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Natural infection with Marek's disease herpesvirus by the respiratory route

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Abstract

Natural infection with MDV occurs after inhalation of feather and skin debris from MDV-infected chickens. Over the last 30 years most studies of MDV pathogenesis and immunity have involved cell-associated MDV, cell-free MDV or MD vaccines, administered by a parenteral route. However, parenteral delivery avoids crucial early events that occur directly after natural infection with MDV via the respiratory tract. In this paper we describe a method for infecting chickens with MDV in its 'native' form in infective dust. We show, for the first time, that chickens become infected with MDV, determined by viraemia and pathological changes, after infective dust is insufflated into the respiratory tract. Cells in the respiratory tract of the chicken are the first to become infected, two days after infection, followed by those in the spleen. Viral replication in the lung is earlier and higher in susceptible compared to resistant chickens and is associated with a more pronounced immune response. Study of the early events in the lung after natural infection is therefore crucial. The method described here is reliable and allows a precisely-timed, measurable dose of 'native' MDV to be delivered to the natural portal of entry, so allowing the sequence of events in early MD pathogenesis to be studied.

Introduction

Marek's disease (MD) is a naturally-occurring lymphomatous disease that afflicts susceptible genotypes of chicken and other species of poultry (61). The causative agent, Marek's disease virus (MDV), is a highly cell-associated α-herpesvirus (57) that shares many of its biological properties with γ-herpesviruses, such as Epstein-Barr virus (EBV). MD is the only neoplastic condition to be prevented effectively by widespread use of anti-viral vaccines (60). MD vaccination has not only benefited the poultry industry but also provided a rationale for programmes to develop vaccines against EBV (23). However, since the introduction of MD vaccination in the early 1970s, widespread and intensive use of vaccines appears to have caused challenge strains of MDV progressively to increase in virulence (59). The phenomenon of vaccine-induced increases in virulence can partly be explained by MD vaccines invoking non-sterile immunity. Challenge viruses still infect, replicate in, and are shed by MD-vaccinated chickens, providing a reservoir of MDV pathotypes that can survive vaccine-induced immune responses and are able to infect other chickens (60). MDV has thereby become widespread in poultry houses and most chicks face challenge within a few days of hatching.

Vaccine-induced changes in MDV virulence not only pose serious problems for the poultry industry but raise questions for those developing vaccines against similar pathogens. Epidemiological modeling studies predict that vaccines which target pathogen replication tend to drive the pathogen to increased virulence (24). In contrast vaccines that interfere at the infection stage, before amplification of the pathogen, may have a neutral, or even negative, effect on the evolution of virulence. These modeling studies fit the situation with MD vaccination, which currently targets viral replication. It is

important, therefore, to gain an understanding of the early events in natural infection with MDV to investigate the possibilities of blocking the early stages in pathogenesis (reviewed in 3, 26).

Natural infection with MDV occurs through inhalation of MDV-infected debris from feathers and sloughed skin flakes (9). The virus has been suggested to be taken up by lung phagocytic cells (1) which could transport it to the lymphoid tissues where it replicates in lymphocytes. MDV is first detected in the splenic lymphocytes and later thymocytes and bursacytes where it causes a lytic infection from about three days after exposure (5). After the initial round of cytolytic infection, MDV latently infects lymphocytes and can transform $CD4^+$ cells causing lymphomas in visceral tissues of susceptible chickens. Chicken that develop clinical MD were shown to amplify the virus to a much higher level (7, 10). However, little is known of the immunological and virological events that take place before MDV replication in lymphoid organs, as experimental infections have mainly relied on parenteral inoculation of cell-associated MDV, thereby bypassing early events in the lung and the bronchial-associated lymphoid tissue. Studies to mimic natural infection with MDV have generally relied on generating airborne suspensions of infective dust in an enclosed space (1, 9, 14, 31, 32) or contact exposure with infected individuals (25, 34, 42, 49, 50). However, none of these studies have addressed the early events after infection.

In the present study, we have investigated early events after natural infection via the respiratory tract to identify the factors contributing to early MDV pathogenesis. We describe a method for infecting individual chickens with a standard dose of MDV-infective dust in its 'native' state. Since early events in infection could influence later

viral replication and clinical disease outcome, we have compared early virological and immunological changes in the lungs and spleens of two lines of chickens that are genetically-resistant or -susceptible to MD lymphoma formation after natural MDV infection (20, 53, 54).

Materials and Methods

Experimental animals. Chickens were obtained from parent flocks of outbred Rhode Island Red (RIR), inbred White Leghorn sub-line 7_2 (susceptible to MD) or White Leghorn sub-line 6_1 (resistant to MD) and maintained in disease-free conditions at the Institute for Animal Health (IAH). The parent flocks were unvaccinated and tested negative for antibodies to a range of poultry pathogens, including MDV, infectious bursal disease virus (IBDV), and chicken infectious anemia virus (CIAV). Their progeny were free of maternal antibodies to MDV. Experimental birds were housed in wire cages in isolation rooms entered through an air lock. Each room was provided with high efficiency particle air (HEPA)-filtered air under positive pressure. Rooms were hosed down daily to prevent the build-up of dust on the floor and walls. Food and water were available *ad libitum* throughout. All animal procedures were performed in accordance with local ethical regulations, and were approved by the UK Home Office.

Dust isolation for infecting chickens. Thirty two-week-old RIR chickens were infected by intra-abdominal injection of 1000 pfu of a very virulent strain of serotype-1 MDV, RB1B (16), from the 11^{th} passage in primary chicken kidney cells. Three weeks after infection, dust was collected from the extract filters of the isolation room. This dust was

coarsely filtered using a household flour sieve followed by a 300µm-steel mesh, desiccated overnight and stored in 2g quantities in sealed tubes below -70°C. Control dust was obtained from the extract filters of a similar room housing uninfected RIR chicks and treated in the same way. The same batches of dust were used throughout since storage at -70°C does not affect MDV infectivity (17, 30).

Experimental design. Known quantities of a suspension of dust were delivered into the lower trachea by carefully inserting the delivery tube of a PennCentury microaerosiliser ™ (NJ, USA) through the glottis and insufflating the dust using 2-3ml air. Infected chickens were killed when clinical signs of MD reached a pre-determined terminal endpoint.

Titration of infective dust. The minimum infecting dose of the batch of MDV-infective dust to induce clinical signs of MD was determined by insufflating a standard amount (5mg) of dust, containing different proportions of the MDV-infective material. There were eight line 7 chickens per dose and 5, 2.5, 1, 0.1, 0.01, 0.002 or 0.001mg MDV-infective dust were insufflated. A further 8 chickens were treated with 5mg of the uninfected dust. Blood samples (usually 50µl) were obtained by venepuncture at 3, 7, 10, 14, 21, 21, 35, 48 and 62 days post infection (dpi) to monitor MDV viraemia by quantitative PCR and on some occasions test for antibodies against IBDV and CIAV by ELISA (Idexx, UK). Chickens were monitored for clinical signs of MD for 63 days after which the experiment was terminated. At 63 dpi all remaining birds were killed and liver, heart, proventriculus, kidney and lung removed, and fixed in buffered formaldehyde-saline (pH 7.5) prior to histological processing and staining with hematoxylin and eosin.

Tissue sections were examined and scored for lymphocyte infiltration using the MD lesion scoring system described by Burgess *et al.* (11).

Early events after natural infection. Twenty-four chickens from each of the resistant (line 6_1) and susceptible (line 7_2) lines were infected by intra-tracheal insufflation of 2.5mg dust at 2 wk of age. Twenty-four age-matched birds from each line were used as controls and given 2.5mg non-infective dust by the same route. Blood samples (50µl) were collected from the wing veins of six infected and six uninfected chickens of each line at 1, 2, 3, 4 and 5dpi for DNA isolation. The spleen and one of the lungs were removed *post mortem* from each chicken and leukocytes isolated for analysis by flow cytometry and for DNA/RNA extraction. The remaining lung was removed and prepared for cryosectioning and immunostaining.

DNA and RNA extraction. DNA and RNA were isolated using a DNeasy© 96 tissue kit (Qiagen, Crawley, UK) or an RNeasy© 96 tissue kit (Qiagen), respectively, following the manufacturer's instructions. All RNA samples were treated with RNase-free DNase I (Amersham Pharmacia Biotech, Amersham, UK). Viral DNA levels were determined using the *meq* primer and probe set. Cytokine and viral transcript levels were determined using quantitative RT-PCR assay (Table 1 lists primer and probe sequences).

Preparation of cell suspensions. Peripheral blood and spleen leukocytes were purified by density gradient centrifugation using Ficoll-Paque Plus (Amersham Pharmacia Biotech). Leukocytes were prepared by enzymatic digestion of the lung, and by passing

spleens through a 70µm cell strainer. Briefly, lungs were washed in PBS and injected with 3-4ml of enzyme cocktail (556µg/ml DNase I (Boehringer Mannheim, Bracknell, UK), 2.2mg/ml collagenase D (Boehringer Mannheim) in Hank's balanced salt solution (HBSS: Sigma, Gillingham, UK)) using a 23G hypodermic needle. After incubation at 37ºC for 15 min the first suspension that contained mainly erythrocytes was disposed of. Incubation was repeated and subsequent suspensions containing lung leukocytes were transferred to 10mM EDTA in HBSS without calcium. Digestions were repeated until the lung tissue had completely disintegrated. The collected cell suspension was filtered using a 70µm cell strainer (Becton Dickinson).

Quantification of MDV and cytokine transcripts. The amount of MDV present in the DNA isolated from the blood was quantified using real-time quantitative PCR assay (Taqman™, Applied Biosystems, Foster City, USA) for the MDV *Meq* gene, essentially as described by Kaiser *et al.* (33). Meq reaction mixtures included primers and probe to an avian gene, ovotransferrin (*Ovo*), labeled with a second fluorescent dye (VIC™, Applied Biosystems). *Ovo* is present as two copies per chicken cell and so can be used to correct the viral signal to the quantity of chicken DNA isolated from the blood. Raw data, expressed as critical threshold (CT; the cycle number when the PCR crossed a threshold value for detection) were normalized to a positive control included in every run to exclude plate to plate variation. Slopes of CT value *vs.* ln-dilution were generated for each gene. Results of individual samples were initially calculated relative to a theoretical reference sample which had threshold levels of each gene:

$$LnR_1 = \frac{CT_{Meq}}{Slope_{Meq}} - \frac{CT_{ovo}}{Slope_{ovo}}$$

The zero point of this scale was then fixed to the LnR_1 value that would be given by a CT_{Meq} value of 40 and a CT_{ovo} value representing the minimum CT_{ovo} of all samples.

$$LnR_0 = LnR_1 - \frac{40}{Slope_{Meq}} - \frac{^{Minimum}CT_{ovo}}{Slope_{ovo}}$$

The zero point of the LnR_0 scale is thus a minimum estimate of the value that would be given by a sample with threshold levels of *Meq* and maximum level of *ovo*. It should be noted that samples ascribed with a CT of 40 have not reached the threshold within 40 cycles of PCR, and an LnR_0 approaching zero indicates an actual value of template between zero and that indicated by a CT of 40. The primer and probe concentrations and sequences are listed in Table 1.

Reverse transcription of isolated RNA was performed using a single-tube single-enzyme system reaction (Taqman EZ RT-PCR Kit, Applied Biosystems), essentially as described by Kaiser (33). Primer and probe concentrations and sequences are listed in Table 1. All cytokine/MDV reactions were corrected for the amount of cellular RNA by using 28S RNA (housekeeping gene) as described for quantitative PCR reactions.

Isolation and measurement of MDV in dust. MDV DNA was extracted from samples of dust for measurement by quantitative PCR assay. A sample (2.5mg) of the dust was added to 10mL TE buffer (1mM EDTA, 10mM Tris-HCl pH 8.0, 2% v/v nonidet P40, 0.05% v/v Tween-20) and incubated overnight, and after sonication (3×30s, Soniprep, New Jersey, USA), digested in proteinase K (1mg/ml) in 1% (w/v) SDS at 45ºC for 8hr. The supernatant was aspirated after centrifugation (300×g, 5min) and DNA extracted using a DNeasy tissue kit (Qiagen). DNA was also isolated from 1000 pfu cell-associated

MDV using the same technique. Viral DNA was quantified using real time quantitative PCR assay using the *meq* gene.

Four-color staining for flow cytometry analysis. Immunofluorescent staining of leukocytes for flow cytometry analysis was used to quantify relative changes in lymphocyte sub-populations. All antibodies were diluted in PBS-BSA-azide (PBA); PBS (pH 7.6 containing 0.1% (w/v) sodium azide and 0.4% (w/v) BSA. 1×10^6 leukocytes were stained with 50µl anti-CD8β-biotin (1:200, 55), followed by streptavidin-allophycocyanin (APC: Southern Biotechnology Associates, Birmingham, USA) diluted 1:200. Following incubation, washing and blocking with 5% normal mouse serum, the cells were incubated with the following fluorochrome-conjugated monoclonal antibodies, each diluted 1:200: anti chicken CD4-fluoroceinisothiocyanate (18), anti-chicken CD3-phycoerythrin (19), and anti-chicken CD8α-peridinin chlorophyll protein (43, 55). Lymphocyte suspensions were examined using a FACScalibur (Becton-Dickinson, NJ, USA) and data analyzed using the FCSExpress software, version 1.0 (DeNovo Software, USA).

Preparation of sections for immunohistochemistry. Tissues sections (6µm) were cut using a cryostat (Leica CM1900), air-dried overnight and fixed in acetone for 10 min. Slides were stored air-tight at -70ºC. Immunostaining followed the methods described by Vervelde & Davison (58) with the following modifications. Primary monoclonal antibodies against macrophages (KUL-01 diluted 1:30, 40) and $CD8\alpha^+$ T cells (AV14 diluted 1:25) were diluted in 0.4% (w/v) BSA in PBS. Sections were further stained with

Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) and visualized using the Vector NovaRed substrate kit (Vector laboratories), according to manufacturer's instructions before counter-staining with hematoxylin (Sigma, Gill no.3). After de-staining and dehydration sections were permanently mounted in Clearium (Surgipath, Peterborough, UK).

Immunofluorescent staining for confocal microscopy. Cryosections were prepared as described above. Non-specific reactivity was blocked by incubation with 5% (v/v) normal mouse serum for 20 min. Sections were then incubated for 60 min with KUL-01 antibody (1:300), or Bu1 antibody (1:200, 47) diluted in 5% (v/v) normal chicken serum. Primary antibodies were removed and sections washed before addition of the Alexa568-conjugated goat anti-mouse IgG H/L (1:300, Molecular Probes). After incubation for 60 min in the dark, sections were washed and Alexa488-conjugated anti-pp38 antibody (1:100, 37) added and incubated for 60 min. Sections were washed and nuclei stained by incubation with 1.09μM DAPI (Sigma) for 10 min. Sections were mounted in Vectashield (Vector Laboratories) and analyzed using a confocal microscope (Leica SP2 with 405, 488 & 568nm lasers). Control sections were treated similarly, but incubated with isotype control antibodies.

Statistical analysis. Mean lesion scores between multiple groups were compared using the Kruskal-Wallis test. Where an overall level of significance was observed, Dunn's test was conducted to determine significance between groups. Mortality data were analyzed by the Kaplan and Meier method. Significance in viral load was analyzed using analysis

of variance, followed by Tukey's test where overall significance was observed. Statistical analysis was performed using Graphpad Prism software version 3.03 (San Diego, USA). Details of individual tests are reviewed by Petrie and Watson (44).

Results

Infecting with MDV-infective dust by the respiratory route. We have previously shown that the fraction of infective dust containing the MDV particles ranged in size from 1 > 9 μm and consisted of dried sloughed flakes of skin and fragments of feather barbules (4, 56). Both the infective and non-infective dust used here contained small numbers of micrococci (gram positive cocci in cluster) and gram negative lactose-positive and negative rods (P. Barrow, IAH, personal communication). Very small numbers of streptomycetes were present in the non-infective dust, but overall there were no major qualitative differences between the two samples. Insufflating dust deep into the trachea using the microaerosiliser appeared to cause only minor interference with respiratory function and some secondary inflammatory changes from which the chicks quickly recovered (Data not shown). After insufflation with either infective or non-infective dust no clinical or serological (IBDV or CIAV) manifestation of any other lymphotrophic virus infection was observed. Preliminary experiments using a range of doses (2.5-20mg) of this batch of MDV-infective dust demonstrated that RIR chickens developed MD (2).

Here, graded mixtures of MDV-infective and non-infective dust were tested for their ability to induce MD in susceptible line 7 chickens as determined by clinical signs, MD lesions and viraemia (Fig. 1). More than 50% of chickens given the higher doses of infective dust (1, 2.5 and 5 mg) developed clinical signs of MD by 10-12 dpi, whereas these were significantly ($P<0.001$) delayed in those chickens given the lower doses (0.001, 0.002, 0.01 mg), which survived to the end of the experiment at 63 days. A dose of 0.1mg infective dust produced an intermediate effect with 50% survival at 29 days.

Tissues removed *post mortem* were examined for presence of lesions. All chickens treated with the MDV-infective dust had significantly ($P<0.05$) greater lymphocyte infiltration into the liver, heart and nerve compared with those birds treated with non-infective dust, but there was no clear evidence that this infiltration was dependent on the treatment dose (data not shown). The lung and proventriculus of the chickens given the non-infective dust both showed a relatively high degree of lymphocyte infiltration.

MDV levels in whole blood were determined by quantitative PCR assay. Viral DNA was not detected in the samples from chickens treated with the non-infective dust (data not shown). There was clear evidence that the onset of a detectable viraemia of groups treated with MDV-infective dust was delayed in those chickens given smaller doses of infective dust (Fig. 2). MDV was detected from 7 dpi onwards in the chickens receiving the higher doses (5, 2.5 and 1mg), but later (21 dpi onwards) in chickens receiving the lower doses of MDV-infective dust. Not all blood samples of chicks treated with MDV-infective dust were positive at all sample points.

Attempts were made to compare the MDV level in the dust to that of a 'standard' dose of 1000 pfu cell-associated virus. Dust obtained from the extract filters in the chicken house was extremely hydrophobic and proved impossible to 'wet' to bring it into contact with, and infect monolayers of chicken kidney cells in order to determine the number of MDV plaques. Several attempts were made to extract virus from a 2.5mg sample of dust using SPG buffer and sonication to disrupt the keratin (13) then co-culturing the extract with chicken kidney cells, but no plaques were detected. Viral loads were therefore compared using quantitative PCR: DNA extracted from both preparations

revealed that there was 16-fold more in 2.5mg dust than there was in 1000 pfu cell-associated virus.

MDV replication in lung and spleen leukocytes. Evidence for early MDV replication was sought after 2.5mg MDV-infective or non-infective dust was insufflated into chickens. DNase-treated RNA from lung and splenic leukocytes was analyzed by quantitative RT-PCR assay using specific primers and probes for immediate early (*ICP4*), early (*pp38*) and late (*gB*) genes. Viral RNA (*pp38*) was first detected in lung leukocytes of susceptible chickens from 2dpi and of resistant chickens from 3dpi (Fig. 3). Both increased with time. Detection of *pp38* in spleen leukocytes was delayed by one day compared with the respective values for lung lymphocytes in both genotypes and these values, like those in the lung, increased with time. From the time of detection, viral replication in the resistant leukocytes remained lower than in the susceptible leukocytes; a significant difference was only evident at 5dpi ($P<0.001$). Similar patterns were observed for *gB* and *ICP4* transcripts, and *meq* DNA (data not shown). *Meq* could not be detected in blood isolated from chickens during the first 5dpi (data not shown).

Changes in CTL populations of the spleen and lung.

The effect of intra-tracheal insufflation of infective dust on proportions and location of $CD3^+CD8\alpha\beta^+$ expressing T cell was investigated. Differences in the proportion of CTL in the lung and spleen of resistant and susceptible chickens during viral replication were analyzed using flow cytometry. The proportion of $CD3^+CD8\alpha\beta^+$ leukocytes increased from 4 days after insufflation in the infected, but not uninfected,

groups, although significance (P<0.05) only occurred at 5dpi with susceptible $CD3^+CD8\alpha\beta^+$ cells (Table 2). Genotype appeared to affect the cellular composition of splenic leukocytes. More T cells were present in the susceptible spleens compared with those of resistant chickens (other sub-populations not shown). Interestingly, a decrease (P<0.05) in the proportion of $CD3^+CD8\alpha\beta^+$ splenic leukocytes from infected compared with uninfected susceptible chickens was evident at 5dpi, the converse of that observed in the lung.

The location of $CD8\alpha^+$ cells in the lung following intra-tracheal infection was investigated using immunohistochemistry. Whereas the influx of the $CD8\alpha^+$ cells was specific for infection in the lungs of resistant chickens, $CD8\alpha^+$ cells were present in the lungs of the uninfected susceptible chickens (Fig. 4). An increase in the proportion of $CD8\alpha^+$ pulmonary leukocytes was evident in both infected chicken lines. The number of positive cells increased with time, especially in susceptible chickens (Table 2), where the $CD8\alpha^+$ cells occupied a large proportion of the lung from 3dpi onwards. The positive cells were usually located adjacent to pulmonary spaces, possibly indicating that cells were moving towards mucosal surfaces of the lung as the infection progressed.

Pro-inflammatory cytokine transcript levels in lung and splenic leukocytes.

As cytokines could play an important role in MDV pathogenesis after intra-tracheal infection, transcripts for IFNα, IFNβ, IFNγ, IL-4, IL-6, IL-18 and TGFβ in lung and splenic leukocytes were measured using quantitative RT-PCR assay. Infection with MDV produced no consistent pattern of change in cytokine transcripts in lung leukocytes from either line, and patterns of change in the different cytokines were similar (data not

shown). The only notable difference was observed for IFNγ, where an increase in transcripts was observed 3-5dpi in the resistant chickens and 5dpi in the susceptible chickens (Fig. 5A). During the first three days after intra-tracheal infection, there was little effect on the transcript levels of IFNα, IFNβ, IFNγ, IL-6 and IL-18 in splenic leukocytes compared with birds treated with non-infective dust (Fig. 5B-F). However, by 4dpi there was an increase (4-8 times) in levels of all inflammatory cytokine transcripts in susceptible leukocytes. This increase was also evident at 5dpi, especially for IFNγ and IL-6, where a 16-fold increase was observed ($P<0.01$ and $P<0.05$, respectively). In the resistant spleen leukocytes this increase was not as pronounced and restricted to IL-6, IFNγ and IL-18, the latter two only at 5dpi. The increase observed with susceptible spleen leukocytes was significantly different ($P<0.05$) from that in resistant spleen leukocytes, where no increase was observed until 5dpi for both IL-6 and IL-18 transcripts. No differences between genotypes or infected/control chickens were observed for either IL-4 or TGFβ in the spleen (data not shown).

Identification of infected cells in the avian lung.

To visualize infected cells in the pulmonary tissue of infected chickens, lungs were analyzed by immunohistochemistry. Recent work has highlighted the role of macrophages in MDV infection and their potential role in the uptake of the virus (8). Macrophages visualized using the KUL-01 antibody (40) were predominantly localized around the pulmonary spaces (Fig. 6). Differences between the lines were apparent: the proportion of macrophages was higher in the lungs of uninfected susceptible compared with uninfected resistant chickens (not shown). Nevertheless, macrophages were present

in higher numbers in the lungs of resistant chickens after intra-tracheal infection, particularly at 1dpi (Fig. 6). The proportion of macrophages decreased with time in the lungs of infected resistant and uninfected susceptible chickens.

Viral antigen was visualized by confocal microscopy using an antibody against pp38. Sections were double-stained for either macrophages or B cells. Viral antigen could only be detected in the lungs from infected susceptible chickens at 5dpi. No co-localization was observed between KUL-01$^+$ cells and pp38$^+$ cells (data not shown). However, the pp38 antigen was expressed in Bu-1$^+$ cells (Fig. 7). pp38-specific staining of these cells was predominantly cytoplasmic, near the plasma membrane and did not co-localize with the Bu-1 staining, which was restricted to the cell-surface (Fig. 7B). Not all infected cells were B cells. Interestingly, infected B cells were surrounded by uninfected B cells.

Discussion

This work was to define a model of infection that allows early events in the pathogenesis of MD to be determined in a way that has not been possible when infection is induced by a parenteral route. Factors that are likely to have a crucial influence on later viral amplification and tumorgenesis could account for the marked differences in viral levels in different chicken genotypes (10, 36), and include: the location(s) within the airways where the virus enters the chicken proper, the state of the virus (free or engulfed in keratin), when and where it crosses the lung epithelial lining, the population of lung phagocytic cells that take up and transport MDV (free or engulfed in keratin) to the lymphoid tissues and the route (blood circulation or lymphatics) by which the phagocytic cells migrate.

We have demonstrated a method for introducing a standard dose of MDV in its 'native' form, associated with feather and squamous epithelial cell debris, by insufflation into the lung. Although other methods for infecting chickens with MDV by the natural route have been described, none of these have succeeded in establishing a reproducible and quantifiable means for infection (1, 9, 29). Calnek *et al.* (15) reported that chickens can be infected with cell-free MDV obtained from sonicated feather tips and suspended in PBS, but details of the method of administration were not described and the virus was not in its complete 'native' form. More recently, Davidson and Borenshtain (21) have described a method for infecting chickens with MDV that involves 'dripping' cell-free MDV into the beak, although it is clear from the results that not all chickens developed clinical MD, presumably because the virus was introduced into the intestinal rather than the respiratory tract.

Insufflating genetically-susceptible chickens with MDV-infective material affected survival in a dose-related manner and caused the development of MD lesions. The observed increase in lymphocyte infiltration was consistent with the chicks having clinical MD (11). The data shown in figures 1 and 3 indicate that viraemia became measurable about the time chickens developed clinical signs of MD. One explanation for the later viraemia in the groups given the three lower doses of MDV-infective dust is that, since chickens were accommodated together, MDV might have been transmitted from those chickens receiving the larger doses of infective dust. MDV DNA and protein have been isolated from the FFE as early as 8 and 11 dpi (38) and it is generally accepted that infectious cell-free MDV is shed from about two weeks after chickens have become infected. However, most chickens given the higher doses of MDV-infective dust developed clinical MD and were euthanized before two weeks of age (Fig. 3). It therefore seems most likely that the later viraemia in those chickens given lower doses of MDV by the intra-tracheal route was a consequence of the dilution of MDV dose.

The intra-tracheal infection method was used to investigate the early virological and immunological events in the lungs and spleens of resistant and susceptible chicken lines that share the B^2 haplotype. Immune responses in these genotypes have been investigated by several researchers and have an important influence on the outcome of infection (reviewed in 41). However, few researchers have investigated the immune responses in the chicken lung, the organ that first encounters the virus, and how these influence pathogenesis during the very early stages after infection. Viral replication was therefore assessed using quantitative fluorescent RT(PCR) for RNA transcripts of representative immediate-early, early and late MDV genes. The data presented here are

the first unequivocal demonstration that viral replication occurs in the lung before the spleen. Viral RNA could be detected as early as 2dpi in the susceptible chickens, whereas detection in resistant birds was delayed by one day (Fig. 3). Similar patterns were observed for *ICP4*, *pp38* and *gB*. Although cell-associated infectivity was reported by Philips and Biggs (45) as early as 24h following the end of three days of contact with infected birds, this methodology did not allow for the determination of the precise time of infection.

That genetic resistance manifests itself so early with respect to viral load is remarkable and the delay and lower viral load could give the resistant line 6 more time to generate an effective immune response. The one-day delay between detection of viral replication in the lung and spleen could be a consequence of the transport of the virus from its site of entry to the major organ where replication occurs. However, no DNA signal was detected in the blood until 5dpi, in contrast with findings of Adldinger *et al*. (1) who reported virus in the 'buffy coat' of infected chickens as early as 18 hours after infection. The number of infected cells migrating from the lung to the spleen could be very small and not detected by the quantitative PCR assay used in the present study. Alternatively, MDV could be transported via the lymph, which was not sampled.

The immune response after infection was investigated by quantitative analysis of cytokine transcript levels by RT-PCR, and of cell sub-populations by flow cytometry and immunohistology. Other than an increase in IFNγ transcripts, no clear cytokine patterns were observed in the lung leukocytes. Clear differences between genotype were observed with the spleen leukocytes. An overall increase in the levels of inflammatory cytokine transcripts was observed 4 and 5 days after intra-tracheal infection in susceptible birds.

Kaiser *et al*. (33) described similar changes for IL-6 and IL-18 transcript levels between the line 6 and 7 chickens later during infection, but reported no differences for IFNγ. The increase of IL-18 transcription in susceptible, but not resistant chickens is surprising, as IL-18 has been shown to be potentially protective to a range of viral infections, including HSV-1, MCMV and EBV (reviewed in 28). Notably, the capacity of IL-18 to induce IFNγ (27) and enhance NK cell activity is not consistent with the results observed after MDV infection. Consistent with earlier reports, a strong increase in cytokine transcript levels in the line 7 birds was observed, suggesting a more profound inflammatory response in these chickens (6, 36).

Changes in the proportions of specific lymphocyte sub-populations as a result of infection were observed in both the spleen and the lungs of line 6 and 7 chickens. Infection caused an increase in $CD3^{+}CD8\alpha\beta^{+}$ T cells in the lung of both susceptible and resistant chickens whilst viral replication was increasing (Table 2). Although cytotoxicity was not addressed in this experiment, a cytotoxic T cell population capable of lysing MDV infected target cells of both lines (B.J.G. Baaten, unpublished data) is generated following MDV vaccination. A decrease in the $CD3^{+}CD8\alpha\beta^{+}$ T cell population in the spleen coincided with extensive viral replication and is most likely a result of cytolytic infection.

The suggestion that macrophages are the first cell type to harbor MDV (12) was investigated using confocal microscopy of lung sections after intra-tracheal infection. Virus was not detected in lung sections from the resistant line 6 chickens, but could be demonstrated from 5dpi in the susceptible line 7 bird, confirming earlier reports (1, 46). The discrepancy between the immunofluorescence and PCR assay is probably caused by

a difference in the sensitivity of these methodologies. Whereas leukocytes were enriched and DNA amplified using PCR, the paucity of infected cells at these early time-points decreases the likelihood of detection by immunofluorescence. No infected lung macrophages were detected and the majority of virus-positive cells were B cells (Fig. 7). However, it may be that macrophages transport the virus from the lung to the spleen without becoming cytolytically infected, and virus is therefore not detectable by the present techniques. Moreover, it cannot be concluded that B cells are the first cells to be infected by MDV in the lung, due to the relatively late detection of infected cells. It is possible that these B cells became infected in the spleen and then returned to the lungs. Indeed, MDV proteins were detected in the lung 5 days after intra-abdominal infection with cell-associated virus (46).

Besides the infected B leukocytes, there were other infected cells that did not express the KUL-01 or Bu-1 antigen. It may be that these cells were activated $CD4^+$ T cells that migrated back to the lung. Several researchers (22, 46, 51) demonstrated that chicken lung epithelial cells themselves can harbor MDV antigen, and Kirisawa and Mikami (35) showed that isolated lung cells can be infected. Epithelial cells in the atria and infundibila have been shown to phagocytose small particles that enter parabronchi in other avian species (39, 48, 52). It is possible that epithelial cells become infected, whilst translocating viral particles from the airside of the lung to the interstitial macrophages in the parenchyma. Macrophages could phagocytose the translocated viral particles, or the cytolytically infected epithelial cells, and transport MDV to the spleen. The location of macrophages adjacent to the air capillaries (Fig. 6) would facilitate this process.

In conclusion, the results confirm that dander composed of dead epithelial cells, cell debris, and fragments of feather barbules released from infected chickens can harbor MDV. This particulate matter can be used to infect chickens when insufflated into the trachea using a microaerosiliser. We suggest that this method offers superior control of dose delivered, precisely-timed initiation of the infection process and delivery to the natural site of infection compared with previously published methods using externally generated aerosols. We have now used this method for introducing MDV infection with three strains of MDV of different pathotypes (G.J. Underwood and T.F. Davison, unpublished data) and shown that it reliably mimics natural infection. This method provides the opportunity to investigate the early events in infection with MDV delivered in its 'native' form and by the natural route of entry. It is evident that differences in viral replication are not limited to the spleens of resistant and susceptible chicken lines, suggesting an important role for the lung in MD pathogenesis. Delay of viral replication in resistant chickens could allow time for priming of a more effective immune response that would be beneficial to disease outcome. The mechanism of MDV transport from the lung to the spleen was not elucidated, but could account for the delay in viral replication. In addition, the larger number of potential target cells and a more profound inflammatory response is more likely to augment the cytolytic infection in the susceptible birds with the eventual loss of leukocytes, leading to immune suppression.

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References

1. **Adldinger, H., and B. Calnek.** 1973. Pathogenesis of Marek's disease: Early distribution of virus and viral antigens in infected chickens. Journal of the National Cancer Institute **50:**1287-1298.
2. **Baaten, B., C. Butter, H. J. Walter, and F. Davison.** 2001. Marek's disease virus: Infection by the 'natural' route. Immunology **104:**131.
3. **Baaten, B. J. G., C. Butter, and F. Davison.** 2004. Study of the host-pathogen interaction to identify sustainable vaccine strategies to Marek's disease. Veterinary Immunology and Immunopathology **100:**165-177.
4. **Baaten, B. J. G., G. Underwood, C. Butter, and T. F. Davison.** 2004. Presented at the Marek's disease symposium, Oxford.
5. **Baigent, S., and F. Davison.** 2004. Marek's disease virus: biology and life cycle, p. 62-77. *In* F. Davison and K. Venugopal (ed.), Marek's disease: an evolving problem. Elsevier Ltd., Oxford.
6. **Baigent, S. J., and T. F. Davison.** 1999. Development and composition of lymphoid lesions in the spleens of Marek's disease virus-infected chickens: association with virus spread and the pathogenesis of Marek's disease. Avian Pathology **28:**287-300.
7. **Baigent, S. J., L. J. N. Ross, and T. F. Davison.** 1996. A flow cytometric method for identifying Marek's disease virus pp38 expression in lymphocyte subpopulations. Avian Pathology **25:**255-267.
8. **Barrow, A. D., S. C. Burgess, S. Baigent, K. Howes, and V. K. Nair.** 2003. Infection of macrophages by a lymphotropic herpesvirus: a new tropism for Marek's disease virus. Journal of General Virology **84:**2635-2645.
9. **Beasley, J. N., L. T. Patterson, and D. H. McWade.** 1970. Transmission of Marek's disease by poultry house dust and chicken dander. American Journal of Veterinary Research **31:**339-344.
10. **Bumstead, N., J. Sillibourne, M. Rennie, N. Ross, and F. Davison.** 1997. Quantification of Marek's disease virus in chicken lymphocytes using the polymerase chain reaction with fluorescence detection. Journal of Virological Methods **65:**75-81.
11. **Burgess, S. C., B. H. Basaran, and T. F. Davison.** 2001. Resistance to Marek's disease herpesvirus-induced lymphoma is multiphasic and dependent on host genotype. Veterinary Pathology **38:**129-142.
12. **Calnek, B. W.** 1986. Marek's disease - a model for herpesvirus oncology. Crc Critical Reviews in Microbiology **12:**293-320.
13. **Calnek, B. W., and H. K. Adldinger.** 1971. Some characteristics of cell-free preparations of Marek's disease virus. Avian Diseases **15:**508-517.
14. **Calnek, B. W., H. K. Adldinger, and D. E. Kahn.** 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Diseases **14:**219-233.

15. **Calnek, B. W., S. B. Hitchner, and H. K. Adldinger.** 1970. Lyophilization of cell-free Marek's disease herpesvirus and a herpesvirus from turkeys. Applied Microbiology **20:**723-726.
16. **Calnek, B. W., K. A. Schat, and J. Fabricant.** 1981. RB-1B, a new highly oncogenic Marek's disease virus. Journal of the American Veterinary Medical Association **179:**270-270.
17. **Carrozza, J. H., T. N. Fredrickson, R. P. Prince, and R. E. Luginbuhl.** 1973. Role of desquamated epithelial cells in transmission of Marek's disease. Avian Diseases **17:**767-781.
18. **Chan, M. M., C. L. H. Chen, L. L. Ager, and M. D. Cooper.** 1988. Identification of the avian homologs of mammalian CD4 and CD8 antigens. Journal of Immunology **140:**2133-2138.
19. **Chen, C. L. H., L. L. Ager, G. L. Gartland, and M. D. Cooper.** 1986. Identification of a T3 T cell receptor complex in chickens. Journal of Experimental Medicine **164:**375-380.
20. **Crittenden, L. B., R. L. Muhm, and B. R. Burmester.** 1972. Genetic control of susceptibility to the avian leukosis complex. 2. Marek's disease. Poultry Science **51:**261-267.
21. **Davidson, I., and R. Borenshtain.** 2003. Novel applications of feather tip extracts from MDV-infected chickens; diagnosis of commercial broilers, whole genome separation by PFGE and synchronic mucosal infection. Fems Immunology and Medical Microbiology **38:**199-203.
22. **Eidson, C. S., J. J. Giambrone, O. J. Fletcher, and S. H. Kleven.** 1980. Vaccination against Marek's disease with cell-free herpesvirus of turkeys by aerosole vaccination. Poultry Science **59:**54-62.
23. **Epstein, M. A.** 2001. Historical background. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **356:**413-420.
24. **Gandon, S., M. J. Mackinnon, S. Nee, and A. F. Read.** 2001. Imperfect vaccines and the evolution of pathogen virulence. Nature **414:**751-756.
25. **Gildow, E. M., J. K. Williams, and C. E. Lampman.** 1940. The transmission of and resistance to fowl paralysis (lymphomatosis). University of Idaho Agricultural Experiment Station Bulletin **235:**1-22.
26. **Gimeno, I.** 2004. Future strategies for controlling Marek's disease, p. 186-199. *In* F. Davison and K. Venugopal (ed.), Marek's disease: an evolving problem. Elsevier Ltd., Oxford.
27. **Gobel, T. W., K. Schneider, B. Schaerer, I. Mejri, F. Puehler, S. Weigend, P. Staeheli, and B. Kaspers.** 2003. IL-18 stimulates the proliferation and IFN-gamma release of CD4(+) T cells in the chicken: Conservation of a Th1-like system in a nonmammalian species. Journal of Immunology **171:**1809-1815.
28. **Gracie, J. A., S. E. Robertson, and I. B. McInnes.** 2003. Interleukin-18. Journal of Leukocyte Biology **73:**213-224.
29. **Higgins, D. A., and B. W. Calnek.** 1976. Some effects of silica treatment on Marek's disease. Infection and Immunity **13:**1054-1060.
30. **Hlozanek, I., and V. Jurajda.** 1972. Cell-free preparations of Marek's disease virus from poultry dust.118-123.

31. **Islam, A. F. M. F., S. W. Walkden-Brown, S. K. Burgess, and P. J. Groves.** 2001. Marek's disease in broiler chickens: effect of route of infection and herpesvirus of turkey-vaccination status on detection of virus from blood or spleen by polymerase chain reaction, and on weights of birds, bursa and spleen. Avian Pathology **30:**621-628.
32. **Jurajda, V., and B. Klimes.** 1970. Presence and survival of Marek's disease agent in dust. Avian Diseases **14:**188-190.
33. **Kaiser, P., G. Underwood, and F. Davison.** 2003. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. Journal of Virology **77:**762-768.
34. **Kenzy, S. G., and B. R. Cho.** 1969. Transmission of classical Marek's disease by affected and carrier birds. Avian Diseases **13:**211-214.
35. **Kirisawa, R., and T. Mikami.** 1983. *In vivo* and *in vitro* infections of chicken lymphocytes with Marek's disease virus or herpesvirus of turkeys. Japanese Journal of Veterinary Science **45:**727-738.
36. **Lee, L. F., P. C. Powell, M. Rennie, L. N. J. Ross, and L. N. Payne.** 1981. Nature of genetic resistance to Marek's disease in chickens. Journal of the National Cancer Institute **66:**789-796.
37. **Li, D. S., P. F. Green, M. A. Skinner, C. L. Jiang, and N. Ross.** 1994. Use of recombinant pp38 antigen of Mareks disease virus to identify serotype 1 specific antibodies in chicken sera by western blotting. Journal of Virological Methods **50:**185-195.
38. **Liu, X., D. Peng, X. Wu, L. Xing, and R. Zhang.** 1999. A recombinant fowlpox virus vaccine expressing glycoprotein B gene from CVI988/Rispens strain of MDV: Protection studies in different chickens. Acta Virologica **43:**201-204.
39. **Maina, J. N., and H. M. Cowley.** 1998. Ultrastructural characterization of the pulmonary cellular defences in the lung of a bird, the rock dove, *Columba livia*. Proceedings of the Royal Society of London Series B-Biological Sciences **265:**1567-1572.
40. **Mast, J., B. M. Goddeeris, K. Peeters, F. Vandesande, and L. R. Berghman.** 1998. Characterisation of chicken monocytes, macrophages and interdigitating cells by the monoclonal antibody KUL-01. Veterinary Immunology and Immunopathology **61:**343-357.
41. **Mikami, T.** 1988. Determinants of host resistance to Marek's disease. *In* S. Kato, T. Horiuchi, T. Mikami, and K. Hirai (ed.), Advances in Marek's disease research. Japanese Association on Marek's Disease, Osaka.
42. **Okamura, H., M. Sakaguchi, K. Yokogawa, E. Tokunaga, S. Abe, S. Tokiyoshi, and K. Mizuno.** 2001. Lack of contact transmission of recombinant Marek's disease virus type 1 expressing the fusion protein of Newcastle disease virus. Vaccine **20:**483-489.
43. **Openshaw, P. J. M., F. J. Culley, and W. Olszewska.** 2002. Immunopathogenesis of vaccine-enhanced RSV disease. Vaccine **20:**S27-S31.
44. **Petrie, A., and P. Watson.** 1999. Statistics for veterinary and animal science. Blackwell Sciences, Oxford.

45. **Philips, P. A., and P. M. Biggs.** 1972. Course of infection in tissues of susceptible chickens after exposure to strains of Marek's disease virus and turkey herpesvirus. Journal of the National Cancer Institute **49:**1367-1373.
46. **Purchase, H. G.** 1970. Virus-specific immunofluorescent and precipitin antigens and cell-free virus in the tissues of birds infected with Marek's disease. Cancer Research **30:**1898-1908.
47. **Rothwell, C., L. Vervelde, and T. Davison.** 1996. Identification of chicken Bu-1 alloantigens using the monoclonal antibody AV20. Veterinary immunology and immunopathology **55:**225-234.
48. **Scheuermann, D. W., E. Klika, M. H. A. De Grodt, I. Bazantova, and A. Switka.** 1997. An electron microscopic study of the parabronchial epithelium in the mature lung of four bird species. Anatomical Record **249:**213-225.
49. **Schmittle, S. C., and C. S. Eidson.** 1968. Studies on acute Marek's disease IV. Relative susceptibility of different lines and crosses of chickens to GA isolate. Avian Diseases **12:**571-576.
50. **Sevoian, M., D. M. Chamberlain, and R. N. Larose.** 1963. Avian lymphomatosis. V. Air-borne transmission. Avian Diseases **7:**102-105.
51. **Sharma, J. M.** 1987. Embryo vaccination of chickens with turkey herpesvirus - characteristics of the target-cell of early viral replication in embryonic lung. Avian Pathology **16:**567-579.
52. **Stearns, R. C., G. M. Barnas, M. Walski, and J. D. Brain.** 1987. Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck, *Anas Platyrhynchos*. Respiration Physiology **67:**23-36.
53. **Stone, H. A.** 1969. Investigations of the genetic control of Marek's disease. Poultry Science **48:**1879.
54. **Stone, H. A.** 1975. Use of highly inbred chickens in research. USDA Technical Bulletin **1514**.
55. **Tregaskes, C. A., F. K. Kong, E. Paramithiotis, C. L. H. Chen, M. J. H. Ratcliffe, T. F. Davison, and J. R. Young.** 1995. Identification and analysis of the expression of CD8-alpha-beta and CD8-alpha-alpha isoforms in chickens reveals a major TCR-gamma-delta CD8-alpha-beta subset of intestinal intraepithelial lymphocytes. Journal of Immunology **154:**4485-4494.
56. **Underwood, G.** 2003. An investigation of early events in the pathogenesis of Marek's disease. PhD. Bristol, Bristol.
57. **Van Regenmortel, M. H. V., C. M. Fauqet, D. H. L. Bishop, E. Carstens, M. K. Estes, S. Lemon, J. Maniloff, M. A. Mayo, D. McGeoch, C. R. Pringle, and R. B. Wickner (ed.).** 1999. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, New York.
58. **Vervelde, L., and F. Davison.** 1997. Comparison of the in situ changes in lymphoid cells during infection with infectious bursal disease virus in chickens of different ages. Avian Pathology **26:**803-822.
59. **Witter, R. L.** 1997. Increased virulence of Marek's disease virus field isolates. Avian Diseases **41:**149-163.
60. **Witter, R. L.** 2001. Protective efficacy of Marek's disease vaccines, p. 57-90. *In* K. Hirai (ed.), Current Topics in Microbiology and Immunology, vol. 255. Springer, Berlin.

61. **Witter, R. L., and A. K. Schat.** 2003. Marek's disease, p. 405-464. *In* Y. M. Saif (ed.), Diseases of Poultry, 11 ed. Iowa state press, Iowa.

Table 1. Primer and probe details for quantitative PCR and RT-PCR

Gene	Probe/primer	Sequence[†]	Final conc.
Ovotransferrin	Forward	CAC TGC CAC TGG GCT CTG T	200
	Reverse	GCA ATG GCA ATA AAC CTC	200
	Probe VIC	AGT CTG GAG AAG TCT GTG	100
Meq	Forward	GGT CTG GTG GTT TCC AGG TGA	400
	Reverse	GCA TAG ACG ATG TGC TGC TGA	400
	Probe FAM	AGA CCC TGA TGA TCC GCA TTG	100
28S	Forward	GGC GAA GCC AGA GGA AAC T	600
	Reverse	GAC GAC CGA TTT GCA CGT C	600
	Probe FAM	AGG ACC GCT ACG GAC CTC	100
IL-6 [‡]	Forward	GCT CGC CGG CTT CGA	400
	Reverse	GGT AGG TCT GAA AGG CGA	400
	Probe FAM	AGG AGA AAT GCC TGA CGA	100
IL-18 [‡]	Forward	AGG TGA AAT CTG GCA GTG	400
	Reverse	ACC TGG ACG CTG AAT GCA A	400
	Probe FAM	CCG CGC CTT CAG CAG GGA TG	100
IFNα	Forward	GAC AGC CAA CGC CAA AGC	400
	Reverse	GTC GCT GCT GTC CAA GCA TT	400
	Probe FAM	CTC AAC CGG ATC CAC CGC TAC	100
IFNβ	Forward	CCT CCA ACA CCT CTT CAA CAT	400
	Reverse	TGG CGT GCG GTC AAT	400
	Probe FAM	TTA GCA GCC CAC ACA CTC CAA	100
IFNγ [‡]	Forward	GTG AAG AAG GTG AAA GAT	400
	Reverse	GCT TTG CGC TGG ATT CTC A	400
	Probe FAM	TGG CCA AGC TCC CGA TGA	100
ICP4	Forward	CGC CAC ACG AGA ACA CAA TG	400
	Reverse	GGT TGG AGT AGA GCT GCA	400
	Probe FAM	CGG CCC AGT ACA GCC TGC GG	100
Pp38	Forward	GAA AAC AGA AGC GGA ATG CG	400
	Reverse	CGA TCC AAA GCG CTC ATC TC	400
	Probe FAM	CCC CGC AT TCT CGC CGT CCT C	100
gB	Forward	GGT TCA ACC GTG ATC CGT CTA	400
	Reverse	CGA TTC CTT CAC CCC ACT	400
	Probe FAM	ACC GCC GCG AAA ATG TCC CG	100

† Primer and probe sequences were designed with Primer Express 1.0 Software (Applied Biosystems). ‡ PCR product spans exons.

Table 2. Percentage $CD3^+CD8\alpha\beta^+$ T cells in spleen and lung of resistant and susceptible chickens after intra-tracheal insufflation of dust.

		Days after infection[†]				
		1	**2**	**3**	**4**	**5**
Lung						
Resistant	Control	ND[‡]	3.61	4.84	6.85	4.59
	Infected	ND	5.40	4.37	12.79	10.54
Susceptible	Control	ND	4.99	5.93	8.96	3.66
	Infected	ND	5.65	6.29	18.20	15.06**
Spleen						
Resistant	Control	10.52	9.82	13.93	14.60	15.98
	Infected	10.67	12.48	10.09**	13.61	15.68
Susceptible	Control	17.06	15.39	16.69	25.40	18.50
	Infected	16.16	14.14	16.59	19.61	12.59*

[†] Percentage $CD3^+CD8\alpha\beta^+$ T cells of live cells analyzed by flow cytometry. [‡] Not determined. Significances between control and infected samples for a particular day are denoted: * $p<0.05$, ** $p<0.01$

Figure 1. Titration of MD-induced mortality after intra-tracheal instillation of 5, 2.5, 1, 0.1, 0.01, 0.002, 0.001 mg of RB1B-infective dust bulked up to 5mg with non-infective dust, or 5mg non-infective dust into susceptible line 7 chickens.

Figure 2. Detection of MDV levels in PBL measured by quantitative PCR after intra-tracheal instillation of various doses of RB1B-infective dust bulked up to 5mg with non-infective dust. Chickens receiving 5mg non-infective dust were negative and are not shown. Dots represent individual birds testing positive for virus for the first time, with the horizontal bar representing the mean value.

Figure 3. MDV pp38 transcripts in lung (A) and spleen (B) leukocytes isolated from resistant line 6 and susceptible line 7 chickens after intra-tracheal infection with RB1B-infective dust. Expression of early gene, pp38, was examined using specific primers and probe in a quantitative RT-PCR assay on DNase-treated RNA. Values shown are means ± SEM (n=6). Significant differences between infected groups are represented as follows: $p<0.01$ = ** , $p<0.001$ = ***.

Figure 4. Effect of intra-tracheal insufflation of RB1B-infective dust on CD8α expressing cells in the lung. Lungs from resistant (A, B) and susceptible (C, D) chickens inoculated with infective (B, D) and non-infective (A, C) dust were removed from birds 4dpi and frozen blocks were prepared. Sections were cut, immunostained (see text) and counterstained using hematoxylin. Most intensely

stained areas are depicted. Some of the parabronchi are indicated by arrows and airways by **a**. The marker bar represents 100μm.

Figure 5. Cytokine transcripts levels in spleen leukocytes of resistant and susceptible birds after intra-tracheal insufflation with RB1B-infective or sham dust. Chicken IFNγ transcripts (A) were measured in RNA extracted from lung leukocytes and IFNα (B), IFNβ (C), IFNγ (D), IL-6 (E) and IL-18 (F) from spleen leukocyte RNA using quantitative RT-PCR and are expressed on a 2log scale. Values shown are means ± SEM (n=6).

Figure 6. Effect of intra-tracheal insufflation of RB1B-infective dust on KUL-01+ cells in the lung. Lungs were removed from control (A) and infected (B) resistant birds 1dpi and frozen blocks were prepared. Sections were cut, immunostained (see text) and counterstained using hematoxylin. Most intensely stained areas are depicted. Some of the parabronchi are indicated by arrows, airways by **a** and blood vessels by **b**. The marker bar represents 50μm.

Figure 7. Viral replication in pulmonary B cells of susceptible chickens 5 days after intra-tracheal infection. Lung sections from line 7 chickens 5dpi were stained by immunofluorescence and studied using confocal microscopy. pp38+ cells (green), Bu-1+ (red) and nuclei were visualized using DAPI (blue). A. White arrows indicate double-stained cells. B. pp38-specific staining in B-cells is predominantly cytoplasmic, near the plasma membrane. The marker bar represents 10μm.

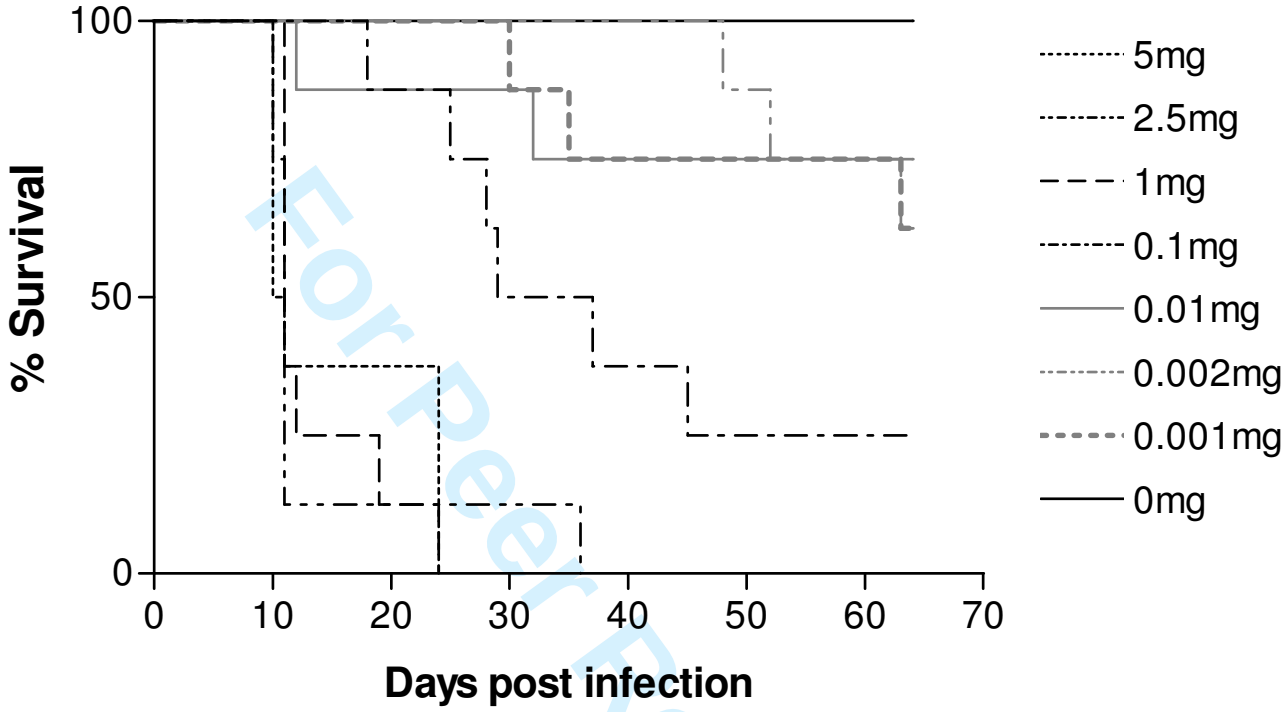

Figure 1.

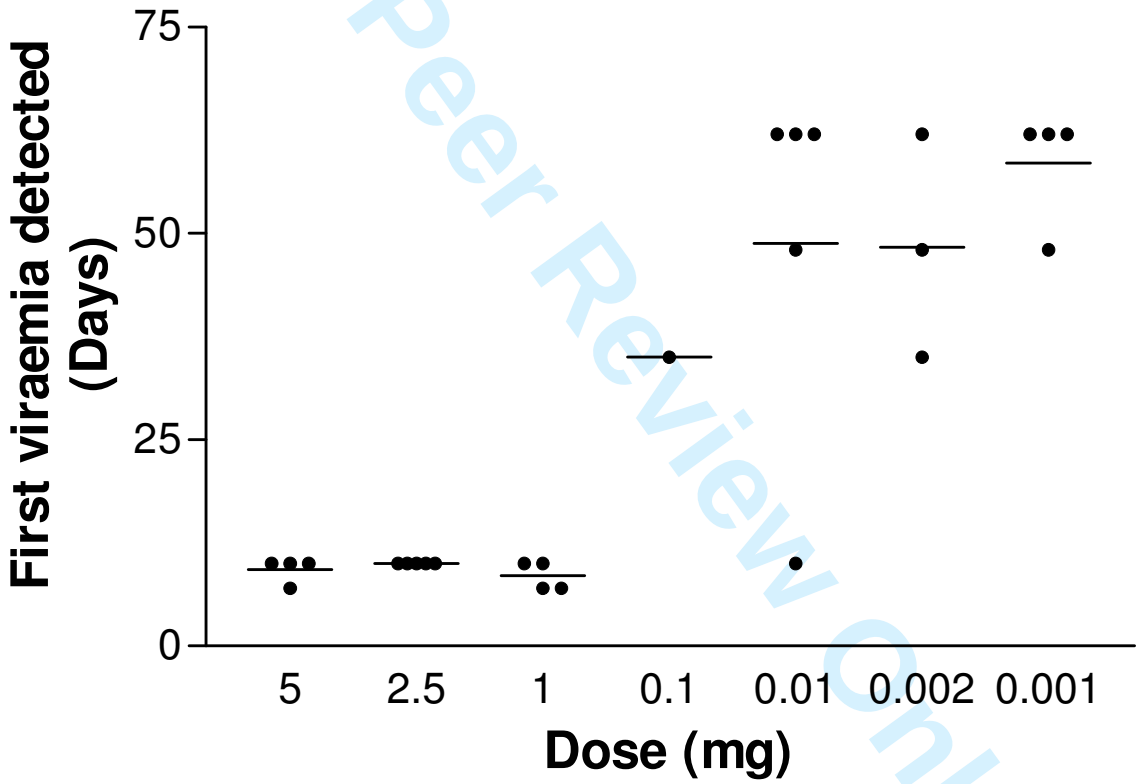

Figure 2.

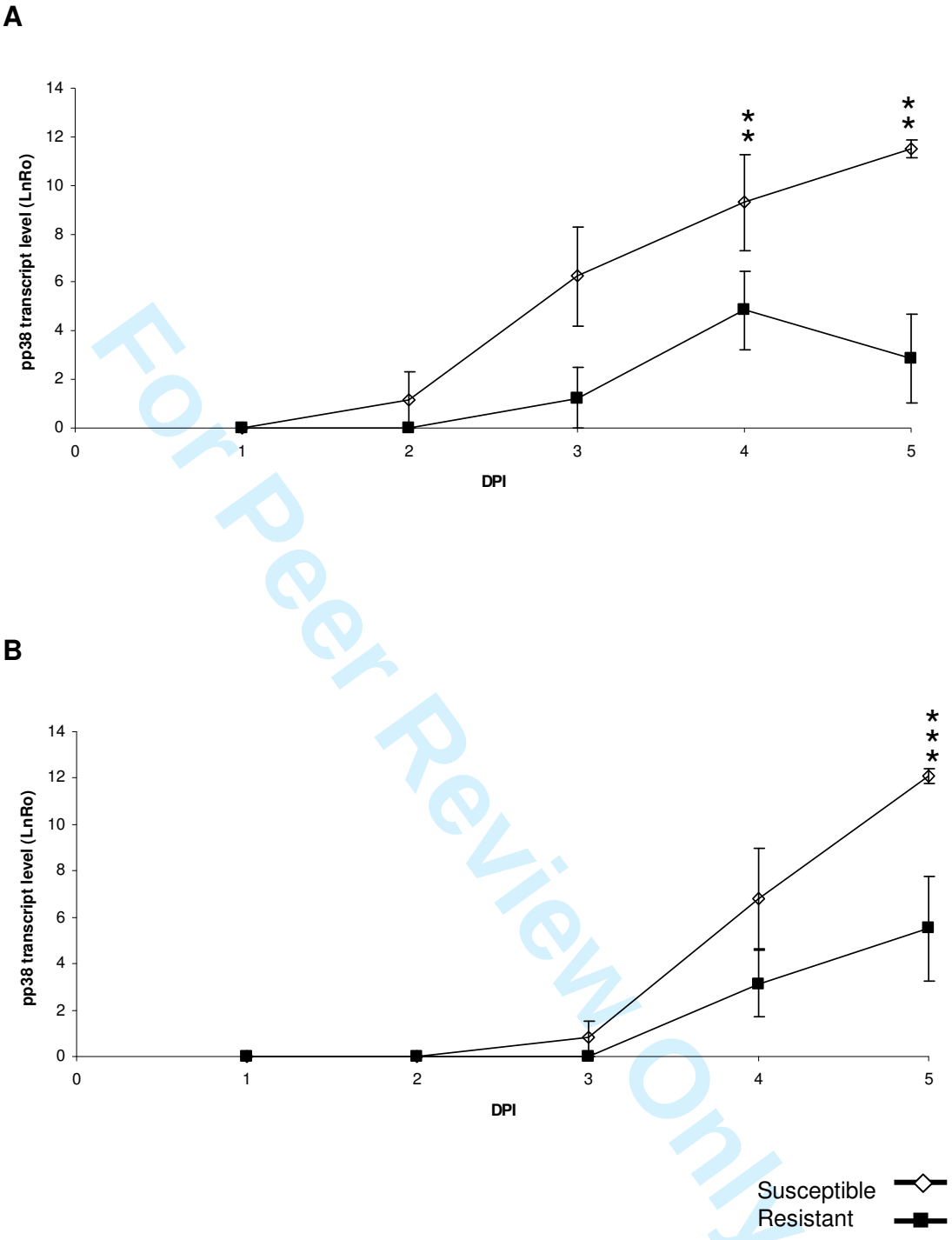

Figure 3.

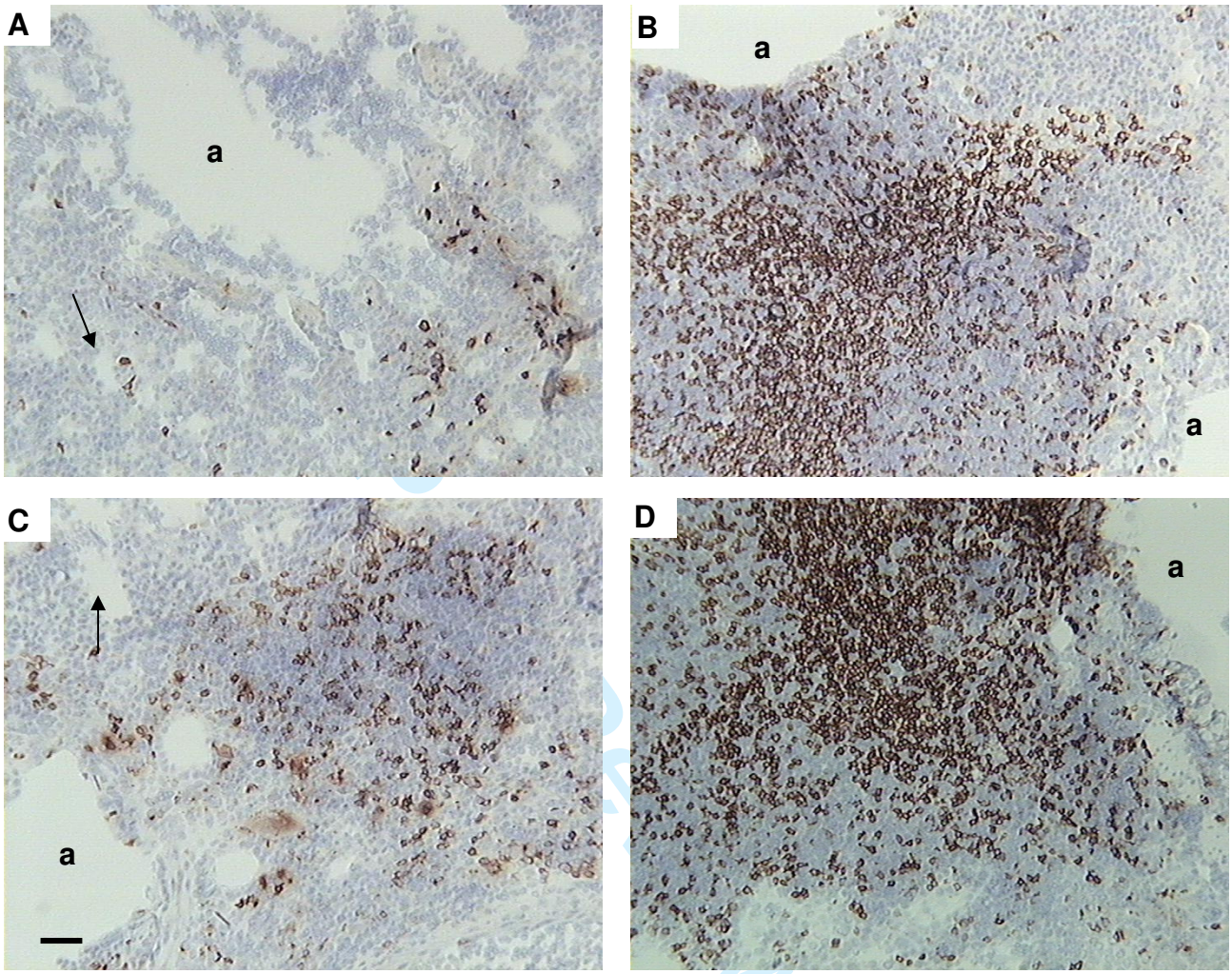

Figure 4.

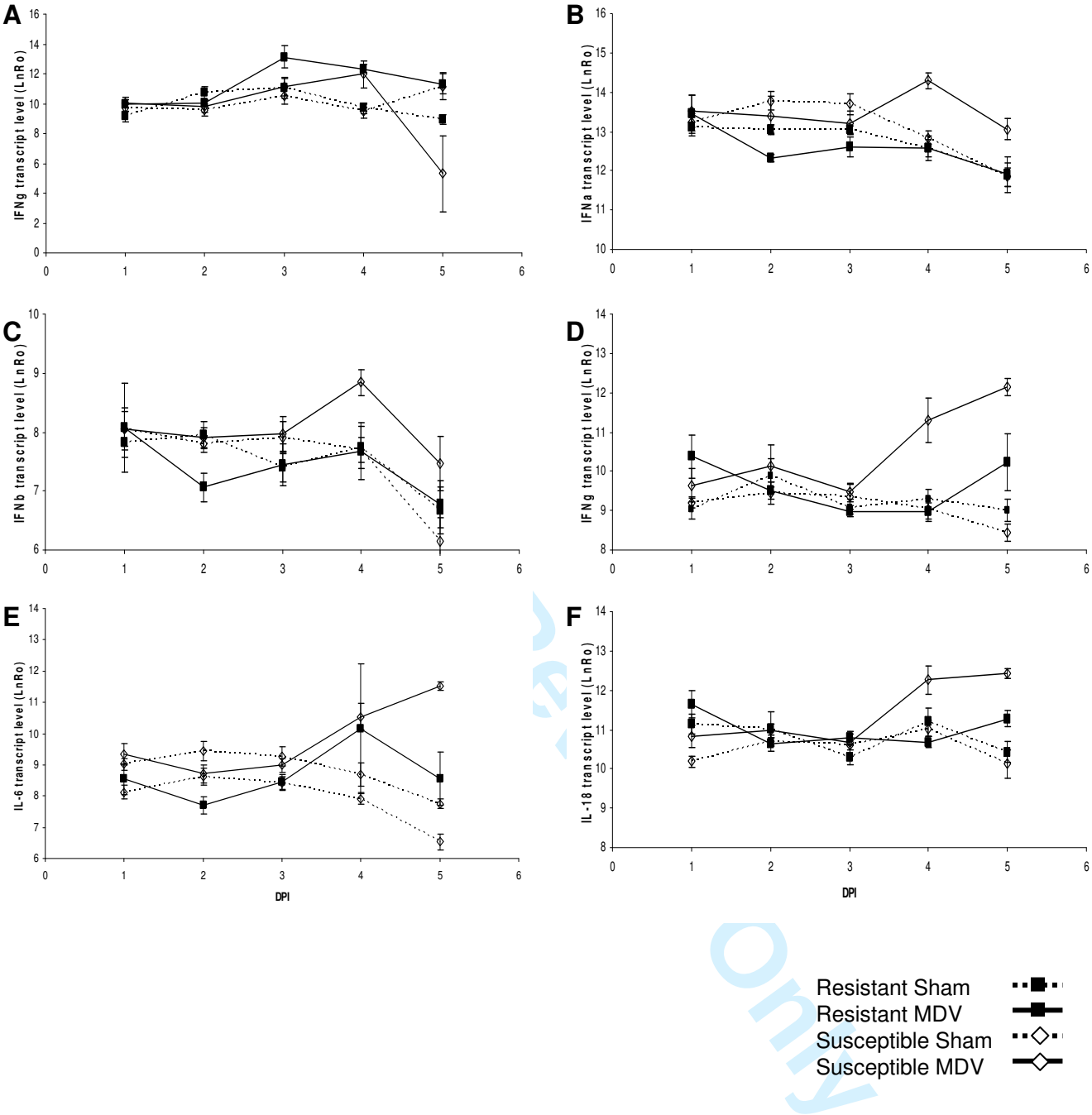
A
IFNg transcript level (LnRo)
B
IFNa transcript level (LnRo)
C
IFNb transcript level (LnRo)
D
IFNg transcript level (LnRo)
E
IL-6 transcript level (LnRo)
F
IL-18 transcript level (LnRo)
DPI
DPI
Resistant Sham
Resistant MDV
Susceptible Sham
Susceptible MDV

Figure 5.

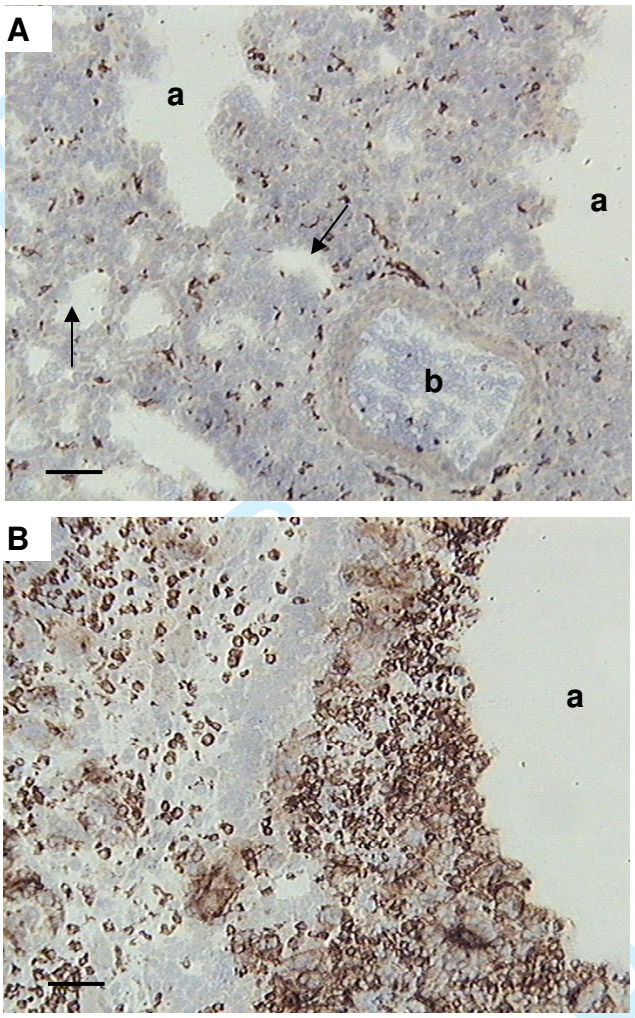

Figure 6.

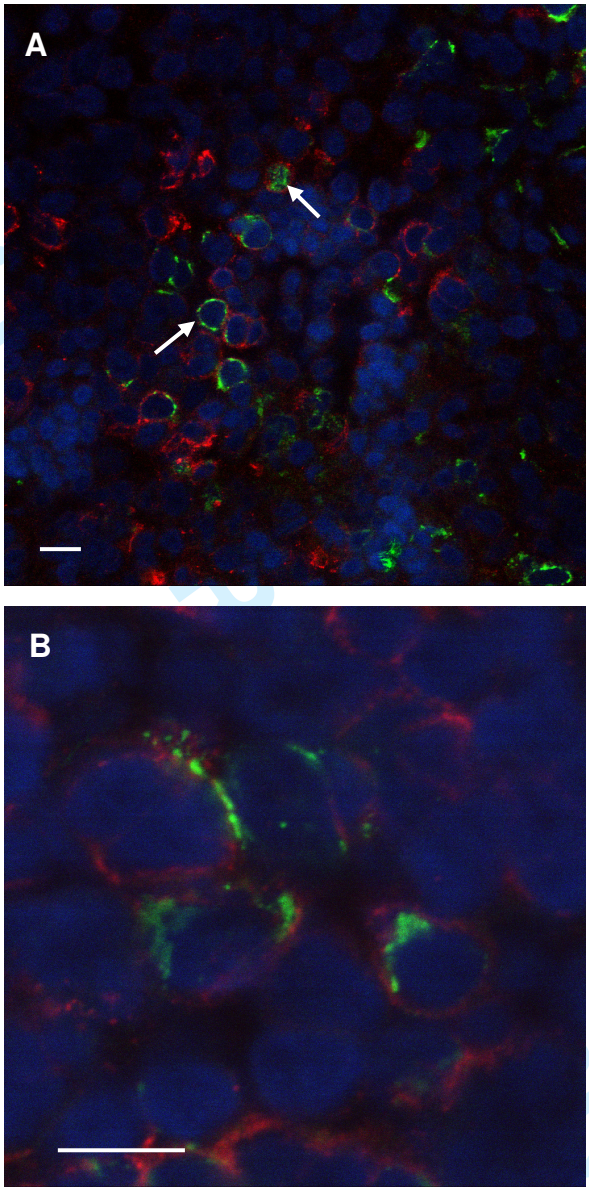

Figure 7.

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Route of challenge is critical in determining the clinical outcome of infection with a very virulent oncogenic herpesvirus, Marek's disease virus.

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Short Title: Infection route is critical to MDV infection

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The majority of experimental studies examining MDV infection have used parenteral injection of cell-associated virus. The aim of this study was to examine whether the route of entry of virus was critical in determining the outcome of infection. Susceptible (L7) and resistant (L6) White Leghorn chickens were infected with a very virulent Marek's disease virus, RB1B, by either the intra-abdominal or intra-tracheal route. Birds infected by the intra-tracheal route had earlier, higher or more sustained blood, spleen and lung viral concentrations than those infected by the intra-abdominal route. L7 birds had higher viral loads than L6 birds infected by the same route. Clinical outcomes reflected these data. Resistant birds infected by the intra-tracheal route had an increased prevalence of tumours and shorter survival times compared with those infected by the intra-abdominal route. Susceptible birds infected by the intra-tracheal route became paralysed 10 days after infection. L7 birds had shorter survival times and increased prevalences of tumours than L6 birds. The pathology and viraemia seen with intra-tracheal infection could not be fully replicated by increasing the dose in intra-abdominal infections. We conclude that instillation of infective dust produces a more aggressive infection that depends on the route of entry and form of virus, and not just on the challenge dose.

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Introduction

Marek's disease virus (MDV) is a highly cell-associated α-herpesvirus (Van Regenmortel *et al.*, 1999) that may cause neoplasia, paralysis and immunosuppression in susceptible genotypes of chickens (Witter & Schat, 2003). As it can cause substantial losses in the poultry industry (Witter, 1971; Powell, 1986), it has been controlled by vaccination, either using herpesvirus of turkeys (HVT) (Purchase & Okazaki, 1971) or attenuated strains of MDV (Churchill *et al.*, 1969). Marek's disease is the best example of a virus-induced neoplasia being effectively controlled by vaccination (Calnek, 1992), although strategies for the control of Epstein-Barr virus (EBV) are being developed using the MDV experience as a model (Epstein, 2001).

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The life-cycle of MDV is complex and is thought to start with the entry of pathogen, in cell-free or "dust-associated" form, through the mucosal surface of the lung, a process facilitated by lung phagocytic cells (Beasley *et al.*, 1970; Adldinger & Calnek, 1973). An early round of viral amplification then takes place in B lymphocytes (Schat *et al.*, 1980; Calnek *et al.*, 1982; Shek *et al.*, 1983) followed by replication in, and in susceptible birds neoplastic transformation of, mainly $CD4^+$ T-lymphocytes (Calnek *et al.*, 1984a; Calnek *et al.*, 1984b; Schat *et al.*, 1991; Ross, 1999; Burgess & Davison, 2002). Cellular immune responses are thought to cause the virus to switch from the cytolytic phase into latency (Buscaglia *et al.*, 1988; Schat & Xing, 2000), with latently-infected T-cells transporting the virus to the feather-follicle epithelium (FFE) (Calnek *et al.*, 1970). Fully-productive replication takes place only in the FFE, from which virus is shed to the environment, presumably in association with keratin particles and possibly also as free virus (Beasley *et al.*, 1970; Calnek *et al.*, 1970). It has been noted that the introduction of MDV vaccines has driven the virus to an increase in virulence, with each successive vaccine being followed by the emergence of more pathogenic strains (Witter, 1998). The theoretical basis for this evolution has been proposed to be

essentially a function of vaccine-induced non-sterile immunity that restricts viral replication within the host, rather than either blocking viral entry or shedding of infective virus into the environment (Gandon *et al.*, 2001).

The resistant L6 (L6) and susceptible L7 (L7chickens used in this study have the B^2 major histocompatibility complex haplotype, but differ markedly in their response to contact-exposure to MDV (Bacon *et al.*, 2001), with L7 birds also producing a higher viraemia than L6 (Lee *et al.*, 1981). Although the majority of experimental studies examining MDV infection have used parenteral injection of cell-associated virus, we have recently demonstrated that replication of virus in the lung occurs a day before replication in the spleen of birds infected by the intra-tracheal route (Baaten *et al.*, submitted for publication). In order better to target vaccination at preventing the initial uptake and early replication of virus, this work tested the hypothesis that the route of entry of virus is critical in determining the outcome of infection.

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Materials and Methods

Viral preparations Chicken embryo fibroblast cultures were used for the propagation of cell-associated virus, by methods previously described (Schat & Purchase, 1998). Infective dust was harvested from air filters in a room housing 60 Rhode Island Red birds, each infected with 1,000 pfu of cell-associated virus. The dust was then coarse-filtered, dried by vacuum, and stored at -80^0C. The vv MDV strain RB1B was used for both preparations of virus, at passage number 12. Birds were either infected by the intra-abdominal route with 100 µl of cell-associated virus diluted to the appropriate concentration, or were insufflated with 2.5 mg of infective dust directly into the trachea using a PennCentury Dry Powder Insufflator (as described by Baaten *et al.,* submitted for publication). Although we estimated, by quantitative

PCR, that the dose of virus given by the intratracheal (I.T.) route was potentially 18x greater than that given by the intra-abdominal (I.A.) route, we were unable to directly determine the infectivity of this virus. These doses of virus were equivalent in their ability to infect birds by the appropriate route, as they were approximately ten fold higher than the minimum dose required to reliably infect all birds.

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Chickens and experimental design Two-week old L6 and L7 birds were obtained from the Compton SPF breeding facility, from parents negative for antibodies to specified pathogens, and were kept in controlled-environment isolation rooms with food and water provided *ad libitum*. All animal procedures were performed in accordance with local ethical regulations, with the approval of the UK Home Office (1986).

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In Experiment 1, 60 chickens from each line were randomly assigned to two groups. One group of each line was infected with 2.5 mg infective dust by the I.T. route, and the other with 1,000 pfu cell–associated virus by the I.A. route. A randomly selected subgroup of 10 birds from each group was identified at the start of the experiment, and these were bled at intervals throughout the experiment.

In Experiment 2, 20 chickens from each line were assigned to two groups of 10 birds and infected with either 10,000 or 100,000 pfu of cell-associated RB1B and bled at intervals. In Experiment 3, groups of 15 birds from each line were infected with either 2.5 mg infective dust or 1,000 pfu cell-associated virus. Birds were killed at 7, 10 and 14 d.p.i. and their spleen and lungs collected for isolation of leucocytes.

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***Post mortem* procedure** All animals were subject to a full postmortem, with the following tissues being removed for examination:bursa, spleen, thymus, lung, kidney, liver, sciatic nerve, brachial nerve, caecal tonsil, skeletal muscle, heart, proventriculus, gonads, adrenal gland and skin.

DNA and RNA isolation. A saponin-lysis method was used to remove haemoglobin from whole blood. Briefly, 50µl of blood was collected from each bird in a equal volume of 3% sodium citrate.A 500µl volume of 1% saponin was added and the sample left for 10 minutes, prior to centrifugation at 650 *g*. (Baaten *et al.*, submitted for publication). Qiagen DNeasy 96-well plates were used for DNA isolation from the resuspended pellet using the manufacturer's instructions for cultured animal cells.

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Spleens and lungs were digested in five changes of HBSS medium containing 556 µg DNase I /ml and 2.2 mg collagenase D (Boeringer Mannheim) /ml. Cells were discarded from the first lung digest, to further deplete any remaining contaminating red blood cells, and then cells collected after each subsequent incubation in HBSS medium containing 10 mM EDTA. The digest and remaining debris were then passed through a cell-sieve, resuspended in PBS and then isolated by under-laying with Ficol-Paque and centrifugation at 650 *g* for 20 min. Cells at the interface were taken and washed in PBS prior to use. A total of 10^6 cells from each organ were used to prepare RNA samples using the Qiagen RNeasy mini-column kits according to the manufacturer's instructions.

Quantitation of genomic MDV and viral transcripts The concentration of MDV genome was measured by real-time quantitative PCR assay (Taqman™, Applied Biosystems, Foster City, USA), using primers and a probe specific for the *meq* gene labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher N, N, N, N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. Primers and a probe labelled with the proprietary fluorescent reporter dye VIC at the 5' end and TAMRA at the 3' end were used to simultaneously determine the concentration of the ovotransferrin gene, two copies of which are found in the chicken genome, in a duplex reaction, thus allowing for standardization of the *meq* assay. The PCR assay used the qPCR Master Mix kit (Abgene). Amplification and

detection of specific products were carried out with the ABI PRISM 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 95°C for 15 min (enzyme activation) and then 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 6 minutes.

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For the quantitation of viral transcripts, primers and probes specific for *meq*, ICP4, pp38 and gB genes were used. Reverse transcription of mRNA was performed prior to amplification in a single tube reaction using the EZ-RT-PCR kit (Roche) with the following cycle profile: 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, followed by 40 cycles of 94°C for 20 seconds and 59°C for 1 min. The reference used for standardization was 28s RNA. Primer and probe sequences are given in Table 1.

Preliminary analysis of the data was used to determine the critical threshold (CT) values. These values were set using standard samples on each plate to eliminate any systematic plate to plate variation. Plots of critical thresholds (CT) against dilution were generated for each test gene. Results of individual samples were then initially calculated relative to a theoretical reference sample which had threshold levels of each gene:

$$Log_2R_1 = \frac{CT_{test}}{Slope_{test}} - \frac{CT_{reference}}{Slope_{reference}}$$

The zero point of this $\log_2$ scale was then fixed to the Log_2R1 value that would be given by a CT_{test} value of 40 and a $CT_{reference}$ value representing the minimum $CT_{reference}$ of all samples:

$$Log_2R_0 = Log_2R_1 - \frac{40}{Slope_{test}} - \frac{^{Minimum}CT_{reference}}{Slope_{reference}}$$

The zero point of the Log_2R_0 scale is thus a minimum estimate of the value that would be given by a sample with threshold levels of test gene and maximum level of reference. It

should be noted that samples described as yielding a CT of 40 have not reached the threshold within 40 cycles of PCR, and an LnR_0 approaching zero indicates an actual value of template between zero and that indicated by CT of 40. For consistency all samples with a CT=40 were assigned a Log_2R_0 value of 0.

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Statistical analysis The statistical comparison of data where some samples failed to reach the threshold value is problematic due to the extreme skewed nature of the data and the arbitrary values attributed to these samples. Where appropriate, analysis of variance was used to determine the significance of the difference between means. Where the assumptions required for parametric analysis were not met, the Kruskal-Wallis test was used to determine the significance of the difference between medians.This test is relatively insensitive for the sample sizes used in this experiment. Fisher's exact test was used to determine the significance of differences between the prevalence of tumours in experimental groups, and Kaplan-Meier analysis was used to determine the significance of the differences in survival. All analyses were performed with Graphpad Prism software (Graphpad, USA).

Results

Viraemia in infected chickens. Concentrations of MDV genome in the blood of L6 and L7 birds infected with either 2.5 mg of dust by the I.T. route, or 1000 pfu of cell-associated RB1B by the I.A. route (Experiment 1), are shown in Figure 1. L6 birds infected by the I.T. route had higher concentrations of MDV genome and a more sustained viraemia than those infected by the I.A. route. The differences in viraemia were significant ($P<0.05$) on most days from 8 d.p.i. until the decline in concentration after 40 d.p.i.. At 5 d.p.i. L7 birds infected by the I.T. route had a detectable viraemia but those infected by the I.A. route, nor any L6 birds had a detectable viraemia. This difference was still evident at 8 d.p.i..The L7 birds infected by

the I.T. route developed paralysis and were euthanased at 10 d.p.i. The concentration of MDV genome in the blood of L7 birds infected by the I.A. route continued to rise to a level approximately 250-fold above that seen at 10 d.p.i. in those birds infected by the I.T. route or the highest levels seen in L6 birds.

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In order to determine whether the differences between the groups were due to a qualitative difference in the route of administration and form of the virus, or were simply due to an effectively increased challenge dose, 10 birds from each line were infected with 10,000 or 100,000 pfu of cell-associated virus by the I.A. route (Experiment 2). The concentrations of MDV genome in the blood of the L7 birds (Figure 2) increased at a faster rate than in those in the birds infected with 1,000 pfu in Experiment 1, but declined after reaching a concentration approximately half that of the peak concentrations seen in these birds. The concentrations of MDV genome in the blood of the L6 birds given these larger doses of virus rose to a peak level higher than those given 1,000 pfu by the I.A. route in Experiment 1, and were comparable to those seen in the birds infected by the I.T. route. This level of viraemia did not appear to be sustained for as long as in the birds infected by the I.T. route, although this difference was not significant.

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Survival of infected chickens. Survival analysis of the birds infected in Experiment 1 showed the groups to have distinctly different profiles (Figure 3). The L7 birds infected by the I.T. route that had paralysis at 10 d.p.i. were also in respiratory distress, and had to be euthanased under the local rules for animal experimentation ethics. Paralysis was not evident in birds from any other group, and the L7 birds infected by the I.A. route had a median survival of 28 d.p.i.. L6 birds infected by the I.T. route had a median survival of 43.5 days, whilst 25/30 L6 birds infected by the I.A. route were still alive at the termination of the experiment at 70 d.p.i. The median survival time of birds in each of the groups was significantly different from those in each of the other groups ($P<0.05$). Survival of the birds

receiving a high dose of virus by the I.A. route is shown in Figure 4. Median survival times of L7 birds receiving 10,000 and 100,000 pfu by the I.A. route (25.5 and 26.5 days post-infection (d.p.i.), respectively) were not significantly different from those of the birds receiving 1,000 pfu. Only one of twenty L6 birds receiving the higher doses of virus did not survive until 60 d.p.i..

Prevalence of tumours. Infection, by the I.T. route, of L6 birds resulted in a significantly higher prevalence of tumours than in the birds infected with 1,000 pfu by the I.A. route ($P<0.0001$), but this could be also be achieved by increasing the dose of virus given intra-abdominally ($P<0.01$). Groups of L7 birds infected by the I.A. route had a higher prevalence of tumours than that seen in the L6 birds (for 1000pfu infective dose $P<.0001$; 10,000pfu $P<.05$; 100,000pfu $P<.001$), Most L7 birds infected by the I.T. route were euthanased before the development of gross tumours.

Transcription of viral genes in the lung and spleen. Viral transcripts were measured in cells isolated from the lungs and spleen of L6 and L7 birds at 7, 10 and 14 d.p.i. by each route. All viral genes (*meq*, ICP4, gB, pp38 and LAT) had very similar patterns of expression, and the concentration of ICP4 and LAT genes transcripts are shown as examples in Figure 5. No significant differences were evident between concentrations of transcripts from any of the viral genes in the two L7 groups. Although the concentrations of viral gene transcripts in the L7 birds infected by the I.T. route were lower at 7 d.p.i. than in the birds infected by the I.A. route, this difference was not significant. Concentrations of viral transcripts in the L6 birds were lower than in the L7 birds, and L6 birds infected by the I.T. route had significantly higher concentrations of all viral transcripts than those infected by the I.A. route from 10 d.p.i. onwards.

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Discussion

In an earlier study we demonstrated the reproducible production of Marek's disease by the intra-tracheal insufflation of infective dust (Baaten *et al*., submitted for publication). Our present results point towards a difference in MDV-induced pathology that is dependent on the form of virus and its route of administration, and not simply on the dose. The high levels of viraemia and prevalence of tumour formation seen after I.T. administration of virus could be partially reproduced by the administration of high titres of virus by the I.A. route. However, intra-abdominal administration of virus did not produce the very early viraemia seen in L7 birds, or the sustained viraemia seen in the L6 birds when infected by the I.T. route. Intra-tracheal administration also produced far earlier mortality in both inbred lines, with an early paralysis uniquely and invariably seen in the L7 birds. Others have reported that approximately one third of susceptible strain 15x7 birds developed transient paralysis when infected with 500 pfu cell-associated RBIB by the I.A. route, while strain TK birds showed no neurological signs (Gimeno *et al.*, 1999). The differences in response seen in the present study may be a consequence of the Line 15 background of the birds used in the previous work producing a more susceptible phenotype, or may reflect differences in pathogenicity between the strain of RB1B virus used in each study.

Yunis *et al.* (Yunis *et al.*, 2004) have recently described the early events in MDV infection after I.A. administration of virus, highlighting the important difference between the appearance of measurable virus in a bird, and the replication of that virus. In their work, an early peak of detectable virus from 1 d.p.i. in splenocytes was not accompanied by viral replication until 4 d.p.i..In our previous work using the I.T. route for infection we demonstrated very early viral replication occurring at 2 d.p.i. in the lung, and at 3 d.p.i. in the spleen.

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The differences in pathology evident between the two routes of infection may reflect differences in the timing or quality of host responses in relation to viral replication. Although increased expression of IFN-γ in splenocytes has been reported at as early as 3 d.p.i. with MDV by the I.A. route (Xing & Schat, 2000; Kaiser *et al.*, 2003), we have shown an increase in transcription of this pro-inflammatory cytokine in lung leukocytes as early as 2 d.p.i. by the I.T. route, but not in splenocytes before 5 d.p.i. (Baaten *et al*., submitted for publication). Whilst the appearance of IFN-γ transcript precedes, or is concomitant with, viral replication in the spleens of birds infected by the I.A. route, or the lungs of birds infected by the I.T. route, there was no measurable expression in the spleens of the birds inoculated by the I.T. route until two days after the initiation of local replication of the virus. It is therefore possible that an mucosal immune response in the lung is unable to initiate an early systemic response to counter viral replication. By contrast, infection by the I.A. route may allow a systemic response to be initiated ahead of viral replication. In effect, the initial dose of cell-associated virus may prime the immune system better to control the later viral replication. The instillation of infective dust may lead to rapid replication, eliminating this possibility. The cell types involved in the immune response in the lung and spleen may also determine the outcome of infection, and the dynamics of viral replication and tropism in these different environments also need to be examined.

The precise nature of the early protective immune responses remains to be elucidated. Natural killer cells may be important, and are known to be activated as early as 4 days after vaccination by the I.A. route (Heller & Schat, 1987), possibly as a result of the administration of MHC-mismatched cells, and not of the virus itself. The MDV-antigen specific cytotoxic T-lymphocyte response that has been reported at 7 d.p.i. in chickens infected by the I.A. route (Omar *et al.*, 1998; Markowski-Grimsrud & Schat, 2002) might be driven by initial priming by the virally-infected cells of dendritic cells, which then respond to antigens produced by subsequent lytic infection.

An early immune response facilitated by I.A. infection with cell-associated virus would explain the lower and less sustained viral loads, and may also be responsible for the lower prevalence of tumour formation, as this appears to correlate with the early levels of viraemia. The pathogenesis of the early paralysis seen in the L7 birds infected by the I.T. route is unclear. Although MD was initially characterized as a paralytic disease, this was due to induction of peripheral nerve lesions by less virulent neurotrophic viruses. These were absent in any of the birds infected with the very virulent RB1B strain. More recently, an acute paralysis has been described following parenteral infection with vv+MDV, and this has been ascribed to lesions within the central nervous system (CNS) (Gimeno *et al.*, 1999; Witter *et al.*, 1999; Gimeno *et al.*, 2001). The immunopathology of the CNS in birds infected by different routes will be an important aspect for further study to enhance understanding of the pathobiology of MDV infection.

The findings described here contrast the results of an artificial route for infection with the known natural route. A similar phenomenon has been noted with two natural routes for infection with the bacterial pathogen *Salmonella*, where infection of the lungs of pigs appears to be 100 times more effective at producing subsequent bacterial replication than infection though the gut (Fedorka-Cray *et al.*, 1995; Gray *et al.*, 1996; Proux *et al.*, 2001). The understanding of immune responses of the lung to pathogenic challenge would now seem central to the rational design of vaccines to better protect at the mucosal surfaces, and eliminate the possibility of subsequent systemic replication.

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References

Adldinger, H. & Calnek, B. (1973). Pathogenesis of Marek's disease: Early distribution of virus and viral antigens in infected chickens. *Journal of the National Cancer Institute, 50*, 1287-1298.

Bacon, L.D., Hunt, H.D. & Cheng, H.H. (2001). Genetic resistance to Marek's Disease. In K. Hirai (Ed.), *Current Topics in Microbiology and Immunology* Vol. 255, (pp. 121-141). Berlin: Springer.

Beasley, J.N., Patterson, L.T. & McWade, D.H. (1970). Transmission of Marek's disease by poultry house dust and chicken dander. *American Journal of Veterinary Research, 31*, 339-344.

Bumstead, N. & Curtis, M.J. (1986). Identification of chicken major histocompatibility complex (B) haplotypes by an immunoblot technique. *Journal of Immunological Methods, 95*, 267-271.

Burgess, S.C. & Davison, T.F. (2002). Identification of the neoplastically transformed cells in Marek's disease herpesvirus-induced lymphomas: Recognition by the monoclonal antibody AV37. *Journal of Virology, 76*, 7276-7292.

Buscaglia, C., Calnek, B.W. & Schat, K.A. (1988). Effect of immunocompetence on the establishment and maintenance of latency with Marek's disease herpesvirus. *Journal of General Virology, 69*, 1067-1077.

Calnek, B.W. (1992). Chicken neoplasia - a model for cancer-research. *British Poultry Science, 33*, 3-16.

Calnek, B.W., Adldinger, H.K. & Kahn, D.E. (1970). Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. *Avian Diseases, 14*, 219-233.

Calnek, B.W., Schat, A.K., Heller, E.D. & Buscaglia, C. (1984a). *In vitro* infection of T-lymphoblasts with Marek's disease virus. Paper presented at *An International Symposium on Marek's Disease*, Cornell University, Ithaca.

Calnek, B.W., Schat, A.K., Ross, L.N.J. & Chen, C.H. (1984b). Further characterization of Marek's disease virus-infected lymphocytes. II. *In vitro* infection. *International Journal of Cancer, 33*, 399-406.

Calnek, B.W., Schat, K.A., Shek, W.R. & Chen, C.L.H. (1982). *In vitro* infection of lymphocytes with Marek's disease virus. *Journal of the National Cancer Institute, 69*, 709-713.

Churchill, A.E., Payne, L.N. & Chubb, R.C. (1969). Immunization against Marek's disease using a live attenuated virus. *Nature, 221*, 744-747.

Epstein, M.A. (2001). Historical background. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 356*, 413-420.

Fedorka-Cray, P.J., Kelley, L.C., Stabel, T.J., Gray, J.T. & Laufer, J.A. (1995). Alternate routes of invasion may affect pathogenesis of Salmonella typhimurium in swine. *Infection and Immunity, 63*, 2658-2664.

Gandon, S., Mackinnon, M.J., Nee, S. & Read, A.F. (2001). Imperfect vaccines and the evolution of pathogen virulence. *Nature, 414*, 751-756.

Gimeno, I.M., Witter, R.L., Hunt, H.D., Lee, L.F., Reddy, S.M. & Neumann, U. (2001). Marek's disease virus infection in the brain: Virus replication, cellular infiltration, and major histocompatibility complex antigen expression. *Veterinary Pathology, 38*, 491-503.

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Gimeno, I.M., Witter, R.L. & Reed, W.M. (1999). Four distinct neurologic syndromes in Marek's disease: Effect of viral strain and pathotype. *Avian Diseases, 43*, 721-737.

Gray, J.T., Fedorka-Cray, P.J., Stabel, T.J. & Kramer, T.T. (1996). Natural transmission of Salmonella choleraesuis in swine. *Applied and Environmental Microbiology, 62*, 141-146.

Heller, E.D. & Schat, K.A. (1987). Enhancement of natural killer cell activity by Marek's disease vaccines. *Avian Pathology, 16*, 51-60.

Kaiser, P., Underwood, G. & Davison, F. (2003). Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. *Journal of Virology, 77*, 762-768.

Lee, L.F., Powell, P.C., Rennie, M., Ross, L.N.J. & Payne, L.N. (1981). Nature of genetic resistance to Marek's disease in chickens. *Journal of the National Cancer Institute, 66*, 789-796.

Markowski-Grimsrud, C.J. & Schat, A.K. (2002). Cytotoxic T lymphocyte responses to Marek's disease herpesvirus-encoded glycoproteins. *Veterinary Immunology and Immunopathology, 90*, 133-144.

Omar, A.R., Schat, K.A., Lee, L.F. & Hunt, H.D. (1998). Cytotoxic T lymphocyte response in chickens immunized with a recombinant fowlpox virus expressing Marek's disease herpesvirus glycoprotein B. *Veterinary Immunology and Immunopathology, 62*, 73-82.

Powell, P.C. (1986). Marek's Disease: a worldwide problem. *World's Poultry Science, 42*, 205-218.

Proux, K., Cariolet, R., Fravalo, P., Houdayer, C., Keranflech, A. & Madec, F. (2001). Contamination of pigs by nose-to-nose contact or airborne transmission of Salmonella Typhimurium. *Veterinary Research, 32*, 591-600.

Purchase, H.G. & Okazaki, W. (1971). Effect of vaccination with herpesvirus of turkeys (HVT) on horizontal spread of Marek's disease herpesvirus. *Avian Diseases, 15*, 391-397.

Ross, L. (1999). T-cell transformation by Marek's Disease Virus. *Trends in Microbiology, 7*, 22-29.

Schat, A.K. & Purchase, H.G. (1998). Cell culture methods. In D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, & W.M. Reed (Eds.), *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* 4th edn (pp. 223-234). Tallahassee: Rose Printing.

Schat, K.A., Calnek, B.W. & Fabricant, J. (1980). Influence of the bursa of Fabricius on the pathogenesis of Marek's disease. *Infection and Immunity, 31*, 199-207.

Schat, K.A., Chen, C.L.H., Calnek, B.W. & Char, D. (1991). Transformation of lymphocyte T subsets by Mareks disease herpesvirus. *Journal of Virology, 65*, 1408-1413.

Schat, K.A. & Xing, Z. (2000). Specific and nonspecific immune responses to Marek's disease virus. *Developmental and Comparative Immunology, 24*, 201-221.

Shek, W., Calnek, B., Schat, K. & Chen, C. (1983). Characterization of Marek's disease virus-infected lymphocytes: Discrimination between cytolytically and latently infected cells. *Journal of the National Cancer Institute, 70*, 485-491.

Van Regenmortel, M.H.V., Fauqet, C.M., Bishop, D.H.L., Carstens, E., Estes, M.K., Lemon, S., Maniloff, J., Mayo, M.A., McGeoch, D., Pringle, C.R. & Wickner, R.B. (Eds.). (1999). *Seventh Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press.

Witter, R.L. (1971). Marek's disease research - History and perspectives. *Poultry Science, 50*, 333-342.

Witter, R.L. (1998). The changing landscape of Marek's disease. *Avian Pathology, 27*, S46-S53.

Witter, R.L., Gimeno, I.M., Reed, W.M. & Bacon, L.D. (1999). An acute form of transient paralysis induced by highly virulent strains of Marek's disease virus. *Avian Diseases, 43*, 704-720.

Witter, R.L. & Schat, K.A. (2003). Marek's Disease. In Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, & D.E. Swayne (Eds.), *Diseases of Poultry* 11th edn (pp. 407-465). Ames: Iowa State Press.

Xing, Z. & Schat, K.A. (2000). Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. *Immunology, 100*, 70-76.

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Yunis, R., Jarosinski, K.W. & Schat, K.A. (2004). Association between rate of viral genome replication and virulence of Marek's disease herpesvirus strains. *Virology, 328*, 142-150.

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Figure Legends

Figure 1. *Mean concentrations of MDV genome in the blood of resistant (L6) and susceptible (L 7) chickens following infection with 1,000 pfu MDV by the I.A. route or 2.5 mg infective dust by the I.T. route.There were 10 birds per group at the start of the experiment, and error bars indicate the standard error of the mean.*

Figure 2. *Mean concentrations of MDV genome in the blood of resistant (L6) and susceptible (L 7) chickens following infection with 10,000 or 100,000pfu MDV by the I.A. route.There were 10 birds per group at the start of the experiment, and error bars indicate the standard error of the mean.*

Figure 3. *Survival of resistant (L6) and susceptible (L 7) chickens following infection with 1,000 pfu MDV by the I.A. route or 2.5 mg infective dust by the I.T. route.*

Figure 4. *Survival of resistant (L6) and susceptible (L7) chickens following infection with 10,000 or 100,000 pfu MDV by the I.A. route.*

Figure 5. *Concentrations of LAT and ICP4 gene transcripts in spleen and lung leucocytes of resistant (L6) and susceptible (L 7) birds following infection with 1,000 pfu MDV by the I.A. route or 2.5 mg infective dust by the I.T. route. Figures are given in a relative $\log_2$ scale. There were 5 birds per group, and error bars indicate the standard error of the mean. All L7 birds infected by the I.T. route had signs of paralysis at 10 d.p.i., and had to be euthanased under local experimentation ethics regulations.*

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Table 1. *Quantitative RT-PCR probe and primer sequences*

Target	Probe or Primer*	Sequence
meq	Probe	5'-(FAM)-AGACCCTGATGATCCGCATTGCGACT-(TAMRA)-3'
	F	5'-GGTCTGGTGGTTTCCAGGTGA-3'
	R	5'-GCATAGACGATGTGCTGCTGA-3'
28S	Probe	5'-(VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3'
	F	5'-GGCGAAGCCAGAGGAAACT-3'
	R	5'-GACGACCGATTTGCACGTC-3'
pp38	Probe	5'-(FAM)-CCCCGCATTTCTCGCCGTCCTC-(TAMRA)-3'
	F	5'-GAAAACAGAAGCGGAATGCG-3'
	R	5'-CGATCCAAAGCGCTCATCTC-3'
gB	Probe	5'-(FAM)-ACCGCCGCGAAAATGTCCCG-(TAMRA)-3'
	F	5'-GGTTCAACCGTGATCCGTCTA-3'
	R	5'-CGATTCCTTCACCCCACT-3'
LAT	Probe	5'-(FAM)-CCATGCCAAACCGGTCCAGGG-(TAMRA)-3'
	F	5'-TCGCAGCCAGGCATTTAAA-3'
	R	5'-GGTGGACGGCCGAGATG-3'
ICP4	Probe	5'-(FAM)-CGGCCCAGTACAGCCTGCGG-(TAMRA)-3'
	F	5'-CGCCACACGAGAACACAATG-3'
	R	5'-GGTTGGAGTAGAGCTGCAACTGT-3'

*F, forward; R, reverse

Accession no. (genomic sequence) for 28S sequence: X59733

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Table 2. *Proportion of animals with gross tumours following infection with increasing doses of MDV strain RB1B by the I.A. route, or by 2.5 mg infective dust by the I.T. route.*

Infection	L6	L7
Intra-tracheal	60%	3.3%*
1,000 pfu I.A.	6.7%	70%
10,000 pfu I.A.	50%	100%
100,000 pfu I.A.	20%	100%

* all animals were euthanased by 10 d.p.i.

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Figure 1

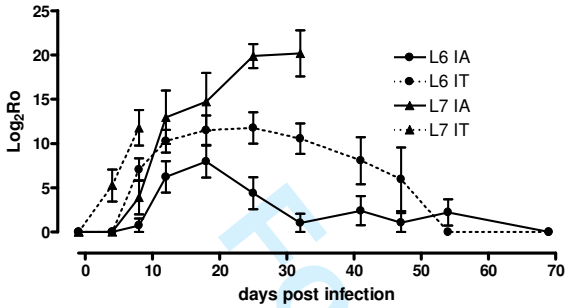

Figure 2

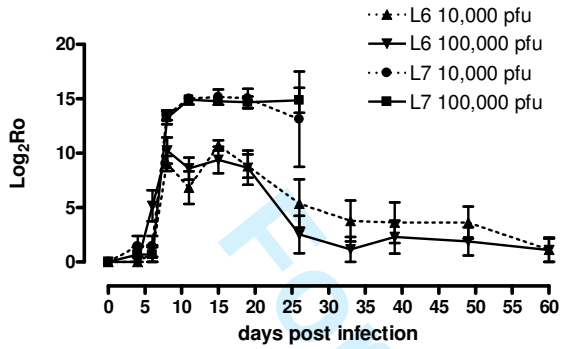

Figure 3

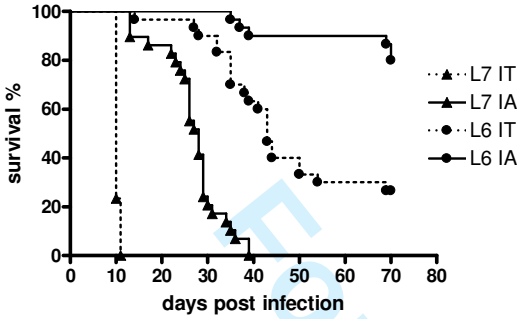

Figure 4

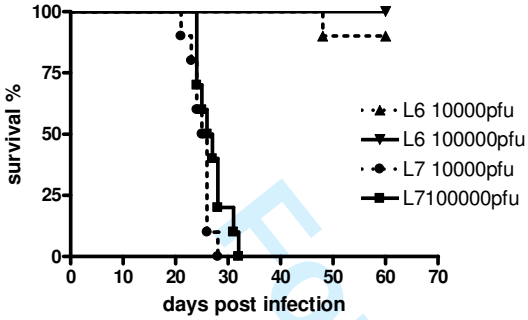

Figure 5