The pathogenicity of serotype 8 fowl adenovirus (FAV) has been reported in field cases of inclusion body hepatitis (IBH) in chickens [7] as well as in experimental reproduction of IBH in specific-pathogen-free (SPF) chickens [6]. Recently, serotype 8 FAV was isolated from gizzards exhibiting gizzard erosion in slaughterhouses [14]. In previous reports, however, gizzard erosion was reproduced in SPF chickens [9, 11] and commercial broiler chickens [12] inoculated with serotype 1 FAV isolated from the gizzards of commercial broiler chickens exhibiting gizzard erosion. Moreover, most cases of gizzard erosion that have occurred frequently in slaughterhouses in Japan were caused by serotype 1 FAV infection [14]. Therefore, it was not clear whether or not serotype 8 FAV was the cause of gizzard erosion.

The purpose of this study was to investigate the pathogenicity of serotype 8 FAV isolated from eroded gizzard by inoculating SPF chickens with the isolates via oral and intramuscular routes.

**MATERIALS AND METHODS**

**Virus:** Two isolates of FAV, isolated from gizzard erosions of slaughtered broiler chickens, were cloned three times by means of the terminal dilution method and designated as the M013 and G0054 strains. These strains were propagated in primary chicken kidney (CK) cells and used for chicken inoculation at the fifth passage level. Both strains had a titer of 10^5.5 median tissue culture infectious doses (TCID_{50})/0.05 ml as determined in CK cell culture. Both strains were identified and classified as serotype 8 by a cross-neutralization test and the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [15].

**Chickens:** Five-day-old (experiment 1) and 1-day-old (experiment 2) specific-pathogen-free (SPF) white leghorn chickens were used for this study. Throughout the experimental period, the chickens were kept in isolated rooms in a negatively pressured house containing filtered air.

**Experimental design:** In experiment 1, 33 5-day-old chickens were used. Fifteen chickens were inoculated with 0.4 ml of M013 strain (group 1) and 14 chickens were inoculated with 0.4 ml of G0054 strain (group 2) via the oral route. Four chickens were not treated (control group). On days 3, 5, 7, 10, and 14 postinoculation (PI), 3 to 4 chickens in groups 1 and 2, and on days 7 and 14 PI, 2 chickens in the control group were killed and necropsied in each period, respectively. All chickens were observed daily for clinical signs.

In experiment 2, 35 1-day-old chickens were used. Twenty-seven chickens were inoculated with 0.4 ml of G0054 strain via the intramuscular (breast muscle) route and 8 chickens were inoculated with 0.4 ml of cell culture medium via the intramuscular route as a control. All chickens were observed daily for clinical signs. Necropsies were scheduled on days 3, 5, 7, and 10 PI and dead chickens and euthanized moribund chickens were necropsied immediately. One to 2 chickens in the control group were killed and necropsied on days 3, 4, 5, 7, and 10 PI.

**Virus isolation:** Virus isolation was attempted from the gizzard, liver, pancreas, and rectum (including feces) according to a method described previously [11]. Briefly, 10% tissue homogenates were inoculated onto CK cell cultures and the cultures were observed in order to detect any
cytopathic effect (CPE). The TCID50 of the virus was determined and titers were calculated.

Serology: Serum antibodies against the inoculated virus were examined by virus neutralization test using sera that were collected when chickens were necropsied. Virus neutralization tests were performed according to constant virus variable serum method on microculture plates with CK cell monolayers.

Histology: Heart, lung, liver, kidney, gizzard, pancreas, spleen, bursa of Fabricius, and gastrointestinal tract were collected and fixed in 20% buffered formalin. All tissue samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin and then examined by light microscopy for pathological changes.

RESULTS

Clinical signs and gross lesions: In experiment 1, no clinical signs were observed in the chickens for the duration of the study. Macroscopically, mild gizzard lesions such as white foci on the keratinoid layer and lesions in gizzard mucosa were observed in one chicken of group 2 on each days 5, 10, and 14 PI, respectively. Other organs such as the liver, pancreas, and spleen showed no gross lesions in any of the chickens.

In experiment 2, 5, 6, and 3 inoculated chickens died suddenly on days 3, 4, and 5 PI, respectively. Four, 3, 1, and 1 inoculated chickens became moribund with severe clinical signs such as ruffled feathers, severe depression, and closed eyes from days 3 to 6 PI, respectively, and yellow-green color feces were observed in the same test period. Two inoculated chickens showed mild to moderate depression on day 7 PI. The chickens in the control group and 2 inoculated chickens necropsied on day 10 PI were clinically healthy. The gross lesions of the dead chickens and euthanized moribund chickens appeared almost the same. The common characteristic of the gross lesion was discoloration of liver. In addition, gizzard erosions were observed in 2 inoculated chickens and pale kidney and spleen were observed in 2 and 1 inoculated chickens, respectively.

Virus isolation: In experiment 1, FAV was recovered from gizzard and rectum (Table 1). From the chickens of group 1, FAV was recovered from gizzard on day 5 PI and from rectum on days 5, 7, and 10 PI. From the chickens of group 2, FAV was recovered from gizzard on day 7 PI and from rectum on days 5 and 7 PI, respectively. FAV was not recovered from the liver and pancreas of any chicken of either group. No FAV was recovered from any sample of the chickens in the control group.

In experiment 2, FAV was recovered from the gizzards, livers, pancreases, and rectums of all inoculated chickens from days 3 to 7 PI (Table 2). From liver and pancreas, especially, FAV was recovered in high titer until day 6 PI and was recovered in low titer on day 7 PI. On day 10 PI, FAV was recovered only from the liver of one inoculated chicken. No FAV was recovered from any sample of the

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Days PI</th>
<th>Gizzard</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M013</td>
<td>5</td>
<td>2/3 (3.63)</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3 (2.33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4 (2.88)</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4 (2.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>2</td>
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<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3 (2.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>2/4 (2.34)</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4 (2.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0/4</td>
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<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

a) No. birds positive in virus isolation/No. birds examined (mean virus titer in birds from which FAV was isolated [logTCID50/0.05 ml] in parentheses).

<table>
<thead>
<tr>
<th>Days PI</th>
<th>Clinical signs</th>
<th>No. of chickens</th>
<th>Gizzard</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Rectum</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>Dead</td>
<td>5</td>
<td>4.35</td>
<td>7.20</td>
<td>6.80</td>
<td>5.15</td>
</tr>
<tr>
<td>4</td>
<td>Dead</td>
<td>6</td>
<td>4.08</td>
<td>7.46</td>
<td>7.11</td>
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<tr>
<td></td>
<td>Severe</td>
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<td>3.42</td>
<td>7.17</td>
<td>6.33</td>
<td>5.25</td>
</tr>
<tr>
<td>5</td>
<td>Dead</td>
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<td>7.33</td>
<td>6.58</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
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<td>2.00</td>
<td>5.75</td>
<td>4.75</td>
<td>4.75</td>
</tr>
<tr>
<td>6</td>
<td>Severe</td>
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<td>2.75</td>
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<td>3.00</td>
</tr>
<tr>
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<td>Mild</td>
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<td>1.50</td>
<td>1.38</td>
<td>1.75</td>
</tr>
<tr>
<td>10</td>
<td>Health</td>
<td>2</td>
<td>0</td>
<td>0.75</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Log TCID50/0.05 ml.
chickens in the control group.

Serology: In experiment 1, serum neutralization antibodies against the inoculated strain were present in the inoculated chickens of both groups from day 7 PI. On day 14 PI, the antibody titer rose to more than 1:256 in the inoculated chickens of both groups. Chickens in the control group were serologically negative for both the M013 and G0054 strains.

In experiment 2, serum antibodies against FAV were present in one inoculated chicken on day 7 PI (titer was 1:32) and two inoculated chickens on day 10 PI (titers were 1:32 and 1:64, respectively). The chickens in the control group were serologically negative for FAV.

Histology: In experiment 1, mild and local lesions were observed in the gizzards of inoculated chickens. Lymphocyte infiltrations in the lamina propria mucosa were observed in one inoculated chicken of group 1 on day 7 PI. Gizzard erosions with edematous degeneration of the keratinoid layer, lymphocyte infiltration in the lamina propria mucosa were observed in one inoculated chicken of group 2 on days 10 and one on 14 PI, respectively. Intranuclear inclusion bodies were observed in the cecal tonsil of 2 inoculated chickens of group 1 on day 7 PI.

In experiment 2, the histological lesions of dead chickens and euthanized moribund chickens appeared almost the same. Multifocal necrosis of hepatocytes and intranuclear inclusion bodies of hepatocytes in the liver (Fig. 2) and multifocal necrosis of glands with inclusions in the pancreas were observed in all inoculated chickens from days 3 to 6 PI. Gizzard lesions such as edematous degeneration of the keratinoid layer and lymphocyte infiltration into the lamina propria mucosa were observed in one inoculated chicken on days 3 and one on day 4 PI, respectively. Intranuclear inclusion bodies were observed in the epithelial gizzard cells of one inoculated chicken on day 4 PI. In addition to these lesions, lymphocyte depletion was observed in the spleen and bursa of Fabricius of all of inoculated chickens except 1 inoculated chicken killed and necropsied on days 10 PI (Fig. 3). Monocyte infiltration into the epicardium and urate deposits in the kidney were observed in 20 and 21 inoculated chickens, respectively.

DISCUSSION

Gizzard erosion associated with FAV in layer chickens, broiler chickens, and quails has been reported [2, 5, 13, 17]. It has been reported that many outbreaks of gizzard erosion associated with FAV occurred frequently in Japanese slaughterhouses and most of FAV isolated from eroded gizzard were of serotype 1 [14]. Moreover, gizzard erosions were reproduced in SPF white leghorn chickens [9, 11] and commercial broiler chickens [12] inoculated with serotype 1 FAV isolated from broiler chickens exhibiting gizzard erosions. Therefore, serotype 1 FAV was regarded as the most general pathogen of gizzard erosion associated with FAV.
In this study, serotype 8 FAV was focussed on as the pathogen of gizzard erosion because this serotype was sometimes isolated from lesional areas of gizzard erosion by means of the terminal dilution method. These results suggested that serotype 8 FAV was the major virus present in the lesional area of gizzard erosion and could be considered the patho-

Fig. 2. Necrosis of hepatocytes and intranuclear inclusion bodies of hepatocytes (arrows) of an euthanized moribund chicken on day 4 PI in experiment 2. H&E. Bar=25 µm.

Fig. 3. Bursa of Fabricius of chickens in experiment 2. (A) Euthanized moribund chicken on days 4 PI. Atrophy of follicles, depletion of medullary lymphocytes were observed. (B) Chicken in control group on day 4 PI. H&E. Bar=250 µm.
gen of gizzard erosion.

In experiment 1, gizzard lesions observed both macroscopically and microscopically in chickens inoculated with the M013 or the G0054 strain were mild. However, the virus was recovered from the gizzards of 2 and 2 inoculated chickens of groups, and the typical histological lesions of adenoviral gizzard erosion were observed. These results suggested that while it might be lower than that of serotype 1 FAV as described in our previous reports [11, 12], these two strains of serotype 8 FAV had some pathogenicity or tissue tropism in the gizzard. In the field, gizzard erosion associated with serotype 8 FAV has also been reported. Tanimura et al. [17] reported a field case of gizzard erosion in which intranuclear inclusion bodies were seen in necrotic epithelial cells of the gizzard, and serotype 8 FAV was isolated from liver samples. In this case, because chicken anemia virus (CAV) was isolated as well as serotype 8 FAV, it was deduced that CAV might have contributed to the outbreak. Immunosuppression caused by infection with CAV or Infectious bursal disease virus (IBDV) as well as various environmental stress aids FAV in producing IBH and other diseases associated with FAV [3, 4, 8]. In our case of gizzard erosion in a slaughterhouse in which the M013 and G0054 strains were isolated from gizzards, these factors might have contributed to the outbreak of gizzard erosion.

In experiment 2, 14 inoculated chickens died suddenly from days 3 to 5 PI and 9 inoculated chickens, even though they did not die on that day, became moribund and showed severe clinical signs. Their livers were discolored and FAV was recovered from the liver and pancreas at high titer levels. Microscopically, moreover, hepatocytic necrosis and intranuclear inclusion bodies of hepatocytes were observed in inoculated chickens. These results are similar to those of previous reports of field cases and experimental reproduction of IBH [8], and indicate that IBH was reproduced by intramuscular inoculation with FAV G0054 isolated from gizzard. Besides the liver, histological lesions such as atrophy and lymphocyte depletion in spleen and bursa of Fabricius were also severe. Saifuddin et al. [16] reported field and experimental cases of IBH with severe depletion of lymphocytes in the bursa, spleen, and thymus. Therefore, it was suggested that virus isolation was not attempted from these organs, however, histological lesions in spleen and bursa of Fabricius in the present study were produced by inoculated FAV. In general, it is difficult to reproduce IBH when FAV isolates are given by natural routes, though some studies have successfully reproduced IBH using unnatural inoculation routes such as intramuscular, intravenous, and intraabdominal routes [8]. In the present study, no typical IBH lesion was observed after the inoculation of the G0054 strain via an oral route in experiment 1. These findings suggested that the G0054 strain was a kind of FAV strain that could be the cause of IBH.

Previous studies have reported that gizzard erosion was observed in chickens in some field cases of IBH or hydropericardium syndrome [1, 10]. Therefore, it was possible that some cases of gizzard erosion detected in the slaughter-

house were caused by not only serotype 1 FAV that could only reproduce gizzard erosion, but also by serotype 8 FAV that could reproduce both gizzard erosion and IBH.

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