Role of viral load in the pathogenesis of chicken anemia virus

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The pathogenesis of strain 3711 of the chicken anemia virus (CAV), propagated in chickens, and two preparations of strain 3711 that had been adapted to grow to high titre in cells of the MDCC-MSB1 line were studied in chicken embryos and/or chickens. Highest viral loads in infected chickens, as measured by a microplate DNA-hybridization assay, were detected in the thymus, clotted blood and pancreas, and the lowest in the duodenum. The CAV DNA copy number in the organs of chicken embryos was significantly lower than in chickens. Route of infection was an important determinant of the course of disease in chickens, with clinical signs appearing earlier in birds infected by the intramuscular than those infected by the oral route; there was a direct relationship between viral load in particular organs and the extent of clinical signs. No reduction in the pathogenicity for chickens was noted for strain 3711 after 65 or 129 passages in the MDCC-MSB1 cell line.

Chicken anemia virus (CAV) is a small, non-enveloped, single-stranded DNA virus, first isolated by Yuasa et al. (1979), and was assigned as the only member of a new genus, Gyrovirus, within the family Circoviridae (Pringle, 1999). The virus causes aplastic anaemia, generalized lymphoid atrophy and increased mortality after infection of day-old susceptible chickens (Bisgaard, 1983; Engström & Luthman, 1984; Yuasa et al., 1987; Vielitz & Landgraf, 1988; Chettle et al., 1989). Infection of older birds with CAV results in subclinical disease and reduced resistance to other pathogens. Dual infection of chickens with other immunosuppressive viruses increases the severity of CAV signs and decreases the resistance of older birds to infection (Yuasa et al., 1980b; Otaki et al., 1988a, b; Rosenberger & Cloud, 1989; De Boer et al., 1994; Cui et al., 1999; Toro et al., 2000). CAV can be transmitted between chickens by both the horizontal and vertical routes. Horizontal spread usually occurs via the oral–faecal route, but infection via the respiratory route has also been demonstrated in experimentally infected birds (Rosenberger & Cloud, 1989). Vertical spread through hatched chickens is considered to be the most important means of dissemination of the virus (Yuasa & Yoshida, 1983; Yuasa et al., 1983; Hoop, 1992, 1993).

The growth of CAV in chicken embryos was first described by von Bülow & Witt (1986). Most studies of CAV pathogenesis have involved inoculation of chickens by the intramuscular (i.m.) route, which has been shown to be the most sensitive for inducing clinical signs in susceptible day-old chickens (Rosenberger & Cloud, 1989; Yuasa, 1989). The size of the CAV inoculum influences the severity of anaemia and other clinical signs and the proportion of chickens infected (Yuasa et al., 1980a; Rosenberger & Cloud, 1989; McNulty et al., 1990). In the present study, the growth of strain 3711, an Australian vaccine virus prepared from infected chickens (Connor et al., 1991), was examined in various tissues of specific-pathogen-free (SPF) chicken embryos by using a microplate DNA-hybridization assay for estimation of the CAV DNA copy number. These studies were extended to SPF chickens, where its growth was compared with that of other preparations of strain 3711 after passage in cells of the MDCC-MSB1 line. The relationship between the occurrence of clinical signs and viral load was also measured.

CAV vaccine strain 3711 was supplied by Dr Gordon Firth, Intervet (Australia) Pty Ltd, as a liver homogenate in PBS after three passages in SPF chickens and was shown to be free from other avian pathogens. Before use, the virus preparation was clarified, sterilized by filtration and diluted in PBS to contain 40 50 % chicken infectious doses (CID50). Strain 3711 was also adapted to grow to high titre (>10^6 TCID50 in 0·1 ml) in cells of the MDCC-MSB1 line (Tannock et al., 2003). Viruses used in the present study were passaged 65 and 129 times.

CAV DNA extracted from tissue samples by the method of Todd et al. (1992) was amplified with the forward primer 5'-CAGTGAATCGCGTCTAGC-3' and the reverse primer 5'-GTCGTCATTGCATCTAACAG-3'. Standard PCR mixtures (50 μl) contained 2·0 μM each dNTP, 2·0 mM MgCl2, 0·2 μM forward and reverse primers, 1·0 U AmpliTaq DNA polymerase (Roche Molecular Systems) and 5 μl template DNA. Each reaction was allowed to take place for 35 cycles, each consisting of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C.
A 266 bp probe within the 452 bp segment was amplified by PCR using primers 5’-GACC GCTAC GGTGTC AG-3’ and 5’-TCGCTTACC GTACTCG-3’. The PCR product was purified and then labelled with Photoprobe biotin (Vector Laboratories), according to procedures described by Forster et al. (1985) and Hibma et al. (1994). The microplate DNA-hybridization assay was based on the method described by Inouye & Hondo (1990). Briefly, the amplified DNA segment (452 bp) was purified (Todd et al., 1992) and coated onto a microplate well at 37 °C for 2 h. After washing three times, 5 ng heat-denatured biotin probe (266 bp) and 4-5 μg salmon-sperm DNA were added per well and incubated at 42 °C overnight. After a further three washes, 100 μl horseradish peroxidase-conjugated streptavidin (1:500) was added to each well and the plate was held at room temperature for 1 h. The wells were again washed and 100 μl TMB substrate (3,3’,5,5’-tetramethylbenzidine; KPL) was added per well and incubated for 30 min. The reaction was then stopped by addition of 50 μl 2 M H2SO4 per well and A450 was read. The DNA-hybridization assay could detect 0.005–0.025 ng of the 452 bp CAV segment; bands containing 5–10 ng of the 452 bp segment could be detected by a standard PCR (Tan, 2002). Novak & Ragland (2001) also demonstrated that a competitive DNA-hybridization assay in microtitre plates for the detection of CAV was more sensitive than in situ hybridization, dot-blot and ELISA assays.

The Mₚ of the 452 bp segment was estimated to be 2.9832 x 10⁵ and its concentration was determined by A₄₅₀ measurement. The copy number of the 452 bp segment was calculated according to the following formula (Mackay, 1999):

\[
\text{DNA copy number} = \frac{\text{Avogadro’s number} \times (6.02 \times 10^{23}) \times \text{mass (g)}}{M_p}.
\]

For internal standardization, serial 10-fold dilutions of the purified 452 bp segment were prepared in parallel. A 50 μl aliquot of each dilution, containing 10⁴–10⁷ copies of the 452 bp segment, was amplified by the standard PCR and the products were quantified by microplate DNA hybridization using the biotin probe. A good linear correlation between A₄₅₀ and copy number for the 452 bp segment was observed (y=0.1751x–0.0561). As each 452 bp segment represented a single CAV genome, the number of CAV DNA templates in samples could be estimated by using the standard PCR to amplify the 452 bp segment, followed by DNA hybridization with the biotin probe and extrapolation from the standard curve. For the assay of tissue extracts, standard preparations containing 10⁴–10⁷ copies of the 452 bp segment were run in parallel. Assays were considered valid if the mean A₄₅₀ of each standard preparation fell within ±1SD of the expected values.

Strain 3711, prepared from infected chickens, was inoculated to 25 5-day-old SPF chicken embryos via the yolk sac. Fifteen eggs were inoculated with PBS as controls and all were examined at days 16–20. Deaths occurred in 12 infected embryos (48 %) and in one from the control group (6 %). Of the 12, four appeared normal, four were stunted and four were stunted with unilateral abnormalities of the eye. The organs from six individual surviving embryos at day 20 were harvested and extracts were prepared for testing for CAV viral load by the microplate DNA-hybridization assay. The CAV copy number for the various organs was 3.6–8.6 log₁₀ (g tissue)⁻¹. CAV DNA could be detected in all thymus and pancreas samples [mean viral loads, 8.6 ± 1.08 and 5.6 ± 0.36 log₁₀ copies (g tissue)⁻¹, respectively]. For the brain, bursa, kidney, liver and lung samples, CAV DNA was detected in one or two of three samples tested [4.85 ± 0.80 or 7.43 ± 0.62 log₁₀ copies (g tissue)⁻¹]. Only one of three duodenum samples tested was positive and the viral copy number [4.60 log₁₀ copies (g tissue)⁻¹] was lower than that in other organs.

A further batch of 25 embryos was infected similarly with another batch of CAV vaccine and 15 chickens (60%) hatched. Three surviving chickens from infected and control groups were euthanized at days 5, 10, 15, 20 and 25 after hatch and individual organ samples were collected and tested for virus load. Hatched chickens inoculated in ovo showed clinical signs, characterized by moderate to severe depression, anorexia, anaemia and dehydration at days 5 and 10 after hatch. One bird from the infected group died at day 15. Fig. 1 shows that the mean body weight of the infected group was lower than that of the controls at day 10 (Student’s t-test; P<0.05), but the greatest differences were seen at days 15–25 (P<0.01). The thymus: and bursa:body-weight ratios of inoculated chickens were significantly lower than those of the control group at days 5–15 (P<0.01). However, for the spleen:body-weight ratio, significant differences were noted only between inoculated and control birds at day 25 (P<0.05).

A significant decline in the haematocrit value (HV) of infected birds compared with controls could be detected at days 5, 10, 15 and 20 after hatch (P<0.01). HV levels had returned to near-normal values (27 %; von Bülow & Schat, 1997; Todd, 2000) by day 25, although, even then, the difference between the two groups was still significant (P<0.05). The decrease in HV preceded the appearance of antibody to CAV by 10–15 days and the highest titre was reached by day 25 (Fig. 1). Macroscopic lesions, including the appearance of pale bone marrow, atrophy of the thymus and paleness of comb and muscle, were present in infected birds. Atrophy was most severe in the bone marrow by days 5 and 10 and in the thymus by days 10 and 15 after hatch. These lesions were evident until day 20, but no subcutaneous or intramuscular haemorrhages were present. Neither clinical signs nor gross lesions were detected in the controls.

Extracted DNA was tested by the microplate DNA-hybridization assay. The results (Table 1) indicate that all tested tissues were positive, but highest viral loads were
Fig. 1. Mean body and organ weights, HV and antibody titre of hatched chickens inoculated as 5-day embryos with strain 3711 prepared in chickens (●) and control (○).

Table 1. CAV load in different organs of hatched chickens infected as 5-day-old embryos with 40 CID<sub>50</sub> strain 3711 prepared by growth in chickens

Values are means±SD log<sub>10</sub> DNA copy number (g tissue)<sup>−1</sup>. The minimum level for detection was 3·1 log<sub>10</sub> copies (g tissue)<sup>−1</sup>.

<table>
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<tr>
<th>Organ</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
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<tr>
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<td>4·76±0·9</td>
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<td>10·26±1·0</td>
<td>9·60±1·7</td>
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<td>Duodenum</td>
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<td>5·60±0</td>
<td>4·10±0</td>
<td>4·60±0</td>
<td>&lt;3·1</td>
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<tr>
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Fig. 2. (a) Mean body and thymus weight, HV and antibody titre of chickens; (b) viral load in different organs of chickens inoculated with strain 3711 prepared in chickens by the i.m. (●) and oral (□) routes and by contact transmission (▲). Results for strain 3711 after 65 (●) and 129 passages (■) in cells of the MDCC-MSB1 line. The limit for detection was a copy number of $3 \cdot 10^1$ log$_{10}$ (g tissue)$^{-1}$.
present in the thymus $[8 \cdot 60 - 11 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$. An unexpected observation was the very high load in the pancreas $[7 \cdot 60 - 10 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$. Organs with moderate loads were the liver $[7 \cdot 85 - 9 \cdot 53 \log_{10} \text{copies (g tissue)}^{-1}]$, spleen $[7 \cdot 60 - 9 \cdot 25 \log_{10} \text{copies (g tissue)}^{-1}]$, bursa $[5 \cdot 60 - 8 \cdot 10 \log_{10} \text{copies (g tissue)}^{-1}]$, kidney $[6 \cdot 60 - 7 \cdot 85 \log_{10} \text{copies (g tissue)}^{-1}]$ and lung $[4 \cdot 60 - 8 \cdot 10 \log_{10} \text{copies (g tissue)}^{-1}]$. Lower loads were present in the brain and duodenum $[3 \cdot 35 - 5 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$. Viral loads in the organs of chickens were significantly higher than in those of embryos. The very high load in clotted blood is partly attributable to viraemia and partly due to the growth of CAV in the bone marrow. The presence of CAV DNA in most organs is probably a consequence of viraemia and not an indication that virus replication had taken place. The high CAV load in the pancreas suggests that it, like the thymus, is a primary target organ. Smyth et al. (1993) described the presence of CAV antigen in the pancreas of infected birds and similar findings have been reported for coxsackievirus B4 virus, which produces infections of the pancreas and a focal myositis of mice (Pallansch & Roos, 2001).

In total, 90 day-old SPF chickens were divided randomly into six groups of 15 chickens. Five groups were inoculated with different CAV preparations by different routes. Chickens in the contact transmission group were wing-tagged and housed together in the same cage with the group that was inoculated with the chicken-propagated strain 3711 by the i.m. route; the sixth control group was inoculated by the i.m. route with PBS.

All birds infected with CAV by i.m. inoculation developed characteristic anaemia by days 10–12 post-infection (p.i.), with peak signs being present at days 15–18. Affected birds appeared anorexic and depressed, with pallor of the comb and wattles and ruffled feathers. Only one bird in the group died, at day 20. Birds in groups inoculated by the oral route developed the same clinical signs by day 15 and no deaths occurred during the experimental period, confirming earlier findings (Rosenberger & Cloud, 1989; Yuasa, 1989) that the i.m. route is most efficient for inducing clinical disease. Neither clinical signs nor deaths occurred in the contact group.

Birds inoculated by the i.m. route showed a significant decline in weight gain after i.m. inoculation at days 10 and 25, compared with the controls (Fig. 2a; $P < 0.05$), and greater differences were seen at days 15 and 20 ($P < 0.01$). Similar results were obtained in the groups inoculated orally at days 15, 20 and 25 ($P < 0.01$), but there was no decline in body weight for the contact group. Only thymic atrophy was observed in i.m.- and orally infected chickens and the thymus:body-weight ratios were greater than for the control group at days 15–20 ($P < 0.01$) and 25 ($P < 0.05$). Lower HVs were observed in groups infected by i.m. inoculation. HVs of $< 27$ % were observed at days 10–20 but, in 53 % of cases, increases to near-normal values had occurred by day 25 (Fig. 2a). For groups inoculated orally, HVs of $< 27$ % were seen up to day 20. Most birds were still anaemic by day 25 and recovery to normal values occurred in one-third of birds. There were no changes in the mean HV of the contact group. Overall, no differences in pathogenesis could be detected between the groups inoculated with chicken- or cell culture-grown viruses.

A blocking ELISA (Tannock et al., 2003) was used to test the antibody status of chickens from each group. Antibody to CAV could not be detected in any group before 10 days. In the group inoculated by the i.m. route, antibody could be detected in 75 and 89 % of birds at days 15 and 20, respectively, and 100 % by day 25. By contrast, only 44 and 67 % of birds in the groups infected by the oral route developed antibody by days 20 and 25 and only one bird from the contact group by day 25 (Fig. 2a).

In the groups inoculated by the i.m. route, CAV DNA could be detected in all organs tested at days 5–25 p.i., with highest copy numbers being present in samples obtained from clotted blood, pancreas and thymus (Fig. 2b). Peak levels $[10 \cdot 10 - 11 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$ were obtained at days 10–20. Intermediate levels $[3 \cdot 35 - 9 \cdot 76 \log_{10} \text{copies (g tissue)}^{-1}]$ were present in samples from the brain, bursa, kidney, liver, lung and spleen and lowest levels $[3 \cdot 10 - 5 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$ from the duodenum. There were no significant differences in load between chicken- or cell culture-passaged viruses (Fig. 2b). Peak loads in the thymus, pancreas and blood of chickens inoculated by the i.m. route occurred at day 15 and by the oral route by day 20, although the extent of each was similar. CAV was present in thymus and clotted blood at day 5 p.i. and other organs of chickens inoculated by the oral route by day 10. Highest loads were present in the thymus, clotted blood and pancreas. In the contact group, CAV DNA could not be detected in any organ before day 10. Fewer birds were positive and the viral loads $[3 \cdot 10 - 7 \cdot 76 \log_{10} \text{copies (g tissue)}^{-1}]$ in individual organs were significantly lower than those in birds inoculated by the i.m. $[3 \cdot 10 - 11 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$ and oral $[3 \cdot 10 - 10 \cdot 60 \log_{10} \text{copies (g tissue)}^{-1}]$ routes.

Apart from route of inoculation, the ability to produce anaemia after experimental infection is related directly to the virus dose (Yuasa et al., 1979; Yuasa, 1989; McNulty et al., 1990). The present study demonstrates, for the first time, a relationship between viral load and the extent of clinical disease. All chickens in groups inoculated by the i.m. and oral routes or by inoculation of embryos showed typical clinical signs. Chickens infected by contact transmission did not show signs and exhibited similar increases in total weight and in the weights of immune organs to those of the controls. Viral loads in all organs of the chickens infected by contact were significantly lower than those infected by the i.m., oral or in ovo routes (Fig. 2b).

There were no differences in pathogenicity between preparations of strain 3711 that were propagated in chickens or passaged to different levels in the MDCC-MSB1 cell
line (Fig. 2a). By contrast, Todd et al. (1995, 1998) found that the Cux-1 strain became substantially less pathogenic after 173 and 320 passages in MDCC-MSB1 cells and that the pathogenicity of attenuated viruses could be restored after 10 passages in young chickens. The reasons for these differences are unclear, but could be related to undefined genetic differences between strain 3711 and the Cux-1 strain. There were also no significant differences with respect to viral load, the appearance of clinical signs, reductions in weight gain, declines in HV or levels of antibody induced between chickens inoculated in ovo at day 5 of embryogenesis and others inoculated at day 1 after hatch (Table 1; Figs 1–2). However, mortality rates following egg inoculation were high (48 and 40%). In support of this, decreases in the bursa : and spleen : body-weight ratios occurred in hatched chickens inoculated in ovo, but not in chickens inoculated at day 1 (Tan, 2002).

There have been several recent reports on the use of real-time PCR for measuring viral loads of CAV in infected chickens (Markowski-Grimsrud et al., 2002) and for the measurement of viral DNA copy number in isolated lymphocytes (Markowski-Grimsrud & Schat, 2003). This test has also been adapted to measurement of CAV-neutralizing antibody (van Santen et al., 2004a). An advantage of the test used in the present study is its low cost and suitability for the simultaneous processing of large numbers of samples without the need for an expensive, dedicated instrument.

Overall, these findings confirm that the bone marrow and thymus are the most important target organs in the pathogenesis of CAV infection. The destruction of haemocytoblasts in bone marrow results in a severe depletion of erythroid and myeloid cells and produces anaemia; the destruction of T-lymphocyte progenitor cells in the thymus results in depletion of mature cytotoxic and helper T cells and induces severe atrophy (Adair, 2000). Virus is spread to other organs by viraemia and lymphoid foci develop in the liver, kidney and lung. However, the reason for higher viral loads in the pancreas is unclear. N.B. A paper on the measurement of peak CAV loads by real-time PCR in chickens infected by the i.m. and oral routes appeared at the time that our paper was submitted, and showed similar results (van Santen et al., 2004b).

References


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