Renal Effects of Coxsackie B4 Virus in Hyper-IgA Mice

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For clarification of the pathogenetic role of viral infection in chronic glomerulonephritis, the renal effects of Coxsackie B4 virus (CB4) were examined in hyper-IgA (HIGA) mice. In experiment 1, HIGA mice (n = 75) were inoculated intravenously with live CB4 and inactivated CB4 once a month from 1 to 12 mo of age. In experiment 2, HIGA mice (n = 45) were inoculated intravenously with live CB4 and inactivated CB4 once at 6 wk of age. In experiment 3, 60 mice were inoculated intravenously with carbon and live or inactivated CB4 once at 6 wk of age. Mice in the control group were inoculated with vehicle. The kidneys were extirpated from five mice of each group killed with time after inoculation for histologic evaluation. The scores for mesangial IgA deposition, PCNA-positive cells, and matrix at 20 wk were higher in mice with live CB4 than in mice with inactivated CB4 or without CB4. On electron microscopic examination, swelling and detachment of endothelial cells from 24 h after inoculation and increase of serum IFN-γ concentration were found in mice with live CB4. Many carbon particles were present in peripheral and central zones of the mesangium from 5 to 10 d in mice with carbon and live CB4. These results suggest that CB4 provokes exacerbation of renal pathologic findings in HIGA mice via endothelial injury, IFN-γ production, and dysfunction of the mesangial pathway.


Primary IgA nephropathy (IgAN) first was reported in 1968 by Berger and Hinglais and is characterized clinically by microhematuria and proteinuria and histologically by the deposition of IgA (1,2). There have been many reports on the relationship between viral infection and renal injury (3–10). Clinically, we often have noted that viral infection provoked transient proteinuria, hematuria, and renal dysfunction. The role of viruses in the pathogenesis and exacerbation of human renal diseases is not well understood, although it is likely that in acute infections, viruses have direct cytopathic effects on the renal tissue, whereas chronic long-standing infections may exert cytopathic effects via immune complexes (11).

Recently, viral antigens were detected in renal tissues from patients with IgAN and viral DNA by PCR or in situ hybridization (12–16). Enteroviruses are established or suspected causative agents in numerous diseases. In particular, group B coxsackie viruses (CB), which are enteroviruses, have been implicated in several diseases, including pancreatitis, insulin-dependent diabetes, myocarditis, and myositis (3–6). They also have been reported to be causative agents in some renal diseases. For example, induction of experimental nephritis by coxsackie B4 virus (CB4) first was described by Sun et al. (17), and subsequent reports indicated relationships of CB4 with renal diseases in humans and experimental animals. However, there have been no reports on exacerbation of nephritis by viral infections or the mechanism of such exacerbation. Conversely, there have been reports on a model mouse of IgAN. Muso et al. (18) established an inbred murine model of IgAN, a high IgA strain (HIGA) of ddY mice, by selective mating of pooled crude ddY mice that have been reported to develop spontaneously mesangioproliferative glomerulonephritis with glomerular IgA deposition. HIGA mice exhibited constantly high serum levels of IgA from 10 to 60 wk of age with polymeric IgA dominant mesangial deposition and enhanced extracellular matrix accumulation. In this investigation, we examine the renal effects of CB4 in IgAN, we evaluated renal injury after viral inoculation in HIGA mice.

Materials and Methods

Disease Model

Animal experiments were performed using female inbred HIGA mice (Japan SLC, Inc., Shizuoka, Japan) that were 5 wk of age. Mice were allowed free access to normal mouse chow and tap water. All animal experiments were performed according to the Institutional Animal Care and Use Committee guidelines of Fukushima Medical University School of Medicine. Virus-infected mice were maintained in separate rooms from control mice.

Virus

CB4 was prepared by methods previously described by Yoshida et al. (19).

Experimental Protocol

Experiment 1: Administration of Live and Inactivated CB4 Once a Month from 2 to 12 Mo of Age. Seventy-five mice were inoculated intravenously with 0.3 ml of 10⁷ TCD₅₀ per 0.1 ml of live CB4 (group A) and inactivated (group B) CB4 once a month from 1 to 12 mo of age. Mice in the control group (group C) were inoculated with the supernatant of Vero cells that were not infected with CB4 in a similar manner.

These mice were killed once every 10 wk from 10 to 50 wk of age.
under chloroform anesthesia. Twenty-four-hour urine samples were collected at 10, 20, 30, 40, and 50 wk of age. Five mice in each group were killed at 10, 20, 30, 40, and 50 wk of age.

Experiment 2: Intravenous Administration of Live and Inactivated CB4 Once to Mice at 6 Wk of Age. Forty-five mice were inoculated intravenously with 6 mg/0.1 ml carbon (Pilot) and 0.3 ml of 107 TCD50 per 0.1 ml of live (group 1) or inactivated (group 2) CB4 once at 6 wk of age. Mice in the control group (group 3) were inoculated with the supernatant of Vero cells that were not infected with CB4 in a similar manner. These mice were killed at 6 h, 24 h, or 5 d after administration under chloroform anesthesia.

Experiment 3: Intravenous Administration of Colloidal Carbon and CB4 Once to Mice at 12 Wk of Age. Sixty mice were inoculated intravenously with 6 mg/0.1 ml carbon (Pilot) and 0.3 ml of 107 TCD50 per 0.1 ml of live (group 1) or inactivated (group 2) CB4 once at 6 wk of age. Mice in the control group (group 3) were inoculated with the supernatant of Vero cells that were not infected with CB4 in a similar manner. Five mice in each group were killed at 12 h, 36 h, 5 d, and 10 d after administration. In all experiments, after cardiac puncture for blood sampling, the kidneys were removed, weighed, cut into portions, and used for assessment by light microscopy (LM), immunofluorescence (IF), immunohistochemical microscopy (IHM), and electron microscopy (EM).

Laboratory Investigation

Serum creatinine (Scr), serum blood urea nitrogen, and urinary creatinine (Ucr) levels were measured. From the Scr values, Ucr, 24-h urine volume, and body weight at sacrifice, the 24-h endogenous creatinine clearance (Ccr) was calculated using the following formula:

\[ \text{Ccr (ml/min per 100 g body wt)} = \frac{\text{Ucr (mg/dl)} \times \text{volume (ml)}}{\text{Scr (mg/dl)} \times 1/1440 \times 1/\text{body wt (g)} \times 1}\]

Histologic Examination

When the rats were killed, one kidney was excised from each rat and divided into three parts for examination by LM, IF, IHM, and EM.

LM

The renal tissue was fixed in buffered formalin and embedded in paraffin for LM examination. Sections 2 to 3 μm thick then were stained individually with hematoxylin-eosin, periodic Schiff, and periodic acid–silver methenamine and observed under a light microscope. Three observers, blind to treatments, semiquantitatively graded extracellular matrix accumulation in each quadrant in 50 glomeruli per kidney on a scale from 0 to 3 using the following scales: 0, no increase in mesangial matrix; 1, slight increase in mesangial matrix; 2, moderate increase in mesangial matrix; and 3, nearly confluent appearance of mesangial matrix. Each score reflects changes in the extent rather than in the density of mesangial matrix staining. Cellular crescent formation score was calculated as the percentage of glomeruli with cellular crescent formation among all glomeruli.

IHM

The paraffin-embedded sections were dewaxed and incubated sequentially with normal goat serum (1:20 dilution) for 20 min, mAb to mouse α-smooth muscle actin (α-SMA; 1A4; Dako, Glostrup, Denmark; 1:50 dilution), mouse PCNA (19A2; Coulter, Hialeah, FL; 1:50 dilution), and mouse macrophage (MCAP497; UK-Serotec Ltd., Tokyo, Japan; 1:100 dilution) for 1 h, and finally with horseradish peroxidase–conjugated goat anti-mouse Ig (EnVision; Dako Japan Co., Kyoto, Japan) for 1 h. The peroxidase reaction product was visualized with 0.5 mg/ml 3’-diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide as a substrate. For all biopsies, negative controls involved substitution of the primary antibody by equivalent concentrations of an irrelevant mouse mAb or normal rabbit IgG. For each biopsy, >50 cross-sections of consecutive cortical glomeruli with diameters of at least 100 μm were evaluated. Mean numbers of MCAP497-positive cells of glomeruli per biopsy were calculated. For evaluation of immunoperoxidase staining for PCNA and α-SMA, each glomerulus was graded semiquantitatively, as described previously (20).
Statistical Analyses

Values are the means ± SD. Statistical analysis was performed on a Macintosh computer with a software package for statistical analysis (Stat View; Abacus Concepts, Berkeley, CA). Differences among groups in laboratory data were assessed by the Mann-Whitney rank sum test or Wilcoxon signed rank test or contingency tables ($\chi^2$). Correlations were evaluated using Fisher r test. Findings of $P < 0.05$ were considered significant.

Results

Results of Experiment 1

Comparison of Pathologic Findings among Groups.

Comparison of IF Findings among Groups. The degree of deposition of Ig (e.g., IgG, IgM, IgA) and C3 are shown in Figure 2. The degrees of deposition of IgA at 20 wk were higher in group A than in groups B and C (2.1 ± 0.5 versus 0.8 ± 0.4 [$P < 0.01$] and 2.1 ± 0.3 versus 0.6 ± 0.5 [$P < 0.01$], respectively). The degrees of deposition of IgA from 10 to 30 wk of age were higher in group A than in groups B and C. The depositions of IgA are shown in Figure 3. Glomerular IgA deposition was significantly shown at 20 wk of age in group A (Figure 3A), and glomerular IgA deposition was almost never shown at 20 wk of age in group C (Figure 3B). The degree of deposition of IgA did not differ between groups B and C. After 40 wk of age, the degree of deposition of IgA did not differ among the three groups.

LM Findings. The proportions of crescent formation from 10 to 50 wk of age were higher in group A than in groups B and C (Figure 4, A and D). The scores for PCNA-positive cells at 20 wk were higher in group A than in groups B and C (1.6 ± 0.3 versus 0.8 ± 0.3 [$P < 0.01$] and 1.6 ± 0.3 versus 0.9 ± 0.3 [$P < 0.01$], respectively). The scores for PCNA-positive cells from 10 to 20 wk were higher in group A than in groups B and C. The scores for $\alpha$-SMA–positive cells at 20 wk were higher in group A than in groups B and C (1.7 ± 0.4 versus 0.8 ± 0.3 [$P < 0.05$] and 1.6 ± 0.3 versus 0.9 ± 0.4 [$P < 0.05$], respectively). The scores for $\alpha$-SMA–positive cells from 10 to 20 wk were higher in group A than in groups B and C. Proportions of crescent formation, scores for PCNA-positive cells, and scores for $\alpha$-SMA–positive cells did not differ between groups B and C (Figures 4 through 6).

Mesangial proliferation and PAS-positive depositions were found more frequently in group A than in groups B and C (Figure 4, D and E).

Matrix scores increased from 40 to 50 wk in each group.

Matrix scores at 30 wk were higher in group A than in groups
B and C (1.6 ± 0.4 versus 1.0 ± 0.3 [P < 0.05] and 1.6 ± 0.4 versus 1.1 ± 0.3 [P < 0.05], respectively). Matrix scores from 20 to 40 wk of age were higher in group A than in groups B and C (Figure 4, C and F). Matrix scores did not differ between groups B and C. The mean number of MCAP497-positive cells at 20 wk was higher in group A than in groups B and C (4.2 ± 1.6 versus 0.6 ± 0.4 [P < 0.01] and 4.2 ± 1.6 versus 0.4 ± 0.4 [P < 0.01], respectively). The mean number of MCAP497-positive cells from 10 to 20 wk was higher in group A than in groups B and C.

Comparison of EM Findings among the Three Groups. The degree of electron-dense mesangial deposition from 10 wk to 30 wk was higher in group A than in groups B and C. The degree of electron-dense mesangial deposition did not differ between groups B and C.

In Situ Hybridization. At 20 wk, strong positive signals were seen in mesangial cells of group A, and positive signals were not seen in mesangial cells of groups B and C (Figure 3C).

Comparison of Laboratory Findings and Serum Cytokine Levels among Groups. Urinary protein from 10 and 50 wk of age was detected in group A and was not detected in groups B and C. At 30 wk, urinary protein (mg/d) increased mildly in group A compared with groups B and C (1.9 ± 0.8 versus 0.1 ± 0.0 [P < 0.05] and 1.9 ± 0.8 versus 0.1 ± 0.0 [P < 0.05], respectively) (Table 1). From 10 and 50 wk of age, hematuria was not detected in all groups. There were no significant differences in body weight and Ccr among the three groups (Table 1, Figure 7).

Serum IgA concentrations (mg/dl) increased markedly after 30 wk in all three groups and did not differ among them (147 ± 19, 156 ± 24, and 151 ± 22 in groups A, B, and C, respectively). Serum IFN-γ concentration increased from 10 to 40 wk in group A, and was hardly detected in groups B and C (Figure 7). Serum IL-4 concentration increased from 10 to 50 wk in groups A, B, and C (Figure 7), with no differences in concentration among the three groups. Serum anti-CB4 virus neutralizing antibody titers in groups A and B were elevated gradually, and no elevation in group C was noted (Figure 7).

Results of Experiment 2

Comparison of Pathologic Findings among Groups. On LM examination, proliferation score and matrix score did not
differ among the three groups. On EM examination, swelling and detachment of endothelial cells from 3 h to 5 d after inoculation were found in all mice of group 1, and none of them from 3 h to 5 d were found in all mice of groups 2 and 3 (Figure 8). Swelling and detachment of endothelial cells was more frequently found in group 1 than in groups 2 and 3 (Figure 8, A and B).
Comparison of body weight and renal function at 40 wk of age among the three groups. Results of Experiment 3

Table 1. Comparison of body weight and renal function at 40 wk of age among the three groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Ccr (mg/min per 100 g body wt)</th>
<th>Urinary Protein Excretion (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>64.8 ± 7.7</td>
<td>0.42 ± 0.13</td>
<td>1.9 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>61.8 ± 8.5</td>
<td>0.46 ± 0.14</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>63.4 ± 6.9</td>
<td>0.43 ± 0.11</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ccr, creatinine clearance.  
<sup>b</sup>p < 0.05.

Discussion

Our findings showed that CB4 promoted IgA deposition in mesangial lesions and increased mesangial cell and matrix proliferation in glomeruli of HIGA mice. Endothelial cell injury and crescent formation were found more frequently in HIGA mice with live CB4 than in HIGA mice without CB4 and HIGA mice with inactivated CB4.

There have been many reports on the relationship between viral infection and renal injury. Clinically, renal signs and symptoms frequently are preceded by episodes of upper respiratory tract infection and/or gastrointestinal infection, suggesting that viral infection as the cause of IgAN.

Gregory et al. (10) revealed that human cytomegalovirus participated in the pathogenesis of IgAN. Iwama et al. (16) reported that Epstein-Barr virus (EBV)-specific DNA in renal biopsies was detected by PCR in seven (58%) of 12 patients with IgAN, three (50%) of six patients with membranous nephropathy, none (0%) of 10 patients with minor glomerular abnormalities, and two (100%) of two patients with focal segmental lesions. We reported positive PCR for the presence of entero-viral RNA for three of 10 patients with IgAN and that entero-viral infection may play a role in the mechanism of onset or evolution of IgAN (12).

CB4, which is in the group of enteroviruses and minimum RNA viruses without lipid, is a common agent of gastrointestinal infection and also is associated with more serious diseases such as myocarditis or myositis. CB4 has been reported as the causative agent in some renal diseases (3,4,17). Yoshida et al. (19) reported that CB4, when inoculated repeatedly into mice, induces lesions that are similar to IgAN. However, HIGA mice are reported as a model mouse of IgAN. Muso et al. (18) developed HIGA mice by selective mating of ddY mice that exhibited high serum levels of IgA. At 25 wk of age, HIGA mice spontaneously develop high levels of serum IgA along with glomerular IgA deposition. In addition, the expression of TGF-β in the kidney was shown to be increased in these mice (21). Moreover, HIGA mice show a remarkable glomerular deposition of matrix components such as fibronectin and collagen IV (22). These findings suggest that HIGA mice may provide a valuable model for studying the mechanisms of chronic sclerosis developing types of IgAN. However, glomerular crescent formation, which sometimes is observed in ddY mice, was decreased in HIGA animals (21). In addition, HIGA mice had no hematuria and no change of GFR and the renal immunohistology did not show increased C3 deposition in contrast to IgAN. Because HIGA mice are an animal model, they may not be the same as typical human IgAN at all.

In this study, we found that CB4 promoted IgA deposition in mesangial lesions and increased mesangial cell and matrix

Figure 6. (A) PCNA-positive cells were found frequently in the glomeruli at 20 wk in group A. (B) PCNA-positive cells were not seen in the glomeruli at 20 wk in group C. (C) α-SMA-positive cells were expressed frequently in the glomeruli at 20 wk in group A. (D) α-SMA-positive cells were not expressed in the glomeruli at 20 wk in group C.

Figure 10C to 10D. Many carbon particles were present in the peripheral and central zones of the mesangium and lacis area at 12 h after administration (Figure 10A) to 36 h (Figure 10B) after administration. Many carbon particles were present in the peripheral zone of the mesangium at 36 h after administration (Figure 10F). Moderate numbers of carbon particles were present in the peripheral zone of the mesangium at 36 h after administration (Figure 10F). Many carbon particles were present in the peripheral and central zones of the mesangium and lacis area at 5 d after administration (Figure 10G). Almost all carbon particles had disappeared from the mesangium and lacis area at 10 d after administration (Figure 10H).
proliferation in glomeruli of HIGA mice. Endothelial cell injury and crescent formation were found more frequently in HIGA mice with live CB4 than in HIGA mice without CB4 and HIGA mice with inactivated CB4. These findings suggested that CB4 infection provoked pathologic changes in HIGA mice.

The mechanism of exacerbation of renal injury by viral infection still is unclear. However, there has been some speculation regarding the mechanism of exacerbation of renal injury by CB4 infection. With recent advances in immunopathologic and molecular biologic technique, it has become possible to clarify the mechanisms of induction of glomerulonephritis by viruses. There are two explanations for the renal injury. First, direct injury by viruses can cause renal injury. In our study, the detachment and swelling of endothelial cells were found more frequently in HIGA mice with CB4 than in HIGA mice without CB4 and HIGA mice with inactivated CB4. These findings showed that CB4 directly injured glomerular endothelial cells. In addition, serum IFN-γ concentration in HIGA mice with live CB4 was higher than those in HIGA mice without CB4 and HIGA mice with inactivated CB4. IFN-γ is the major cytokine secreted from T-helper 1 cells and promotes cellular immunity including macrophage and natural killer cell (23). CB4 promoted IgA deposition in mesangial lesions and mesangial cell matrix proliferation in glomeruli of HIGA mice, and crescent formation and infiltration by macrophages were observed.
more frequently in HIGA mice with live CB4. IFN-γ might have caused these pathologic changes. Therefore, direct injury and mediators such as IFN-γ that are produced by viral infection in the glomerulus may play important roles in the cause of these pathologic changes.

Second, dysfunction of the mesangial pathway that is induced by viruses can cause renal injury (24,25). As to a functional pathway between the mesangium and juxtaglomerular apparatus, Leiper et al. (24) reported that iron-dextran complex as tracer particles initially was taken into the matrix channels of the mesangium from which it progressed over the course of 8 h to the matrix of the juxtaglomerular apparatus and intercellular spaces of the macula densa and mentioned mesangial pathway, which is a continuous functional pathway from the glomerular capillary lumen to the macula densa cells of the distal tubule for material that is taken up by the mesangium.

In our study, many carbon particles were present in peripheral and central zones of the mesangium from 5 to 10 d in HIGA mice with live CB4 after administration. These findings suggest that mesangial pathway function was decreased in HIGA with CB4. In addition, IgA concentrations did not differ among the groups. These findings suggest that low clearance of IgA by CB4 infection might cause an increase in deposition of mesangial IgA.

There has been some speculation concerning renal injury by immune complexes (17,19). Immune complexes consist of anti-
gen and antibody, and the antigen may be virus or renal tissue that is injured by viral infection. Deposition of these immune complexes in the mesangium and basement membrane can cause renal injury. However, in our study, results of pathologic examination did not differ between HIGA without CB4 and HIGA with inactive CB4. Therefore, immune complexes that are induced by inactive CB4 may not cause renal disease in our model.

**Conclusion**

CB4 provokes exacerbation of renal pathologic findings in HIGA mice via endothelial injury, IFN-γ production, and dysfunction of the mesangial pathway.

**References**

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**Figure 10.** Comparison of the proportion of grades S, M, and L between groups 1 and 3. (A) Large carbon particles were present in surrounding capillary lumina from 12 h after administration in group 1. (B) Large carbon particles were still present in capillary lumen, and mesangium contained small amounts of carbon particles at 36 h after administration in group 1. (C) Many carbon particles were present in the peripheral and central zones of the mesangium from 5 d after administration in group 1. (D) Many carbon particles were still present in the peripheral and central zones of the mesangium from 10 d after administration in group 1. (E) Large carbon particles were present in surrounding capillary lumina at 12 h after administration in group 3. (F) Moderate numbers of carbon particles were present in the peripheral zone of the mesangium at 36 h after administration in group 3. (G) Many carbon particles were present in the peripheral and central zones of the mesangium and lacis area at 5 d after administration in group 3. (H) Most carbon particles had disappeared from the mesangium and lacis area at 10 d after administration in group 3.
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