Advances in Tumor Virology

REVIEW

The Knowledge that Human Tumor Virology can Gain from Studies on Avian Tumor Viruses

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Abstract: Avian tumor viruses are important, economically significant pathogens in the poultry industry, and can provide scientific insight in topics that cannot be experimented in humans. The present review will present viral-induced tumors in poultry and will emphasize the specific findings that can be taken from their study, regarding (a) multiple virus infections in vivo, (b) molecular interactions in vitro and in vivo between avian oncogenic viruses, (c) creation of new viable viruses with altered biological properties by the integration of two retroviruses in vivo, (d) the diversity of clinical signs that appear in viral-infected poultry prior to tumor formation. The inter-viral molecular interaction were studied in vitro and in vivo, in natural infections and in experimentally-infected birds. About 25% of flocks were double-virus-infected, and molecular integrations occurred spontaneously in about 5% of the samples, as by the chimeric molecules detection. In experimentally-infected birds the chimeric molecules rate was higher, while in herpes- and retroviruses co-infected tissue cultures, recombinant virus creation was efficient, leading to a new virus with altered biology. Spontaneous inter-viral recombination occurred between retroviruses, lead to the emergence of a delirious new virus, avian leukosis virus, subgroup J, which actually caused great economic losses to the poultry industry.

Keywords: avian oncogenic viruses, Marek's disease virus, avian leukosis virus, reticuloendotheliosis virus, molecular recombination, avian tumors

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Avian Tumors: Viral- and Non-Viral Induced

Neoplastic diseases in poultry consist of a variety of conditions that can be divided into two categories, depending on whether the etiologic agent is known or not. Tumors in poultry are caused either by infection with avian oncogenic viruses, or by unknown etiology. Most attention and study on poultry tumors was dedicated to the viral-induced tumors and much less to the non-virally induced tumors, because their incidence appears to be low, and because they usually appear at advanced ages. The life span of commercially raised chickens and turkeys is generally short, and probably less than required for the development of non-virally induced tumors. In contrast, most human cancers have unknown etiology, while only about 15% of the human cancers have virus origins.

Viral induced tumors in poultry have considerable economic importance for the poultry industry; they cause a decreased productivity, causes increased morbidity and mortality of birds during growth and increased condemnation at slaughter, because of esthetic reasons, as the tumor viruses are not zoonotic and do not have any public health significance.

In the study of avian tumors, attention has been focused on those of viral etiology, not only from the standpoint of their economic importance, but also as potential models applicable to tumors in humans.[1,2] In contrast to the study of viral-induced avian tumors, experimentation to elaborate various aspects are not possible in humans. As no experimental infection trials are feasible and only a limited knowledge regarding the etiology of human tumors is available.

The Avian Oncogenic Viruses

The avian oncogenic viruses of chickens include one herpesvirus, the Marek's disease virus (MDV),[3] and three retroviruses. The three avian retroviruses consist of the reticuloendotheliosis virus (REV),[4] avian leukosis/sarcoma viruses[5] (including ALV-J),[6] and the avian retrovirus that is responsible for the rare neoplastic disease of turkey known as lymphoproliferative disease (LPD),[9] which is distinctly different from REV and ALV avian leucosis and avian leucosis virus subgroup J (ALV-J).[6] Turkeys are susceptible to MDV[7,8] and REV and to the relatively rare retrovirus, LPDV (Table 1).

MDV is a dsDNA avian herpesvirus that causes tumors in chickens and turkeys, and is one of the most economically important pathogen that affects the poultry industry profitability. MDV transforms T-lymphocytes, leading to the formation of skin and visceral tumors, but also causes immunosuppression and a variety of symptoms until tumors became visible, including neurological symptoms and eye lesions. MDV is widely disseminated in poultry worldwide, therefore considered as ubiquitous. Natural MDV isolates of a variable virulence have been isolated, including very virulent plus, very virulent, virulent, mild, belonging to serotype 1 and avirulent strains, belonging to serotypes 1, 2 and 3. Avirulent viruses have been adapted to serve as effective vaccines against virulent viruses, and prevention of disease. In that regard, the MD comprises a unique example in nature, as it is the first

Table 1. The avian oncogenic viruses, detection and characterization.

Virus	Acronym	Family	Affected species	Cell type for transformation	PCR amplicon for differential diagnosis	Reviewed in reference no.
Marek's diseases virus	MDV	Herpesvirus	Chickens, turkeys	T (Chickens) T, B? (Turkeys)	Bam H1 D/H 132 bp tandem repeat	3
Reticuloendotheliosis virus	REV	Retrovirus	Chickens, turkeys	T, B	Proviral LTR	4
Avian Leukosis virus	ALV	Retrovirus	Chickens	B	Proviral LTR	5
Avian leucosis virus, subgroup J	ALV-J	Retrovirus	Chickens (meat type)	Myelocytes	Env gene	28
Lymphoproliferative disease virus	LPDV	Retrovirus	Turkeys	?	Proviral LTR	9

naturally-occurring malignant disease that is caused by a herpesvirus, and can be effectively controlled by vaccination using naturally-isolated avirulent MDVs. Furthermore, MDV is the first herpesvirus in which an oncogene has been discovered and characterized. The virus replicates in the feather follicle epithelium cells and spreads horizontally in the poultry houses. The MDV environmental spread of skin stratified squamous epithelium cells, which commonly detach with molted feathers or skin renewal, via dust and dander causes the virus to be ubiquitous. The feature of MDV replication in feather follicle epithelium motivated numerous studies that utilized the feathers to detect and isolate the virus, as reviewed by Davidson.[10,11]

Retroviruses replication within chicken cells employs a rapid transition to DNA by a reverse transcriptase step, following which, the viral genome is incorporated into the cellular genome. The spread of retroviruses occurs mainly vertically, as proviruses integrated into the host cellular genomes are transmitted from infected birds through their eggs to their offspring. However, horizontal dissemination of retroviruses from bird to bird by direct or indirect contact, occur as well. The horizontal spread is mainly facilitated by feces and other excretions from infected birds, although the stability of retroviruses in dry substances is very limited.[11]

Avian leukosis viruses, called also as leukosis/sarcoma viruses, the L/S group, cause a group of leukoses, sarcomas and related neoplasms. ALV is a lymphoproliferative disease of chickens affecting primarily the bursa of Fabricious and visceral organs, transforming the B-type lymphocytes. However, with the recognition of subgroup J ALV infection, myelocytomatosis was frequently diagnosed during the 1990s, particularly in affected meat-type chicken breeders, as ALV-J transforms myeloid cells, and the tumors tend to be formed on bones.

REV causes a group of disease syndromes that are unrelated to the L/S group of viruses and transforms pre-B and pre-T lymphocytes, causing bursal and T-cell lymphomas in chickens and turkeys. The most common REV-induced syndromes are chronic lymphomas and an immunosuppressive runting disease. The virus is widespread and often infects asymptomatically chickens, turkeys, ducks, geese, pheasants, quails and other avian species, causing often contamination of biological products.

A neoplasm of turkeys, known as lymphoproliferative disease of turkeys (LPD) was sporadically reported in Europe and Israel, and is induced by a distinct retrovirus and most virus and disease features are unknown, including the target cell for transformation.

Differentiation between Tumors Caused by Avian Oncogenic Viruses

The tumors are the most prominent clinical sign manifested in poultry following infection with avian oncogenic viruses. The clinical signs caused by infection with the five avian oncogenic viruses overlap and are of a low degree of pathognomy; REV induced T-cell lymphomas are similar both macroscopically and microscopically to those caused by MDV, while REV induced B-cell lymphomas resemble those caused by ALV. Generally, but not exclusively, the enlargement of the peripheral nerves and proventriculus, as well as visceral tumors characterize MDV involvement; the presence of bursal tumors in birds older than 16 weeks is characteristic to lymphoid leukosis; the presence of nerve lesions, bursal tumors, pancreas and intestines suggest REV oncogenesis, and the appearance of tumors on bones is characteristic to infection with ALV-J.

The lack of distinctive clinical markers for infection with the different oncogenic viruses motivated us to develop differential diagnostic assays by PCR.[12,13]

The Diversity of Clinical Signs Prior to Tumor Formation

Infection with avian oncogenic viruses in poultry can be presented with typical clinical signs, but as these viruses cause slow and chronic infections, sometimes the infection has a sub-clinical appearance and the accurate diagnosis is very complex. Although it is tempting for poultry clinicians to diagnose the disease, a precise diagnosis should include laboratory confirmation. Many of the tumor viruses appear to have multipotent characteristics. Most often infection with oncogenic viruses leads to tumor development following a relatively long period of time. During this period, and even before, several non-specific symptoms develop and persist in the presence, or absence of tumors. The associated symptoms are slow weight gain, immunosuppression, uneven growth and enhanced mortality and morbidity. These symptoms can appear separately or in various combinations, and with various degrees

of severity. Various stress factors, such as hormonal changes, multiple viral infections, inferior management conditions help promote tumor formation. It is not trivial to assign various health conditions in commercial flocks with infections with oncogenic viruses, due to the plurality of symptoms that they cause, and multiple forms of morbidity in affected flocks.

To evaluate the importance of the various clinical signs of the PCR-positive flocks, we assessed the differential diagnosis by PCR employed in a 13 year-study of more than 1000 commercial chicken flocks and correlated the PCR findings to the clinical signs, as reported at the sample submission.[14] The study demonstrated the need to substantiate the differential diagnosis on two pillars side by side, the clinical signs and the laboratory diagnosis. Neither of these two components can stand alone, as clinical diagnosis is not definitive without laboratory confirmation, while the latter is insignificant without at least one virus-specific clinical sign.

Multiple Virus Infections—Clinical Manifestations

Poultry flocks are exposed to multiple pathogens and stressors during rearing. The actual health condition in poultry houses reflects the cumulative clinical effects of diverse combination of pathogens. While some pathogens can cause acute or chronic diseases with distinctive clinical signs and lesions, others cause subclinical infections that may sometimes intensify the severity of clinical signs caused by supplementary pathogens. The veiled pathogens may alter biological properties of co-infecting pathogens, including transmission patterns, immunosuppression, and even molecular interactions between the co-infecting viruses. The consciousness of multiple pathogen infection of poultry is not trivial and is not always evident to poultry breeders and to growers; accordingly, the treatment and control of poultry diseases might be sometimes inadequate and/or insufficient, as being directed only towards the identification of part of the causative pathogens.

The multiple virus infections might be overseen because the clinical signs of infection with various oncogenic viruses often overlap and are not pathognomic. MDV also possess many features that promotes it as a common partner in multiple pathogen poultry infections; (a) MDV worldwide spread and ubiquitous nature, (b) MDV pathogenicity pattern, implying that often tumor development require a relatively long period of time, during which, non-specific symptoms develop, (c) vaccination which does not induce sterile immunity. As a result, MDV is often present as a hidden pathogen, although it impairs cellular immune responses to various pathogens, due to its cytolytic activities.

Although more than one oncogenic virus can create a tumor, their presence in a bird or in a tumor does not necessarily reflect on the cause of a particular tumor, and the disease severity could not be predicted. Each virus is immunosuppressive and oncogenic by itself, but they also could jointly influence the deregulation of cellular genes, transactivate each other's regulatory genes, and also retrovirus sequences might modify the original MDV properties by integration into the MDV genome and altering the gene transcription at the insertion site.

Synergism in pathogenicity was suspected in experimental trials, and in commercial flocks, however the documentation is poor. The evident reason for the aggravation of the disease severity by a double virus infection might reside in the lymphotropic nature and the independent transforming and immunosuppressive potentials of each of these viruses. The first report on dual infection of birds with MDV and REV under natural conditions was as early as 1967.[15] In the following years it was found that several MDV strains influenced both ALV and REV pathogenicity. To assess the in vivo effect of mixed infection with MDV and REV, we performed an experimental trail with MDV and REV as the infecting viruses. Main findings were a doubled mortality rate and decreased weight for birds co-infected with both MDV and REV, compared to single virus-infected groups.

Not only MDV-1 was implicated in the aggravation of the retroviral infection symptoms, but also MDV of serotype 2 augmented the development of bursal lymphomas induced by ALV and REV. In chickens vaccinated with the bivalent vaccine (SB1 + HVT) it was found that the incidence of spontaneous bursal lymphomas was significantly higher. The MDV-2 influence occurred at the stage of enhancing the hyperplastic follicles formation of the bursa of Fabricius, as both herpes and retroviruses replicated in the same ALV-transformed bursal follicles and cells.[16–18] A molecular analysis revealed that MDV-2 increased the ALV-gene expression by the transactivation of LTR promotors in chicken embryo fibroblasts[19,20] and by the apoptosis inhibition of the retroviral-transformed B cells.[21]

Multiple Virus Infections as Revealed by the Molecular Differential Diagnosis

Israeli commercial flocks, which were submitted for molecular diagnosis of avian oncogenic viruses that was performed between the years 1993 to 2000, served to evaluate the incidence and the prevalence rate of flocks and birds that carry a multiple infection.[12–14] The survey of natural co-infections with MDV and each of the three avian retroviruses (REV, ALV and ALV-J) included a total of 306 chicken and 59 turkey commercial flocks, of which, about a quarter of the tumor-bearing commercial flocks carried a mixed MDV and retrovirus-infection.[22] A total 2926 DNA samples were analysed, including 2428 chicken and 498 turkey DNA samples. Of these, 991 DNAs originated from 25% of the flocks which carried a multiple virus-infection. Multiple virus sequences were detected by PCR in 103 DNA preparations from that group (103/991—10.4%), including 38 and 56 DNAs from chicken blood and tumor tissues, respectively, and 9 samples from turkey blood.

The high prevalence (25%) of chicken and turkey flocks with multiple virus infections was unforeseen, and was of scientific interest. Multiple virus infections have a double biological significance, as the clinical and pathological signs of each virus might differ from those typical of each in separate, and because of the multiple viral infection of the same host, a biological interaction between them might occur. In further studies we showed that in chicken cells, which were infected with dsDNA viruses and retroviruses, molecular recombination between the two viruses happened, leading to their increasing genetic variability.[23]

Multiple Virus Infections—Intracellular Consequences

In cases of multiple virus infection of one particular cell, both viruses can interfere for replication, like in cases of avian leukosis viruses[5] in a single or mutual mode, or inversely, they would not impede each other's replication. In cases that the replication of both viruses are not inhibited, their dual presence in the same cell might lead to their interaction on various levels, either on the genomic level, or on the protein activity level. One of the possibilities of genomic interactions is gene exchanges between the viruses that infect the same cell, involving viruses of the same, or of different species. Genomic exchanges between viruses can occur between two RNA viruses, between two DNA viruses, or between DNA and RNA viruses. Moreover, viruses can recombine either in vitro or in vivo in cases of intentional or of native dual virus infections. The review will present below the main findings according to that classification.

Molecular recombination between viruses of the same species

Molecular interactions between RNA viruses

The most outstanding example for in vivo recombination of avian RNA viruses, led to the creation of the new avian leukosis virus, subgroup J (ALV-J). ALV-J emerged following a spontaneous recombination between exogenous and endogenous retroviral sequences in a meat-type breeder chicken, and it was revealed in the course of a survey intended to discover novel retroviruses.[6] Very soon after its unveiling, the new virus disseminated worldwide by the extensive international trade of genetic breeds, including the specific genetic material in which ALV-J was created.

Severe economical outcome emerged as a consequence of the massive worldwide poultry infection with ALV-J.[24–26] In the US the prevalence of ALV-J-infected broiler breeder flocks reached up to 87%.[27] The new virus differed from its ALV ancestors in the in vivo avian cell that it infected, myelocytes, instead of B-lymphocytes, in the clinical form of tumors that it caused, tumors on the bone surfaces and not bursal lymphomas, and in the extent of morbidity and mortality that it caused. As a result of the unexpected, vast and delirious economical damage that the new recombinant virus caused, breeding companies initiated massive and very costly genetic selection activities that were aimed to clean the genetic lines from ALV-J infections. The success of the ALV-J eradication program could be credited to the extensive blood-testing that were aimed to keep non-infected birds of the breeding lines of chickens free of ALV-J infection, while the infected birds were excluded from the genetic pedigree.

ALV-J genetic sequence revealed several recombination events that happened between the exogenous ALV gag and pol genes and the env gene of the endogenous avian erythoblastosis virus.[28] In the respect of

viral evolution through genetic recombination, ALV-J represents a viable recombinant that occurred spontaneously, in vivo between exogenous and endogenous avian retroviruses, ALV and AEV, respectively. Analysis of the sequence of an infectious clone of the complete proviral genome indicates that HPRS-103, the U.K. original ALV-J isolate, is a multiple recombinant of at least five avian leukosis virus sequences and one EAV (endogenous avian retroviral) sequence. The HPRS-103 env was most closely related to the env gene of the defective EAV-E51 but divergent from those of other ALSV subgroups. While the LTR, gag and pol genes were highly homologous with other ALV subgroups, the env gene has only 40% identity with other exogenous ALV env genes, but 75%–95% homology with env-like genes of the endogenous avian retroviruses (EAV) family.[5] The sequence comparisons of the US ALV-J isolates revealed that while the subgroup J envelope gene includes some regions that are related to those found in env genes of the A to E subgroups, the majority of the subgroup J gene is composed of sequences either that were more similar to those of a member (E51) of the ancient endogenous avian virus (EAV) family of proviruses or that appear unique to subgroup J viruses. These data led to the suggestion that the ALV-J env gene might have arisen by multiple recombination events between one or more endogenous and exogenous viruses.[29]

A number of other in vivo recombination events between ALVs have been described; an ALV-J encoding an ALV-A envelope,[30,31] an acutely transforming isolate of ALV-J,[32] a recombinant ALV containing the ALV-J sequence uncovered examples of in vivo recombination events between RNA viruses which commonly infect same lymphocytes in the chicken, as lately documented for the Australian breeding flocks which were co-infected with ALV-A and ALV-J.[33]

Molecular interactions between DNA viruses

Multiple viral infections of chickens with DNA viruses are probably the ground on which genetic exchanges between these viruses occur. To our knowledge, two studies documented natural dual infections of chickens, employing FPV[34] and Infectious laryngotracheitis[35] (ILTV) virus[36] and FPV and MDV.[37] These events might facilitate, in a yet unknown mechanism, transfer of genomic fragments between DNA viruses. Although the rate of these DNA movements are supposed to be even lower than events which involve RNA viruses, Brunovskis and Velicer[38] (1995) provided evidences that several FPV genes have homologs in the MDV genome.

Molecular interactions between DNA and RNA viruses

Herpes and retroviruses

In vitro studies

Integration of the retroviral sequences into the herpesvirus genome was documented in vitro by co-infecting CEF cultures with MDV and the retroviruses REV and ALV[39–44] and reviewed by Kawaguchi and Mikami,[45] Brunovskis and Kung[46] and Kung et al.[47] By co-cultivating MDV and REV in the same tissue culture dish Jones et al[43] created the first recombinant virus, RM1, which was characterized both molecularly and biologically as having an altered in vitro replication and in vivo biological properties.[48] The RM1 was named by the initials of its two progenitor viruses, REV and MDV. However, co-cultivation of MDV and one of the retroviruses were not the only mechanism by which retroviruses recombine with MDV; Sakaguchi et al[49] and Endoh et al[21] reported retroviral long terminal repeat (LTR) integrations into MDV not as a result of co-cultivation of both viruses, but instead, as a result of culture maintenance or the presence of avian endogenous viruses in the host cells.

The retrovirus recombination process with MDV occurs because retroviruses integrate into any double stranded (ds) DNA for replication, and in a MDV-infected cell, the integration can occur into the cellular or the dsMDV, or other DNA virus genome. The documented inserts of avian retroviral sequences, were mainly the LTR, and those were gathered at the junctions between the unique (long or short) MDV fragments and the terminal or internal repeated MDV fragments (TR_L and TR_S and IR_L and IR_S) (reviewed by Brunovskis and Kung).[46]

In vivo studies: The complexity of in vivo systems versus in vitro systems

Having experienced the relatively efficient creation of recombinant viruses in vitro, we questioned at the Kimron Veterinary Institute, Bet Dagan, Israel,

whether retrovirus integrates into DNA viruses also in vivo, in the bird, in multiple viral infections. If such process would occur, serious consequences might follow; recombinant MDV might possess altered biological properties, and relatively known features of these viruses will turn into unknown and unpredictable patterns. Putative features, whose changes might be of biological significance are, pathogenicity, virus spread, antigenicity and immunogenicity leading to changes in the ability of specific vaccines to protect against diseases.

Our below detailed studies exposed for the first time, that molecular recombination events between two viruses occur also in vivo, in natural infections, in multiple virus-infected birds; retroviruses can integrate into MDV and form recombinant viruses, which might differ biologically from their ancestors. That finding might reflect on general virology, as herpes and retroviruses co-exist in many animals, including humans, where no trials are feasible, while poultry are a natural big laboratory, where low-rate events can be depicted. Avian tumors reflect virology without any alterations, which might be imposed by laboratory conditions.

Our studies were original as they attempted to avoid artifactual creation of recombinant viruses that might occur in the course of virus replication in systems that differ from their natural host, i.e. in vitro. Accordingly, we analysed the in vivo integration events within the original organs, and not in viruses that were re-cultured in vitro, in order not include artifacts created by virus replication in vitro within cell cultures. Frequent genetic changes occur upon in vitro virus-replication processes, as evidenced by Robinson and Gagnon,[50] showing that most of the retroviral genome, except the LTR, was excluded from the cellular chromosome.

As Jones et al[43] demonstrated that the retroviral LTR of the experimentally-created recombinant virus RM1 undergo duplication during replication in cell cultures, we strictly avoided further replication of the viruses which contained chimeric molecules in cell cultures, process which might have increased the amounts of the recombinant viruses.[22,51–53] For that reason, several experimental difficulties were met compared to the studies performed in tissue cultures. However, in spite of all difficulties, we showed that retroviruses could integrate into the MDV genome as exemplified by the detection of chimeric molecules, directly within the DNA that was purified from the tumor-bearing chicken.[22,53]

Unlike in vitro, where recombinant viruses were separated by several rounds of plaque purifications and limiting dilutions, the in vivo situation differs; many different events occurred simultaneously in the same bird as each cell produces many herpes virions. As various molecules were formed and were detected by us in the same DNA preparation, recombinant virus isolation was problematic. Only a biological advantage would enable a recombinant virus to dominate in an infected bird.

By comparing the in vivo situation with previous methods employed to study in vitro created recombinant viruses, we realized that these were not adequate for the study of samples taken directly from the bird. As we aimed to reflect the true in vivo status of the viruses, without further molecular rearrangements, to detect the chimeric molecules that were created by the two viruses, the Hot Spot-Combined PCR (HS-cPCR)[54] was developed to amplify recombinant molecules that were present in the clinical sample. The development was based on the molecularly known RM1 virus.

Although the pulsed field gel electrophoresis, that was used for tissue cultured-MDV separation was inefficient for separating MDV from organs, it was useful with feather tips as a source of the original MDV that was infecting the bird.[22,55] Much attention was dedicated to feathers, because if a recombinant virus would be formed in vivo, its biological significance would be evident by horizontal dissemination through the feathers.[10,11,56] Major findings were: a) not only in vitro, but also in vivo MDV and retrovirus co-infections lead to LTR integrations into MDV, shown by the detection of chimeric molecules. These appeared in low quantities and as quasispecies, thus interfering with sequence analysis of cloned gel-purified chimeric molecules.

In addition, the in vivo herpes-retro recombination issue differs and is rather more complex than in vitro co-cultivation of the two virus types; the cells in the in vitro co-infection were fibroblasts, whereas in vivo, the target cells are mostly lymphocytes and monocytes. While the recombination rate in vitro was rather high, the in vivo formation of viable recombinant viruses was lower, depending on different factors, such as the presence of immune responses of the host

and tissue affinities. We concluded therefore, that both situations cannot be extrapolated, however, showing that commercial poultry co-infections, not only have the potential for a collective clinical influence, but also can result in the emergence of recombinant viruses, possibly with unexpected biological properties.

In vivo studies—The systems that were studied and main findings

The issue of retroviral sequence integration into herpesviruses in vivo, in cases of double virus-infection is of a wide significance in general virology and veterinary medicine and also represents a special case of gene transposition. We aimed to determine occurrence of such integrations in vivo by following the presence of chimeric molecules. Several conditions were analysed:

a) Commercial birds that acquired naturally a mixed infection;
b) Experimentally infected chickens with prototype strains of MDV and ALV-J;
c) Commercial chickens infected experimentally with virus inoculae obtained from commercial cases of double infection with MDV and ALV-J, in the same flock or the same bird.

In the two first categories we found that integration events happened at various rates, depending on the experimental system used. While in commercial flocks the event was limited (about 2.5% of the 2926 DNA samples),[22] it reached a 30%–50% rate in experimentally-infected birds with prototype viruses, and was undetectable in experimentally-infected birds with that were inoculated with a field isolate. It seemed that by increasing the virus adaptation to laboratory conditions, the rate of retrovirus LTR integration into MDV increases, as judged by the extent of chimeric molecules.

By the HS-cPCR assay we revealed that about 2.5% of the total blood and tumor tissue DNAs, prepared from birds with a double MDV and retrovirus infection, contained chimeric molecules. Actually, that rate might be even higher, as the HS-cPCR method was selective for relatively short chimeric molecules, as based on MDV genetic locations that are proximal to the MDV primer sites and were shown in vitro to serve as the hot spots for LTR integration.[46]

For the first time, it was demonstrated that not only in vitro, but also in vivo, co-infections with MDV and each of the three avian retroviruses (REV, ALV and ALV-J) lead to the process of retroviral LTR integration into MDV.[22] Unlike demonstrated with the in vitro created recombinant virus RM1, we were not able to determine whether viable viruses were formed, but demonstrated the presence of a variety of chimeric molecules that tracked the integration events that happened in the birds. The amplification of the nested REV- and the ALV-LTR fragments from the chimeric molecule amplified DNA validated that a large fragment of LTR was inserted into MDV. That finding also supported the notion that these integrations were recent and might be a direct consequence of the bird co-infection with the two viruses. These inserts were not only the traces of ancestral LTR integration into MDV, as shown earlier by Isfort et al[40] to be represented by the presence of short (20 bp) LTR stretches with a 70% homology or more into various MDV strains, but most probably they illustrated recent recombination events that occurred in the mixed-infected bird.

Also, in each DNA preparation a variety of chimeric molecule types were detected, indicating the in vivo formation of molecular quasispecies in dually-infected birds. The chimeric molecule heterogeneity found now might indicate that several integrations occurred in one double virus-infected cell or reflects the events in several cells, as each DNA preparation originated from numerous cells. As such, each DNA sample amplified by HS-cPCR might differ in the molecular population content. The diversity of viral quasispecies in a host might result also from the natural and vaccination selective pressures.[57]

A further implication of this study concerned recombination events between MDV, or avirulent MDV vaccine strains and endogenous retroviruses. About 5%–7% of the mammalian and human genome was found to be comprised of endogenous retroviruses.[58,59] A similar feature was reported for many endogenous retroviruses residing in the chicken genome,[60] and these are also prone to shuffle their LTR into the MDV genome.

Recently Cui et al[61,70] demonstrated the spontaneous creation of MDV and retroviruses in Chinese commercial flocks. These viruses have been disseminated to commercial chicken flocks, where enhanced pathogenicity was observed. The recombinant viruses were collected and re-isolated in tissue cultures and

characterized molecularly to contain retroviral LTR inserts of 540 bp, and to cause an apparently more severe thymus and bursa of Fabricius atrophy than expected.

Pox and retroviruses

Fowlpox virus (FPV) is worldwide distributed in poultry and wild birds[34] and consists an additional natural example of recombination between viruses. Reticuloendotheliosis virus (REV) fragments were demonstrated in all poultry FPVs and implicated in virulence alteration. REV integration occurred also in FPV vaccine strains, leading to their immediate elimination from use.[62] The linkage between FPV and REV was evident by the wide prevalence of REV antibodies in conjunction with FPV lesions, as the REV envelope gene functions as the immunodominant protein and is responsible for elicitation of REV antibodies.

Molecular descriptions of FPV isolates with REV genomic insertions were accumulated from various parts of the world.[62–65] These studies demonstrated that REV-inserted fragments into wild type field FPV isolates were variable in content, although they included the REV long terminal repeats (LTR) of various lengths, and also additional sections of the proviral genome. Although the REV genomic integrations into the FPV genome has been demonstrated only in the last 10 years, the analysis of previous field isolates indicated that such events occurred even 50 years ago.[64]

The analysis of the REV integration site in wild type field FPV isolates and in FPV vaccine strains revealed that all events occurred in a hot spot of the FPV genome, located between the FPV ORF 201 and FPV ORF 203.[63,65] The inserted REV fragment into the FPV differed in composition between wild type FWPV and FWPV vaccine strains. While all the field isolates contained complete REV provirus or various fragments of the 3' and 5' REV-LTRs, in addition to other REV fragments, including the REV env gene, recent FPV vaccine strains included only the REV LTR.[63,65–67]

Recently, we questioned the universality of the phenomena and analysed 128 poultry flocks and birds collected during the last 10 years.[68] Various fragments of both viruses were amplified and sequenced at the FPV integration site, located between FWPV open reading frames 201 and 203. Seven isolates were found to contain no REV insertion, including fragments of the REV env, gag and 5' REV-LTR. We demonstrate the existence of poultry FPVs without REV inserts for the first time, showing only remnant REV-LTR and no REV envelope gene fragments in the FPV genome. In most other FPV isolates the REV inserts were heterogeneous in sizes, indicating the occurrence of various recombination events that led to the creation of the present prevalent FPV isolates.

Summary

Chickens provide a unique study model as they can be utilized experimentally, but also possess the advantage that they are grown as commercial chicken flocks, where viruses cause natural infections in the natural host. Moreover, the stress and environment conditions are natural and not artificial. Also, the population of study is large, thus allowing the study of rare events. Studies on avian oncogenic viruses, but also on other viral diseases, like circovirus infection,[69] can be used to better understand viral infections in human with viruses of similar families. Moreover, as the multiple virus infections in poultries can be dissected and studied experimentally, situation that is not feasible in humans, much knowledge from poultry systems can serve to expand consciousness in human virology. For example, as a consequence of a multiple viral infection of poultry with several oncogenic viruses, molecular recombination between DNA and retroviruses might lead to the emergence of new MDVs with altered properties, including tropism, spreading pattern, pathogenicity and protection by vaccination.

Moreover, for human tumors, studies on avian tumor viruses could provide an animal model to understand the consequences of dual infections in human. Since most human population carry ubiquitous infections with the 8 human herpesviruses (HSV1, HSV2, VZV, CMV, EBV, HHV6, HHV7 and HHV8 and some, are dually infected with retroviruses, like the human immunodeficiency virus (HIV), multiple virus infections can occur in the same individual. One example of co-infection with a pathogenic consequence in humans is the Kaposi sarcoma, where both HHV8 and HIV were implicated in the development of the disease. Also, HHV6 and HIV were proposed as co-factors for the development of the acquired immunodeficiency syndrome in HIV carries. In other study HHV6 and

HHV7 were implicated as major opportunistic viral infections in AIDS patients. By extrapolation of human situations to the avian oncogenic viruses, similar events might happen also in humans, where as a result of the infection of a person with retroviruses, like HIV, and asymptomatic herpesviruses, a recombinant herpesvirus might emerge, which might disseminate HIV genomic information horizontally.

Disclosure

The authour reports no conflicts of interest.

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