SUMMARY

Type-8 avian adenoviruses were isolated from chickens in a commercial flock suffering an outbreak of inclusion body hepatitis. Serum-neutralizing titer to this type, but not to 7 other types of avian adenovirus, was more than 4 times as high in convalescing chickens as in chickens from the flock bled 2 weeks previously, during the disease outbreak. A disease similar to that in the commercial flock and to inclusion body hepatitis as described in the literature was produced by intra-abdominal inoculation of a type-8 isolant, AMG 5 (2a), into 1-day-old specific-pathogen-free chicks. Pathologic features of the disease included necrotizing hepatitis, pancreatitis, and severe lymphoid depletion of the bursa of Fabricius, thymus, and spleen. It was concluded that type-8 avian adenoviruses were involved in the etiology of the naturally occurring outbreak of inclusion body hepatitis.

INTRODUCTION

An inclusion body hepatitis (IBH) of unknown etiology was described by Helmboldt and Frazier (11) in 1963. Other early reports of field outbreaks of a similar disease include those of Howell et al. (12) and Pettit and Carlson (18). Subsequent studies (1,4,20) incriminated an avian adenovirus (AAV), designated as inclusion body hepatitis virus, Tipton strain (IBHV) (a type-5 AAV), in the etiology. Table 1 summarizes the clinical, pathologic, and hematologic findings described in those reports.

The work reported here isolated a type-8 AAV (16) from a field outbreak of IBH and investigated its role in the etiology of this disease.

---

AOn study leave from the Queensland Department of Primary Industries, Animal Research Institute, Yeerongpilly, Queensland, Australia 4105.
MATERIALS AND METHODS

**Virus isolation.** Lung and trachea (pooled), liver, and cecum were collected from chickens at necropsy and homogenized to prepare 10% suspensions in phosphate-buffered saline containing 2% agammaglobulinemic calf serum, potassium penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), and gentamicin (200 μg/ml). Suspensions were centrifuged at 900 × g for 10 minutes, and the supernatant was incubated at 37 C for 1 hour. Preformed chicken kidney cell (CKC) monolayers in 60 × 15-mm dishes (10) were inoculated with 200 μl of supernatant and examined daily with an inverted microscope for cytopathic effects (CPE).

**Serology.** Virus-neutralization (VN) tests were performed in microtiter plates by procedures described previously (9), and were used to titer serum antibody and to serotype viruses. Briefly, two-fold dilutions of serum were made in the wells of plates with microdiluter loops, 200 median tissue culture infective doses (TCID₅₀) of indicator virus were added to each well, virus-serum mixture was incubated for 1 hour at 37 C, and CKC suspension (10) was dispensed into wells of plates. AAV 112, 685, SR-49, KR-5, TR-22, CR-119, YR-36, and TR-59 were used as prototypes of 8 AAV serotypes, designated FA 1-8 (16). Tests were read with an inverted microscope for the presence of CPE in monolayers after 6 days. Virus-neutralizing titers were reported as the reciprocal of the highest dilution of serum, calculated as the mean of readings of duplicate rows of wells, that completely neutralized indicator virus.

Precipitating antibodies were detected by double immunodiffusion using viral antigens in the central well and antiseraums in peripheral wells. AAV antigens were concentrated cell-culture fluids derived from type-1 or type-5 (16) AAV-infected CKC. Infectious bursal disease (IBD) virus antigen was prepared by homogenizing bursas from specific-pathogen-free (SPF) chickens infected with IBD virus. Gels consisted of 2.4-mm-deep layers of 0.7% Noble agar (Difco Laboratories) containing 8% NaCl and 0.01% Thimersal (Nutritional Biochemicals) and into which were cut six 4-mm cylindrical wells 9 mm apart, center to center, in a hexagonal pattern, and a central 4-mm well. Serums were read as positive if lines of identity were obtained between a known positive antiserum and the test serum in an adjacent well.

**Pathology.** Chickens were necropsied and gross lesions recorded. Samples of liver, heart, kidney, spleen, duodenum, pancreas, skeletal muscle, lung, trachea, thymus, and bursa of Fab-
Table 1. Clinical, pathologic, and hematologic findings described for inclusion body hepatitis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age of chickens (days)</th>
<th>Mortality (%)</th>
<th>Course (days)</th>
<th>Macroscopic lesions</th>
<th>Microscopic Lesions</th>
<th>Hematology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L&lt;sup&gt;a&lt;/sup&gt; K M M B BM Other</td>
<td>L K B BM S P Other</td>
<td>PCV (%)</td>
</tr>
<tr>
<td>11</td>
<td>35-49</td>
<td>1-2</td>
<td>2-10</td>
<td>+ &lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ trachea</td>
<td>- - - trachea</td>
</tr>
<tr>
<td>12</td>
<td>21-84</td>
<td>1-8</td>
<td>21</td>
<td>+</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>13</td>
<td>35</td>
<td>0.1-7</td>
<td>7-10</td>
<td>+ + + + + +</td>
<td>+ + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1-6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20</td>
<td>21</td>
<td>+ -</td>
<td>+ -</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23-25</td>
<td>10-17</td>
<td>+ +</td>
<td>+ +</td>
<td>20-25</td>
</tr>
</tbody>
</table>

<sup>a</sup>L = liver; K = kidney; M = skeletal muscle; B = bursa; BM = bone marrow; S = spleen; P = pancreas; PCV = packed cell volume.

<sup>b</sup>Blank spaces indicate that tests were not done or not reported.

<sup>c</sup>Experimentally inoculated chickens.

Table 2. Serum antibody of chickens from a flock affected with inclusion body hepatitis.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Neutralizing antibody&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Precipitating antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>685&lt;sup&gt;b&lt;/sup&gt; (Type-2)</td>
<td>TR-59 (Type-3)</td>
</tr>
<tr>
<td>Acute&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Convalescent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>120</td>
<td>1900</td>
</tr>
</tbody>
</table>

<sup>a</sup>Serum antibody titers to prototype avian adenoviruses (AAV) 112, SR-49, KR-5, TR-22, OR-119, and YR-36 and to AAV IBHV were equal to or less than 50.

<sup>b</sup>AAV 685, TR-59, CELO and IBHV; IBDV = infectious bursal disease virus. Virus-typing nomenclature is based on McFerran et al. (16).

<sup>c</sup>Results are the mean titers of eight 5-week-old chickens.

<sup>d</sup>Results are the mean titers of five 7-week-old chickens.
ricius were fixed in 10% buffered neutral formalin. Histologic sections, 8 μm thick if embedded in paraffin and 2 μm thick if embedded in plastic (6), were prepared and stained with hematoxylin and eosin (H & E).

**Naturally occurring disease.** Ten 5-week-old broiler breeder replacements were submitted for laboratory examination from a flock suffering an outbreak of IBH (diagnosed by Dr. R. B. Davis). A round-cell CPE-producing agent isolated from the liver of one chicken was cloned by making 3 successive plaque picks from terminal dilutions, characterized as a type-8 AAV (9,10,16), and designated as AMG 5 (2a).

**Transmission trial.** Chicks, hatched from eggs purchased from a commercial supplier of SPF chickens, were bled when 1-5 days old, and serums were tested for neutralizing titers to 8 AAV serotypes. Eggs from a flock which was negative for all 8 serotypes were used as a source of 35 one-day-old chicks. Five chicks were bled to obtain preinoculation serum samples which were titered by neu-

Fig. 1. Pale swollen petechiated liver of a 5-week-old chicken affected with inclusion body hepatitis.
Table 3. Clinical, pathologic, and serologic findings observed in chickens inoculated intra-abdominally at one day old with avian adenovirus AMG 5(2a).

<table>
<thead>
<tr>
<th>Days postinoculation</th>
<th>Number of birds</th>
<th>Morbidity and mortality</th>
<th>Gross lesions</th>
<th>Microscopic lesions</th>
<th>Hematocrit (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Body wt. (g)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralizing antibody to AMG 5(2a)</th>
<th>Precipitating antibody&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CELO</td>
<td>IBHV</td>
</tr>
<tr>
<td>Virus-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CELO</td>
<td>IBHV</td>
</tr>
<tr>
<td>inoculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CELO</td>
<td>IBHV</td>
</tr>
<tr>
<td>3–4</td>
<td>6</td>
<td>dead</td>
<td>L,S,H,B,P,D</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>NR&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>5–7</td>
<td>8</td>
<td>moribund</td>
<td>L,S,H,B,D</td>
<td>29.6 ± 6.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
<td>64 ± 11&lt;sup&gt;g&lt;/sup&gt;</td>
<td>40&lt;sup&gt;i&lt;/sup&gt;</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>9–11&lt;sup&gt;H&lt;/sup&gt;</td>
<td>6</td>
<td>-</td>
<td>L,I,B,F</td>
<td>35.8 ± 3.5</td>
<td>-</td>
<td>140&lt;sup&gt;i&lt;/sup&gt;</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>35.2 ± 2.3</td>
<td>107 ± 6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&lt;20</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Results expressed as 95% confidence limits.
<sup>b</sup>CELO = chicken embryo lethal orphan virus; IBHV = inclusion body hepatitis virus; IBDV = infectious bursal disease virus.
<sup>c</sup>Organs with lesions. L = liver; S = spleen; H = heart; B = bursa; P = pancreas; D = duodenum; M = skeletal muscle; T = thymus; K = kidney.
<sup>d</sup>Not done.
<sup>e</sup>No results as serum controls were positive for round-cell CPE-producing agents.
<sup>f</sup>Negative.
<sup>g</sup>Significantly different from control results at the 5% level using Student's t-distribution.
<sup>h</sup>Four chickens tested for antibody to avian adenovirus prototypes 112, 685, SR-49, KR-5, TR-22, CR-119, and YR-36 were negative.
<sup>i</sup>Results of chickens necropsied 9–11 days postinoculation.
neutralization for antibody to 8 AAV serotypes. Each of 20 chicks was injected in the abdominal cavity with 0.2 ml of diluent containing about \(10^6\) plaque-forming units of fourth-passage AMG 5 (2a), and each of 10 chicks was injected similarly with diluent alone. Virus-inoculated chicks were housed in Horsfall units separate from control chicks. Samples collected from chicks included blood from the wing vein for microhematocrit determination, heart blood for serologic tests, and tissues for virus reisolation and histopathologic examination.

RESULTS

Naturally occurring disease. Two chickens were dead when submitted for laboratory examination, four were lethargic, one had nervous signs, and three had no detectable clinical signs. Lesions observed at necropsy included swollen fatty livers with multiple subcapsular petechiae (Fig. 1), enlarged pancreases with isolated petechiae, small bursas of Fabricius, pale swollen petechiated kidneys, severe mucoid to necrotic enteritis, petechiation of spleens

Fig. 2. Liver of a 5-week-old chicken affected with inclusion body hepatitis. Coalescing foci of vacuolated and necrotic hepatocytes (light areas) between remnants of hepatic cords (dark areas). Paraffin-embedded. H & E. 55X.
and adipose tissue attached to skeletal muscles, serosanguineous exudate in the peritoneal cavity, and dark-red musculature. One chicken had gizzard erosions. All birds had feed in the crop, proventriculus, and gizzard.

Histopathologic examination was performed on the organs of 4 birds. Liver lesions (Figs. 2, 3) were extensive and included multiple coalescing foci of swollen vacuolated hepatocytes, focal necrosis and hemorrhage, bile duct hyperplasia, and eosinophilic intranuclear inclusions in hepatocytes (Fig. 4). Basophilic inclusions completely filling the nucleus, causing it to be distended, were observed in a few hepatocytes (Fig. 5). Lymphoid depletion had occurred in the spleen and bursa of Fabricius. Bursal follicles were atrophied, some contained necrotic lymphocytes, and many were surrounded with fibrous tissue, lymphocytes, and histiocytes (Fig. 6). Necrosis of the villi of the duodenum was observed. Sporadic necrotic foci and histiocytic accumulations were present in the pancreas, myocardium, and kidney. No lesions were detected in the lung, trachea, thymus, or skeletal muscle.

Neutralizing-antibody titers to 2 AAV serotypes and precipitating antibody to IBD virus were detected in the serums of

Fig. 3. Liver of a 5-week-old chicken affected with inclusion body hepatitis. Hepatic architecture is disrupted. Many hepatocytes have vacuolated cytoplasm and distended nuclei with marginated chromatin and eosinophilic inclusions (arrows). Paraffin-embedded. H & E. 725×.
Fig. 4. Liver of a 5-week-old chicken affected with inclusion body hepatitis. Hepatocytes are vacuolated and contain eosinophilic, intranuclear inclusions (arrows). Plastic-embedded. H & E. 1800X.

Fig. 5. Liver of a 5-week-old chicken affected with inclusion body hepatitis. Basophilic inclusions (arrows) distend the nuclei of two hepatocytes. The cytoplasm of many hepatocytes contain vacuoles of various size. Paraffin-embedded. H & E. 1800X.
chickens submitted for laboratory examination (acute, Table 2) and in the sera of a subsequent group of chickens from the same flock sampled 2 weeks later (convalescent, Table 2).

Round-cell CPE-producing agents were isolated from the cecum of 9 chickens, the liver of three, and the trachea of one chicken. All but 3 isolants were obtained on the first passage in CKC. Two cecal isolants and one isolant from each of the liver and trachea, obtained from 4 chickens, were classified as type-8 AAV using AAV TR-59 as the prototype. The other 9 isolants were not typed. Syncytial-cell CPE-producing agents, which failed to hemagglutinate chicken erythrocytes, were isolated from the cecum of 2 chickens. These were not further characterized.

Transmission trial. Antibody to AAV TR-59, but not the other 7 AAV prototypes, was detected in sera of chicks from 2 of the SPF flocks tested, the mean VN titers of 10 chicks from each flock being 180 and 1035, respectively. Progeny of the third flock were negative to all 8 AAV prototypes (titer less than 40), and were used in this trial.

High mortality and morbidity occurred in the chickens inoculated with virus, and lesions were observed in a number of organs (Table 3). Necropsy examination of dead and moribund chickens revealed swollen, friable yellow-orange petechiated livers; yellow nodular pancreases; serosanguineous fluid in the pericardial and peritoneal cavities; pale swollen kidneys with urate-distended ureters; small bursas and thymuses; small pale spleens; flaccid gastrointestinal tracts which contained feed in the crop and gizzard but gas and pinkish exudate in the intestines; petechiation of leg muscles and fat surrounding visceral organs; and generalized congestion of subcutaneous tissue and skeletal muscles. Histologic lesions included necrotizing pancreatitis and hepatitis, severe lymphoid depletion of the bursa, spleen, and thymus, nephrosis of the proximal convoluted tubules, necrotic duodenitis, and isolated foci of Zenker's hyaline degeneration in the myocardium. Both basophilic and eosinophilic intranuclear inclusions were numerous in epithelial cells of pancreatic acini, liver, kidney, and duodenum. Chickens which survived 9-11 days post-inoculation (Table 3) had similar but less extensive lesions. Only eosinophilic intranuclear inclusions were found at this stage. Lung, trachea, and skeletal muscles did not appear to be affected histologically. Detailed pathologic findings are to be reported in a subsequent manuscript.
Virus was reisolated on first passage from pooled lung and trachea, liver, and cecum of birds injected with virus and sampled within 5 days of inoculation, but was reisolated from the cecum only of chickens sampled after this time. Avian adenovirus VN antibody, but not precipitating antibody to AAV or IBD virus, was detected in the serums of chickens inoculated with virus (Table 3).

VN antibody to 8 AAV was not detected in preinoculation serums. Control chickens were negative for clinical signs of disease, tissue lesions, virus, and AAV VN and precipitating antibody.

DISCUSSION

Necropsy examination of chickens submitted from a commercial flock revealed lesions similar to those described for IBH (Table 1). Numerous round-cell CPE-producing agents were isolated from the tissues of the chickens, and 4 isolants selected for characterization were type-8 AAV. Serologic examination indicated that a current infection with a type-8 AAV was occurring in the flock since the titer was more than 4 times as great in convalescent serums as in acute serums (Table 2). On the basis of these pathologic, virologic, and serologic findings, the possibility was further investigated that a type-8 AAV was involved in IBH.

Fig. 6. Bursa of Fabricius of a 5-week-old chicken affected with inclusion body hepatitis. Atrophied follicles are surrounded by fibrous tissue and mononuclear inflammatory cells. Paraffin-embedded. H & E. 180x.
When inoculated by the intra-abdominal route into 1-day-old chicks, an isolant of type-8 AAV, AMG 5 (2a), produced 70% mortality and a range of lesions similar to those found in birds submitted from the commercial flock in which IBH was diagnosed. A statistically significant depression of packed cell volume was detected. Rosenberger et al. (20) obtained similar results with type-5 AAV (IBHV and DPI-1) (Table 1). However, these results probably do not warrant a diagnosis of anemia since normal hematocrit values reported for chickens range from 20 to 40% (7). Values as low as 7% as reported by Klopp et al. (10) for naturally occurring cases of IBH were certainly not produced. No histopathologic examination was made of bone marrow since no macroscopic abnormalities were detected. However, future evaluations of disease induced by AAV should probably include more extensive investigation of hemopoietic function since experimental infections with type-5 AAV have produced conflicting effects on bone marrow (1,4,20; Table 1).

Infectious bursal disease virus has been incriminated in the etiology of adenoviral diseases (5,21). A positive correlation between lack of immunity to IBD virus in breeder flocks and increased susceptibility of progeny to hemorrhagic-aplastic-anemia syndrome was reported (21), and prior infection of chickens with IBD virus enhanced the pathogenicity of IBHV (5). In the case reported here, precipitating antibody to IBD virus was detected in both groups of chickens submitted for examination (Table 2), suggesting that infection with IBD virus occurred in the flock. Another possible interpretation of these findings is that maternal antibody was detected, but that seems unlikely since it has been shown that maternally-derived precipitating antibody to IBD virus cannot be detected after 3 weeks of age (21). It has been suggested that bursal lesions described in naturally occurring outbreaks of IBH may have been due to IBD virus (21). That possibility was considered an unlikely explanation for the lesions observed in chicks inoculated with AMG 5 (2a) since they were derived from an IBD virus-negative SPF flock, were housed under strict isolation conditions, and had no detectable precipitating antibody to IBD virus in their serums. It appears from these results that AAV can produce lymphoid depletion of lymphoid organs essential for humoral and cell-mediated immunity.

Pancreatitis has not been a common finding in IBH, although it is often not apparent whether the pancreas was examined patho-
Type-8 adenovirus in inclusion body hepatitis

logically (Table 1). Lesions of the pancreas were reported in an IBH outbreak in Italy (19). A type-1 AAV (18) and adenoviruses isolated from horses (14) and monkeys (2) were shown to produce pancreatitis experimentally.

Virus-neutralization testing of serums of progeny chicks indicated that 2 of the 3 SPF flocks from which fertile eggs were obtained had been exposed to at least one serotype of AAV. Quality-control tests by the SPF egg supplier included an agar gel test for AAV performed on serums of breeder hens. Results were negative, suggesting possible deficiencies of agar-gel tests for detecting AAV-infected flocks. Similar problems were reported by McFerran et al. (16). It has been suspected that AAV can be egg-transmitted (3,4,22,23), and that maternal antibody can protect against disease in chicks inoculated with AAV (4). Evaluation of preinoculation serums by neutralization tests for all known AAV serotypes would appear to be a particularly important test to AAV researchers under these circumstances.

Type-8 AAV (16) have not been firmly incriminated in the etiology of IBH. However, MacPherson et al. (17) reported the isolation of a type-8 AAV (H131) (15,16) from several outbreaks of IBH in England. Although mortality and hepatitis were produced in birds experimentally infected with H131, those workers considered that they had failed to reproduce "typical lesions." In addition, virus-neutralization tests with H131 indicated that birds unaffected by clinical IBH had levels of neutralizing antibody similar to those of birds affected with the disease. Those findings influenced those workers to suggest that the "adenovirus isolated was not the sole agent in the etiology of the natural disease" and to conclude that it was of "doubtful significance" in the etiology.

The fact that a current systemic type-8 AAV infection of chickens affected with IBH was demonstrated by virologic and serologic means in the work reported here, and that lesions similar to those found in the naturally occurring outbreak were reproduced in chicks inoculated with a type-8 AAV, under appropriately controlled conditions and in the absence of other known pathogens, indicates that type-8 AAV were involved in the etiology of the field outbreak of IBH.

REFERENCES


ACKNOWLEDGMENT

The authors thank Penny Walker, Becky Escoe, Nancy Underwood, and Ralph Ashley for competent technical assistance.