specific combination of two CRegs, Crry and CD59, in the peritoneum, acute and transient peritonitis was induced. Although anti-CRegs might in themselves cause some C activation, this is unlikely to be significant because neither single nor any other combination of two anti-CRegs caused any significant inflammation in the peritoneum. Local production of complement has been reported in peritoneum [11–14], and CRegs likely regulate local C activation. The inflammation induced by blockade of Crry and CD59 was prevented by systemic C depletion with CVF, confirming its C dependence. We could not distinguish local C production from systemic C delivery, both local and systemic C likely contribute to the peritoneal injuries. Systemic effects of i.p. injected mAbs anti-Crry and anti-CD59 were minimal as demonstrated by lack of binding of mAbs and no microscopic injuries in other tissues.

These findings showed that C regulation by CRegs contributes to the maintenance of homeostasis in the normal peritoneum, as shown in other tissues. It was interesting that neutralization of no single CReg induced inflammation in rat peritoneum, only simultaneous neutralization of Crry and CD59 induced inflammation, suggesting that homeostatic C regulation was achieved through a combination of Crry and CD59 in rat peritoneum. Although CD55 is also a C3 level C regulator, Crry is the dominant regulator of this step; consequently, blocking CD55 alone or a combination of CD55 and CD59 did not induce peritoneal injuries while Crry remained active. The peritoneal cavity is usually a sterile environment. However, there are opportunities, particularly in the context of PD, for the environment to be contaminated by microorganisms. It might therefore be essential for tick-over low-level complement activation to occur in the normal peritoneum to maintain homeostasis and provide the primary defense against microorganism invasion. In support of this concept is the trace C3b distribution along the normal peritoneal surface. Collaboration of CRegs in the peritoneum would then be important to regulate this physiological C activation. At the C3 level in rat peritoneum, Crry is the key CReg because neutralization of Crry was essential to induce peritoneal inflammation, but additional neutralization of CD59 was required to develop peritonitis. CD55, another C3 level

Fig. 6. C3 and C5b-9 deposition in parietal peritoneum at 24 h after neutralization of CRegs. C3 deposition is shown in frames A to D and I to L, while C5b-9 deposition is shown in frames E to H and M to P. The pairs of frames show A/E, Group 1 (vehicle); B/F, Group 2 (anti-Crry); C/G, Group 3 (anti-CD55); D/H, Group 4 (anti-CD59); I/M, Group 5 (anti-Crry + anti-CD55); J/N, Group 6 (anti-CD55 + anti-CD59); K/O, Group 7 (anti-Crry + anti-CD59); and L/P, untreated control. The peritoneal surface was labelled as ‘*’. Arrowheads show the positive binding sites. Original magnification is × 200. The bar in the left-upper corner in frame A is shown as scale of 100 μm.
CReg expressed in peritoneum, was not critical to peritoneal integrity, as its neutralization, alone or with either Crry or CD59, did not result in peritoneal injury.

Our in vitro results using rat mesothelial cells support the in vivo results, showing that regulation both in the activation pathways and the terminal pathway is required for homeostasis, a finding that may have relevance for the therapeutic use of C inhibitors. The findings imply that spontaneous C activation in peritoneum is essential in defense against infection and that low C levels in peritoneal fluid, for example in liver cirrhosis [25], might predispose to disease, particularly spontaneous bacterial peritonitis [26,27].

We investigated the effects of Crry and CD59 neutralization under four different conditions: PBS (isotonic and neutral pH), NPD4 1.5% (moderate high osmotic and close to neutral pH), PD4 1.5% and PD4 4.25% (high osmotic and low pH). Peritonitis induced by neutralization of Crry and CD59 in PBS was clearly milder than that induced by the same antibodies in either NPD4 1.5%, PD4 1.5% or PD4 4.25%; the latter caused the most severe peritoneal inflammation. In support of these findings, acidic PD solution caused more C3 deposition on cultured peritoneal mesothelial cells exposed to anti-Crry, anti-CD59 and RS than neutral solution, supporting the proposition that acidic pH induced higher C activation, revealed by blocking Crry and CD59.

New generation formulations of PDF are improved in terms of pH (near neutral) and/or glucose degradation products (GDPs) because low pH and high GDP levels in PDF were found to directly cause mesothelial injuries [8,28]. However, many PDFs still in clinical use are low in pH and often, because of ultrafiltration failure, hypertonic PDFs must be used in some patients. PDFs with neutral pH and low GDPs improve local peritoneal homeostasis [28]. This is in part because both low pH [8] and GDPs can activate the C system, the latter because glycated IgG enhances C activation [29]. Furthermore, high glucose PDF can cause hyperglycemia, which in turn can cause glycated LDL activation of CD59 and impaired C regulation [30], or loss of CD55 and CD59 from cell membranes in vitro [31]. High glucose content in PDF likely causes local inactivation of CD59, further contributing to injury. From those reports, it is clear that PDF can enhance C-dependent acute peritonitis and that biocompatible dialysate is likely beneficial, as shown in vitro and in vivo in animal and human studies [32–34].

Recently, we showed that the C activation enhanced inflammation in fungal peritonitis in a rodent PD model, leading to severe and chronically progressive peritoneal inflammation [18]. The present study confirms that peritoneal C regulation is important not only to suppress peritoneal inflammation during PD therapy but also to maintain homeostasis in the peritoneal cavity.
mesothelial cells treated with the different mAb combinations and was seen (D). Figure 8E quantifies the degree of C3 deposition on mesothelial cells treated with anti-Crry and anti-CD59 and incubated with RS. When mesothelial cells treated with anti-Crry and anti-CD59, was small and similar to that seen with 2% RS without mAb (as an example, compare anti-Crry treated in A and controls in C). Frame B shows abundant C3b deposition on rat mesothelial cells after incubation with 2% rat serum (RS). C3 deposition following incubation with heat-inactivated rat serum (RS). When mesothelial cells treated with neutralization of complement regulators in rat peritoneum 1829

Conflict of interest statement

The authors thank Suzuki, Asano and Fujitani for technical help. This work was supported in part by grant-in-aid for Scientic Research from the Ministry of Education, Science, and Culture, Japan (#19590649 and #21591054) and by the 2008 research grant from the Aichi Kidney Foundation. Morgan is supported by The Wellcome Trust (Programme no. 068590).

Supplementary data

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

Acknowledgements. The authors thank Suzuki, Asano and Fujitani for technical help. This work was supported in part by grant-in-aids for Scientic Research from the Ministry of Education, Science, and Culture, Japan (#19590649 and #21591054) and by the 2008 research grant from the Aichi Kidney Foundation. Morgan is supported by The Wellcome Trust (Programme no. 068590).

Conflict of interest statement. None declared.

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*Received for publication: 8.4.10; Accepted in revised form: 14.10.10*