COMPARISON OF TWO SEROTYPE 1 MDV ISOLATES

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Summary. – We compared the RB1B and T. King (TK) serotype 1 isolates of Marek's disease virus (MDV) *in vivo*. Body and organ weights, mortality, and lesions indicated that the TK inoculum established early infection more efficiently than RB1B and did greater damage to the bursa of Fabricius and thymus. Subsequent studies showed that the TK inoculum that we used contained chicken infectious anemia virus (CIAV). Therefore, pathogenicity profiles shown here should be interpreted with the presence of CIAV contamination in the TK stock in mind.

Key words: Marek's disease; MDV; herpesvirus of turkeys; herpesvirus

Introduction

In recent years, MDV isolates with apparently increased virulence have been reported. In North America, two reports (Ficken *et al.*, 1991; Rosenberger and Cloud, 1993) described strains that caused a high incidence of Marek's disease (MD) in vaccinated flocks. Other reports have described isolates with increased virulence isolated in South America (Buscaglia *et al.*, 1995), Europe (Kross *et al.*, 1996; Venugopal *et al.*, 1996) and Australia (Zerbes *et al.*, 1994).

Witter (1997) evaluated 31 serotype 1 MDV isolates obtained from the United States between 1987 and 1995 and compared them to JM/102W (vMDV) and Md5 (vvMDV). The 31 strains were found to represent a continuum of pathotypes based on protection against them by herpesvirus of turkeys (HVT) alone or in combination with SB-1. Strains that produced significantly higher levels of MD gross lesions in bivalently-vaccinated chickens compared to MD5 were provisionally designated as vv+ MDV and appeared to be on the high end of the virulence continuum. Recently, Calnek *et al.* (1998) compared isolates of all three pathotypes, and this work indicated that increased cytolytic infection and atrophic changes in the primary immune organs were associated with the vv+ isolates. Preliminary studies in our laboratory indicated that the MDV ICP4 immediate early gene was expressed earlier in chickens infected with a recent MDV isolate, designated TK, compared to those infected with RB1B. This suggested that the increase in virulence of newly emerged MDV strains might be due to more robust early cytolytic infection.

As a first step toward addressing this question, we compared RB1B and TK *in vivo*. Data on immediate early viral gene expression and lymphoid cell populations will be presented elsewhere. Here we discuss our findings with regard to virus isolations from spleen and peripheral blood, body and organ weights, mortality, and lesions.

Materials and Methods

Viruses. MDV strain RB1B (Schat *et al.*, 1982) was obtained from K. A. Schat. Following isolation from commercial layer breeder chickens, this isolate had been clone-purified in chicken kidney cells (CKC) six times and passed once in specific-pathogen-free (SPF) chickens to amplify the virus before a sample was sent to us. The TK isolate was obtained from J. Rosenberger and S. Cloud. It was originally isolated from four-week-old broilers and passed four times in SPF chickens before a sample was given to us. We then passed both the RB1B and TK isolates twice in SPF chickens to obtain enough inocula for the planned experiments. Stocks were titered in both CKC and chicken embryo fibroblasts (CEF) prior to and on the day of inoculation.

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Table 1. Mortality and tumor incidence in RB1B- and TK-inoculated chickens

Inoculum	No. of chickens[a]	Early mortality[b]	Total mortality[c]	Tumor incidence[d]
Expt. 1				
None	35	1 (3%)	1 (3%)	0
RB1B	26	2 (8%)	21 (81%)	23 (88%)
TK	27	19 (70%)	27 (100%)	9 (33%)
Expt. 2				
None	20	0	0	0
Mock	20	0	0	0
RB1B	16	0	5 (31%)	16 (100%)
TK	21	20 (95%)	20 (95%)	16 (76%)

[a]Number of chickens found dead plus live chickens remaining at 42 DPI.
[b]Number of chickens found dead through 21 DPI.
[c]Number of chickens found dead through 42 DPI.
[d]Number of chickens with grossly evident MD at necropsy.

Experimental chickens. SPF chickens were obtained from SPAFAS (Storrs, CT) and housed in glove-port isolators.

Testing stocks for extraneous viruses. Seroconversion of five-week-old SPF chickens inoculated with either untreated or chloroform-treated (Feldman and Wang, 1962) samples of our stock viruses was evaluated. Sera were collected prior to and at three weeks post-inoculation and evaluated using commercial enzyme-linked immunosorbent assay (ELISA) kits (IDEXX, Westbrook, ME) for avian leukosis virus, infectious bursal disease virus (IBDV), reticuloendotheliosis virus, and CIAV. Hematocrit depression in chickens simultaneously inoculated with IBDV and the test samples at one day of age was also evaluated (Rosenberger and Cloud, 1989). Polymerase chain reaction (PCR) assays were done on DNAs purified from the various stocks using three sets of primers; namely, National Veterinary Services Laboratory primers (unpublished), University of Delaware primers (unpublished), and primers described by Soine *et al.* (1993).

Experimental design. For the first experiment, one-day-old SPF chickens were inoculated intraabdominally with RB1B (550 PFU), TK (430 PFU) or left as uninoculated controls. At six, nine, twelve, and fifteen days post inoculation (DPI), samples were collected from 15 to 24 birds per treatment group and used for DNA analysis, RNA analysis, and virus isolations. At 42 DPI, the remaining chickens were killed and examined for gross lesions. At all time points, body weight and lymphoid organ weights were obtained, and tissues were collected for histopathology. For the second experiment, one-day-old chickens were inoculated with RB1B (138 PFU), TK (126 PFU), uninfected spleen tissue (mock controls), or left as uninoculated controls. At 28 DPI, remaining birds were killed and examined for gross MD lesions.

Statistical analysis. Body weights and relative organ weights were compared by analysis of variance (ANOVA) for a single criterion of classification for any number of groups with unequal replication. Where significant differences were found, paired comparisons were analyzed by the least significant difference (Steel and Torrie, 1980). Mortality and tumor incidence were compared using Chi-square analysis. Differences were considered significant when $P < 0.05$.

Results and Discussion

Mortality

Although there was not an obvious difference in final mortality in chickens inoculated with the two virus isolates in Expt. 1 (81% for RB1B and 100% for TK), the difference in early mortality was notable (Table 1). Cumulative mortality at 21 DPI was significantly greater for TK inoculates (70%) compared to RB1B inoculates (8%). The results were similar, but more striking, in Expt. 2 when lower doses were used. In Expt. 2, no mortality was seen in the RB1B inoculates at 21 DPI, while 95% of the TK inoculates had died. It was interesting that a greater percentage of the RB1B inoculates developed gross lesions (88% and 100% in Expts. 1 and 2, respectively) compared to the TK inoculates (33% and 76% in Expts. 1 and 2, respectively; see also Table 1). These results suggest that TK is more cytolytic than RB1B; i.e., susceptible individuals tend to die early not surviving long enough to develop tumors.

Body and lymphoid organ weights

Body weights were reduced in virus-inoculated chickens relative to controls (Fig. 1A). This effect was more pronounced for the TK inoculates than for the RB1B inoculates. Relative spleen weights were higher for both TK- and RB1B-inoculated chickens compared to control birds (Fig. 1B), indicating that hypertrophy of the spleen occurred in infected chickens. At 9, 12, and 15 DPI, relative weights of the bursa of Fabricius and thymus were lower for both TK- and RB1B-infected chickens compared to controls, indicating that atrophy of these organs had occurred (Fig. 1C and 1D). It was interesting that for RB1B-infected chickens, relative bursa and thymus weights increased over time after day 6; whereas, for TK-inoculates, they continued to decrease throughout the course of the experiment. These results indicated that the TK inoculum caused greater early mortality and damage to the primary lymphoid organs compared to the RB1B inoculum.

Presence of CIAV in the TK stock

After completion of two experiments, we checked our inocula to be certain they were not contaminated with other immunosuppressive or tumor-inducing viruses. No increase in ELISA antibody titers for avian leukosis virus, IBDV, or reticuloendotheliosis virus were found. However, most of the chickens inoculated with TK, but not RB1B, had antibodies to CIAV. Hematocrit depression following simultaneous inoculation of one-day-old chickens with IBDV and test stocks was also positive for the TK stock but negative for the RB1B stock (data not shown). Subsequent PCR anal-

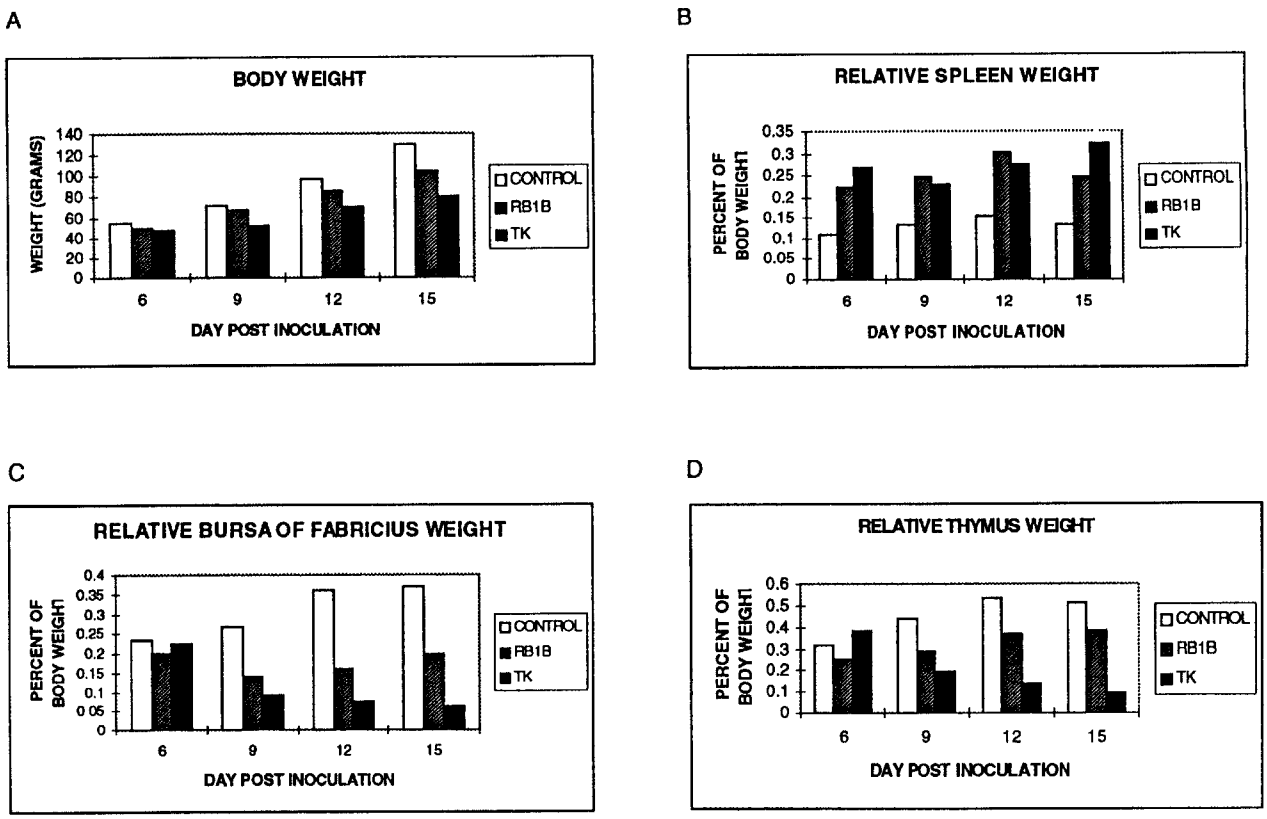

Fig. 1
Average body weights (A) and average organ weights (B-D)
Average body weights of chickens in the indicated groups. Average organ weights are presented as a percentage of body weights. Data are from experiment 1.

ysis confirmed that spleens of chickens inoculated with TK, but not those from chicken inoculated with RB1B, contained CIAV. Of the three primer sets used, NVSL and the University of Delaware primers resulted in positive PCR for the TK sample. In contrast, the primers originally described by Soine *et al.* (1993) failed to result in a positive PCR for our TK sample, although they did direct the production of an appropriate product from a positive control plasmid that contained the CIAV genome. Sequence differences between the CIAV contaminating the TK sample and the CIAV isolate used to design these primers may explain why they were negative in our tests. Our experience indicates that sole reliance on PCR to ensure that MDV stocks are CIAV-free may be risky; biological assays (seroconversion or hematocrit depression in IBDV-treated chickens) are more reliable tests for the presence of CIAV.

At this point, we are unsure as to what, if any, influence the CIAV contamination of the TK inoculum might have had on our results. The influence could be minimal since we used very high doses and MDV-induced immunosuppression and early mortality may have overshadowed any influence of the CIAV. On the other hand, the CIAV could have had significant effects on the results. It is important to note that our pathogenicity results are strikingly similar to results published by Calnek (1998) using CIAV-negative vv+ MDV isolates.

We plan to repeat these *in vivo* experiments using a CIAV-negative vv+ strain tested using biological assays. In addition, we are planning to spike samples of our CIAV-free MDV stocks (both RB1B and a vv+ isolate) with a known amount of CIAV to see if the presence of CIAV significantly alters MDV pathogenicity at the dose levels we are using.

Acknowledgements. We gratefully acknowledge financial support for this research from the Delaware Research Partnership and Intervet International BV.

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