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Marek's disease herpesvirus vaccines integrate into chicken host chromosomes yet lack a virus-host phenotype associated with oncogenic transformation

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ABSTRACT

Marek's disease (MD) is a lymphotropic and oncogenic disease of chickens that can lead to death in susceptible and unvaccinated host birds. The causative pathogen, MD virus (MDV), a highly oncogenic alphaherpesvirus, integrates into host genome near the telomeres. MD occurrence is controlled across the globe by biosecurity, selective breeding for enhanced MD genetic resistance, and widespread vaccination of flocks using attenuated serotype 1 MDV or other serotypes. Despite over 40 years of usage, the specific mechanism(s) of MD vaccine-related immunity and anti-tumor effects are not known. Here we investigated the cytogenetic interactions of commonly used MD vaccine strains of all three serotypes (HVT, SB-1, and Rispens) with the host to determine if all were equally capable of host genome integration. We also studied the dynamic profiles of chromosomal association and integration of the three vaccine strains, a first for MD vaccine research. Our cytogenetic data provide evidence that all three MD vaccine strains tested integrate in the chicken host genome as early as 1 day after vaccination similar to oncogenic strains. However, a specific, transformation-associated virus-host phenotype observed for oncogenic viruses is not established. Our results collectively provide an updated model of MD vaccine-host genome interaction and an improved understanding of the possible mechanisms of vaccinal immunity. Physical integration of the oncogenic MDV genome into host chromosomes along with cessation of viral replication appears to have joint signification in MDV's ability to induce oncogenic transformation. Whereas for MD vaccine serotypes, a sustained viral replication stage and lack of the chromosome-integrated only stage were shared traits during early infection.

1. Introduction

Marek's disease (MD) is a lymphotropic disease of chickens characterized by fatal lymphoma development in the visceral organs, paralysis and blindness in susceptible birds. The causative alphaherpesvirus [1,2], Marek's disease virus (MDV aka gallid herpesvirus type 2 or serotype 1), is known to integrate into chicken telomeres [3–5]. The MDV genome contains homologous host genes and telomeric repeats, which were presumably acquired through recombination and/or integration and reemergence events during the course of the virus' evolutionary history [6–8]. As a herpesvirus, the life cycle of virulent strains involves transitioning from a stage of cytolytic replication in the episomal form to a latent stage, by which MDV evades host immune responses [9–14]. Furthermore, latent host CD4+ T lymphocytes become transformed after serotype-1 oncogenic MDV infection, between 14 and 21 days post-infection (dpi), resulting in lymphomas [15–19]. Telomeric integration of MDV is temporally related to latency [4,5]. Furthermore, latently-infected MD cell lines consist of primarily host genome integrated MDV, rather than extra-chromosomal viral DNA [3,20]. MDV telomeric repeats facilitate integration into chicken telomeres [21,22], although the precise mechanism of integration is not fully known. The viral *Meq*

Abbreviations: MD, Marek's disease; MDV, Marek's disease virus; HVT, herpesvirus of turkey; dpi, days post-infection; vv, very virulent; MHC, major histocompatibility complex; IR_L, inverted repeats long; IR_S, inverted repeats short; TR_L, terminal repeats long; TR_S, terminal repeats short; TR, telomerase RNA; vTR, viral telomerase; ΔMeq, *Meq*-deleted; ADOL, Avian Disease and Oncology Laboratory; BAC, bacterial artificial chromosome; DIG, digoxigenin; FITC, fluorescein isothiocyanate; FISH, fluorescence *in situ* hybridization; DAPI, 4′,6-diamidino-2-ph enylindole; PCR, polymerase chain reaction.

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oncogene and viral telomerase (vTR) gene, among others, play critical roles in host cell transformation [23–25]. Interestingly, a *Meq*-deleted (ΔMeq) MDV strain that does not induce MD lymphomas *in vivo* [26] can integrate into the chicken genome, but lacks a transformation-coupled viral-host phenotype in lymphocytes [5]. Osterrieder and colleagues presented experimental data suggesting that vTR is involved in telomeric integration of MDV [9].

MD can be controlled by selective breeding for increase genetic resistance, however, in commercial production systems, the primary control method is widespread vaccination [16]. An effective MD vaccine, the related herpesvirus of turkey (HVT; meleagrid herpesvirus type 1 aka serotype 3) [27,28], was first employed in the early 1970s. Field strain virulency increased (very virulent (vv) to vv+ ratings) through the late 1970s resulting in more outbreaks until bivalent vaccine [SB-1 (gallid herpesvirus type 3 or serotype 2) + HVT] and, later, the attenuated serotype 1 Rispens/CV1988 vaccine were employed [29,30]. The Rispens vaccine provides the highest level of protection against vv+ MDV, although its protective efficiency varies with bird major histocompatibility complex (MHC) haplotype [31]. These non-oncogenic, vaccine strains have been detected in a latent form *in vivo*, but do not induce MD lymphomas [32]. Current vaccination strategies do not elicit sterilizing immunity [18]. The specific mechanisms of vaccine-related immunity are not known, although reduced growth rate of MDV [33], increased late reactivity of peripheral blood lymphocytes to mitogens (in the case of HVT) [34] and prevention of immunosuppressive effect in the host [35] may contribute in addition to vaccine strain(s) interference with the infection process of oncogenic MDV in T and B lymphocytes [36,37]. However, supplementary mechanisms are certainly involved [9,15] and an explanation for the anti-tumor effect of vaccination has not yet been established. Furthermore, the underlying features of the vaccinal protective mechanism undoubtedly vary among the vaccine serotypes.

Although the genomes of oncogenic and vaccine strains are highly similar, key differences exist [38] (*see Table 1 p.2*). A shared aspect among serotypes is the presence of telomeric repeats between the inverted repeats long (IR_L)/short (IR_S) and terminal repeats long (TR_L)/short (TR_S) of the viruses. The presence of telomeric repeats in both oncogenic and vaccine MDV strains suggests that the vaccines could be capable of integrating into telomeres of host lymphocytes, despite differences in or the absence of, multiple disease-associated genes (*Meq*, vTR, vIL8, etc.) [39]. Our central hypothesis is that MD vaccines integrate into the chicken genome at the telomeres. This vaccinal interaction with the host genome, in addition to various other behaviors that contribute to the vaccinal protective mechanism, may disrupt aspects of infection, latency, and/or the transformation cycle of the oncogenic MDV strain(s). Here we sought to test our hypothesis by investigating interactions of MD vaccines with the host at the level of the genome (*i.e.*, study virus chromosomal association and integration profiles) via cytogenetic methods.

2. Materials and methods

2.1. Genetic lines and vaccination/challenge

Experimental birds were progeny of a cross between two highly inbred, MD susceptible lines 7_2 and $15I_5$ from the USDA, ARS, Avian Disease and Oncology Laboratory (ADOL), cared for under approved animal care protocols by trained staff. All institutional (ADOL Institutional Animal Care and Use Committee) and national (USA National Research Council of the National Academies) guidelines for the appropriate care and use of laboratory animals were closely followed throughout the experiments. One week post-hatch, F_1 chicks were intra-abdominally vaccinated with 5000 pfu of HVT, SB-1, or Rispens alone, or challenged with GA oncogenic MDV alone or received no treatment. Chicks were hatched and maintained in Horsfall-Bauer isolation chambers at the USDA facility.

2.2. Spleen sample collection and processing

Mitotic chromosome preparations were harvested from spleen samples collected at 1, 4, 7, 14 and 21 days post-infection (dpi). Spleen cell suspensions were treated with 100 μl of colcemid (10 μg/ml, Invitrogen, Waltham, MA, USA) in cell culture medium for 30–60 min. Single cell suspensions were generated from each spleen and exposed to 0.56% KCl hypotonic solution (30 min) and then fixed in 3:1 methanol and glacial acetic acid (several fixes over one to three days) according to standard procedures. The chromosome spreads were applied to slides using the air-dry method after the third fixative change, as described by Delany et al. [40]. All chromosome slides were stored according to Delany et al. [40] until further use in FISH experiments, where they were left at room temperature in a slide box for at least 24 h to allow thawing and for condensation to be absorbed by fresh desiccant.

2.3. MDV-specific probes and probe labeling

The DNA probe utilized for hybridization to MDV genome was an Md11 bacterial artificial chromosome (BAC) (Md11gDc1.2 [7]). The probes were labeled using nick translation with Digoxigenin (DIG) dUTP through a DIG Nick Translation kit (Roche Applied Science, Indianapolis, IN, USA). An anti-digoxigenin secondary antibody (Roche Applied Science) conjugated with Fluorescein isothiocyanate (FITC, green) was then applied. Due to the presence of chicken telomeric repeats ($TTAGGG_n$) in the Md11 BAC probe, telomeric DNA sequence in the chicken genome was blocked with approximately 280 ng/uL of custom telomere oligonucleotide ($TTAGGG_7$), also called "cold telo."

2.4. Fluorescence in situ hybridization (FISH)

Slide hybridization was completed by standard FISH procedures as described by Delany et al. [40] with adaptations for labeling MDV; (1) the Md11 BAC probe was applied; (2) cold telo was utilized to block host telomeric DNA. The preparations were counterstained with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) diluted 1:9 (DAPI:VS). Prior to analysis, the slides were stored flat at 4 °C and image capture occurred within 24 h of FISH and DAPI counterstaining.

2.5. Cytogenetic analysis

Metaphase and interphase cell images were collected using an Olympus BX41 epifluorescence microscope equipped with an automatic filter wheel (Chroma Technology 82000, DAPI/fluorescein isothiocyanate/tetra methyl rhodamine isothiocyanate filter set), X-Cite 120 Series metal-halide fiber optic lamp, and Applied Imaging software (CytoVision 7.4 GENUS, Leica Biosystems, Buffalo Grove, IL, USA). Between 10 and 80 spleen cell images were analyzed per sample (representing an individual spleen from a bird) in each FISH experiment. Negative control (no treatment) samples were incorporated in most MDV FISH experiments to ensure that the Md11 BAC probe was hybridizing specifically to the MDV genome, as indicated by the absence of FITC signals from all terminal and interstitial telomeres, as described by Robinson et al. [4]. All captured mitotic metaphase cells were categorized as one of four cytogenetic viral phenotypes as previously described [4,38] and

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