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Original article

EFFECTS OF CHICKEN ANAEMIA VIRUS ON EXPERIMENTAL LEUKOSIS, INDUCED BY AVIAN MYELOCYTOMATOSIS VIRUS MC29

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Summary

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The effects of concomitant infection with chicken anaemia virus (CAV) on the incidence, clinical manifestation and mortality from leukosis, induced by the avian myelocytomatosis virus strain Mc29 were studied. Experimental one-day-old 15 I line White Leghorn chickens were inoculated simultaneously with Mc29 and CAV or with Mc29 alone and observed daily for clinical signs and mortality. Both groups of chickens inoculated with Mc29 virus strain alone or in combination with CAV developed tumours and died within 57 days. Necropsy has been performed on all dead birds following the standard protocol. Organ samples from thymuses, spleens, bone marrow, and livers were collected and histopathologically investigated. Neoplasms detected included myelocytomas, nephroblastomas and hepatocellular carcinomas. In addition, 50% of the CAV/Mc29-inoculated chickens developed epithelial type thymomas. However, no such lesions were found in chickens infected with Mc29 alone. No significant differences in the clinical course of leukosis between the two experimental groups of chickens were observed. The results indicated that CAV infection did not affect substantially the incidence and mortality from avian leukosis, induced by myelocytomatosis virus strain Mc29, but contributed to greater variety of the induced tumours.

Key words: chicken anaemia virus, co-infection, Mc29 virus strain, myelocytomatosis

INTRODUCTION

The chicken anaemia virus (CAV) currently belongs to the *Gyrovirus* genus, *Anelloviridae* family (Rosario *et al.,* 2017) and causes a disease characterised by depression, aplastic anaemia, subcutaneous and muscular haemorrhages, and increased mortality of susceptible chickens up to 3 weeks of age (Schat, 2003). In

older chickens the infection is mainly subclinical. The virus destroys the pluripotent haematopoietic progenitor cells in the bone marrow and lymphocyte precursors in the thymus. As a result, severe depletion of the helper (predominantly CD4+) T-cell and cytotoxic (CD8+) T-cell populations, and impairment of cytokine production with consequent immunosuppressive effects occurs (Adair, 2000, Ragland *et al.*, 2002; Markowski-Grimsrud & Schat, 2003). The detrimental effects of the concurrent CAV infection with different viruses, e.g. Marek's disease virus, infectious bursal disease virus, infectious bronchitis virus, reoviruses, Newcastle disease virus, reticuloendotheliosis virus, and adenoviruses have been demonstrated (Todd, 2000). However, the interference, if any, between CAV and avian leukosis viruses (ALVs) has not been investigated. The former represent a distinct group of alpharetroviruses classified into 6 subgroups (A to E and J) according to the properties of viral envelope glycoprotein. These viruses are known to induce a variety of non-neoplastic disorders and neoplastic diseases in affected flocks (Payne, 1998).

Although many neoplasms utilise mechanisms leading to suppression and subsequent escape from eradication by the immune system of the host, the latter is an integral part of tumour biology and is determined by the ability of immune system to recognize and respond to tumours (Dunn *et al.,* 2004**)**. It is, therefore, reasonable to anticipate that the immunosuppressive viruses, including CAV, having the potential to destroy cells with tumoricidal activity or affect lymphokine production, might influence the frequency, clinical manifestations and mortality from avian retroviruses-induced leukoses.

Tumour growth, a common consequence of the avian retrovirus infections depends on the balance between two opposing processes, namely cell proliferation, and tumor cells death, realised mainly by apoptosis. Jeurissen *et al.* (1992) provided evidence that CAV exerts its cytopathogenic effect in hematopoietic and lymphoid progenitor cells via apoptosis. This process has been later ascertained to be mediated by the non-structural viral protein VP3 (Apoptin) (Noteborn *et al.,* 1994).

In a series of experiments it has been found that *in vitro* Apoptin induces apoptosis selectively in malignant or transformed cell lines of animal or human origin by interacting with specific set of factors, characteristic for or being produced by the transformed cells (Zhou *et al.,* 2012). The anti-neoplastic effects of Apoptin have been studied *in vitro* after transfection of Apoptin expressing vectors in neoplastic cells and *in vivo* by intratumour delivery of VP3 gene (Natesan *et al.,* 2006; Lian *et al.,* 2007).

However, the delivery of Apoptin into tumour cells via natural CAV infection has not been investigated to date. Thus, presuming both immunosuppressive and anti-tumour properties of CAV and its strong specificity for bone marrow cells and lymphoid tissues, which are the main targets for retrovirus-induced transformation, we designed this study to examine whether a simultaneous infection with CAV could modify the frequency of tumour development, the pattern of the induced tumours, the clinical course and the survival rate of animals with experimental leukosis, induced by the Mc29 strain of avian myelocytomatosis virus.

MATERIALS AND METHODS

Cells and viruses

The Del Rose strain of CAV (Rosenberger & Cloud, 1989) at 12th *in vitro* passage, generously provided by W. Ragland (Ruđer Bošković Institute, Croatia), was used in the experiment. The virus was propagated in MDCC MSB1 cells, grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (Bio Witthacker) and antibiotics in usual concentrations, and cultivated at 38 ^{o}C in a CO_2 incubator. An inoculum of $0.2\times10^{6.5}$ $TCID_{50}$/mL was used to infect the chickens.

MC29 avian myelocytomatosis virus is an acute leukaemia virus and the prototype of the MC29-subgroup of the avian leukosis-sarcoma retroviruses (Stoye *et al.,* 2012). The Mc29 virus-producing cell line LSCC-SF (Mc29) (Alexandrova, 2009) established from a transplantable chicken hepatoma was used as a source of virus for the experiments. The cells were cultured in Dulbecco's minimal essential medium (DMEM), supplemented with 10% FBS and antibiotics – penicillin (100 UI/mL) and streptomycin (100 µg/mL). For induction of tumour growth, cell-free supernatants from 48-hour LSCC-SF (Mc29) cell cultures were used to inoculate susceptible 15 I line White Leghorn chickens. The Mc29 stock contained $10^{3.5}$ to 10^{4} colony-forming units per mililiter (CFU_{50}/mL), as determined after titration in primary chicken embryo fibroblasts (CEFs) cultures.

Chickens and experimental design

Thirty five one-day-old chickens were used in the experiments. They originated from a parent flock of 15 I White Leghorn line, considered free of CAV infection based on a negative results from a serological testing by Chicken Anemia Virus Antibody Test Kit (IDEXX Laboratories, USA). Birds from this line were used because of their high susceptibility to ALVs (Filchev *et al.,* 1993). The experimental chickens were separated into three groups of 10 birds each and one group of 5 birds and placed in isolation units. The first group of chickens was inoculated intramuscularly (i.m.) with $0.2\times10^{6.5}$ $TCID_{50}$ of Del Rose strain of CAV. Birds of the second group received intravenous (*i.v.*) injections of 0.1 mL tissue culture fluid from LSCC-SF (Mc29) cells, while the chickens from the third group were simultaneously inoculated with Del Rose and Mc29 by *i.m.* and *i.v.* route, respectively. The birds from the fourth group (5 chickens) were kept as uninoculated controls. All experimental groups were observed daily for clinical signs of disease and the day of death, and the number of deaths was recorded. Fourteen days post inoculation (d.p.i.) blood was collected from all experimental birds and haematocrit (packed cell volume) values were determined by centrifugation in heparinised capillary tubes. Three birds from the control uninoculated group were euthanised on 32nd, 38th and 40th d.p.i., respectively. The last two birds were sacrificed on day 57 from the beginning of the experiment. All samples from this group of birds were tested in parallel with samples from virus inoculated chickens. The experiment was performed in line with biosafety, animal welfare and ethical rules applicable in the EU.

Histopathology

Samples of thymus, spleen, liver, bone marrow and kidneys were collected for routine histopathological examination. The tissue specimens were dehydrated and embedded in paraffin according to the

standard histopathology protocol, sectioned (4 μm thick) and routinely stained with haematoxylin and eosin (HE).

DNA extraction and PCR

To document the presence of CAV DNA at the day of death, thymuses from one chicken from a group 1 (CAV), dead on the 24th d.p.i., nine chickens from group 2 (Mc29), six chickens from group 3 (CAV/Mc29) and four chickens from the control group were collected and assayed by PCR. Organs were collected using individual sterile sets of instruments for each bird. Thymuses from control birds, as well as those infected only with Mc29 were PCR assayed as a pool in order to ensure that they have not been inadvertently exposed to CAV. DNA was extracted using Animal and Fungi DNA preparation Kit (Jena Bioscience) according to manufacturer's instructions. All extracted DNA samples were tested with a PCR assay using primers targeting a fragment of 583 bp (positions 485–1067) of the published genome of the reference strain Cuxhaven-1 of CAV (Tham & Stanislawek, 1992). The amplification and visualisation of the products were performed as described elsewhere (Simeonov *et al.,* 2014), with the exception that the reaction volume was 25 μL.

Statistical analysis

Descriptive statistics were used to determine minimum, maximum, mean, median, standard deviation values of the studied parameters and the non-parametric Mann-Whitney U-test to determine significant differences of the Hct values in the groups (due to the small and different number of the birds tested in the groups).

RESULTS

Clinical signs and mortality

Four birds from group 1 and six birds from group 3 developed clinical manifestations, including moderate to severe depression, ruffled feathers and reduced appetite, consistent with CAV infection between the 11th and 18th d.p.i. Two of them, one bird from a group 1 and one from group 3 got worse and died at the 19th and the 24th d.p.i., respectively. All other birds apparently become stable in the next 5–7 days. Despite of the lack of clinical manifestations in the rest of the chickens, at the day 14 after inoculation the mean haematocrit values of group 1 (CAV) and group 3 (CAV + Mc29) infected birds were significantly lower, compared with group 2 (P=0.00073) and control group (P=0.00466, P= 0.00133), respectively. Neither clinical signs, nor mortality were found at this time in the group 2 (Mc29-infected) and group 4 (uninoculated controls). The first Mc29-related signs of illness were observed 28 d.p.i. in a bird from group 3. From the 30th d.p.i. all nine birds from this experimental group started to show clinical symptoms (depression and anorexia accompanied in most cases by hemiparesis) which quickly increased in severity and all birds died until the 42nd d.p.i. (mean survival time =35.22±4.52 days, min-max range 30–42 days). In group 2 (Mc29), nine of 10 chickens showed identical clinical signs and died between 32nd and 41st d.p.i., whilst one bird became ill and died on the 57th d.p.i. (mean survival time 38.6±6.98 days, min 31-max 57 days). Neither significant difference in the mortality rates (100% in both groups), nor in the clinical manifestation of the disease were observed between co-infected chickens of group 3 and these from group 2.

Table 1. Haematocrit values, incidence of tumour development and organ distribution in chickens experimentally infected with CAV, Mc29/CAV, Mc29, and uninoculated controls.

	Experimental groups			
	Group 1 (CAV)	Group 2 (Mc29)	Group 3 (CAV/Mc29)	Group 4 (Control)
Number of chickens	10 (9)[a]	10	10 (9)[a]	5
Haematocrit [L/L]	0.266±0.054 **, □□	0.354±0.026	0.271±0.046 **, □□	0.374±0.023
Tumours in:				
kidney	0/9	10/10	9/9	0/5
spleen	0/9	0/10	0/9	0/5
liver	0/9	3 /10 [b]	3 /9[b]	0/5
thymus	0/9	0/10	5/9	0/5

[a]The birds having died due to the CAV-related anaemia are excluded; [b] Detected at histopathological observation;** significant difference from control group ($P<0.01$); □□ significant difference from group 2 ($P<0.001$).

Gross pathology

Macroscopic lesions, including pale bone marrow, atrophy of the thymus and paleness of the carcasses, were present in the two birds from group 1 and 3, that died early in the experiment on 19th and 24th d.p.i., respectively. The results from gross pathology observations of all experimental birds are summarised in Table 1.

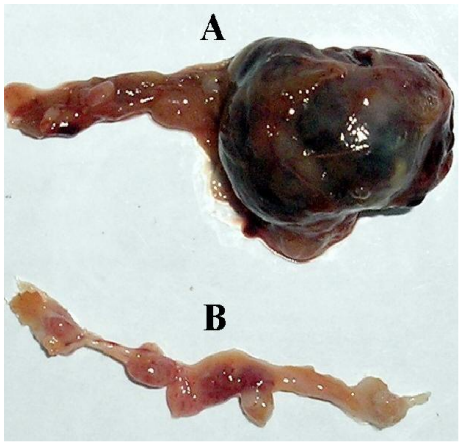

Fig. 1. Tumour formation in the thymus of CAV/Mc29 inoculated chicken (A); control (B).

At postmortem examination, all birds were in poor body condition with atrophic muscles and in most cases with anaemic appearance. Strikingly enlarged kidneys, showing yellowish-grey lobulated masses of different size were the most pronounced findings in all necropsied birds. Along with the tumours in kidneys, five birds from group 3 showed significantly enlarged thymus lobes with oedematous and haemorrhagic appearance (Fig. 1). No tumour nodules on livers were observed; however, in some birds these appeared yellowish and slightly enlarged.

Histopathological findings

Moderate lymphocyte depletion in the thymic cortical zone and marked to complete bone marrow aplasia with fully depletion of the erythrocytic and granulocytic series were observed in the birds from groups 1 and 3, that died on 19th and 24th d.p.i, respectively. Histological examination of kidneys of the birds inoculated with either Mc29 or with CAV/Mc29, died at the 30th d.p.i. and thereafter revealed the presence of nephroblastomas. Although no solid tu-

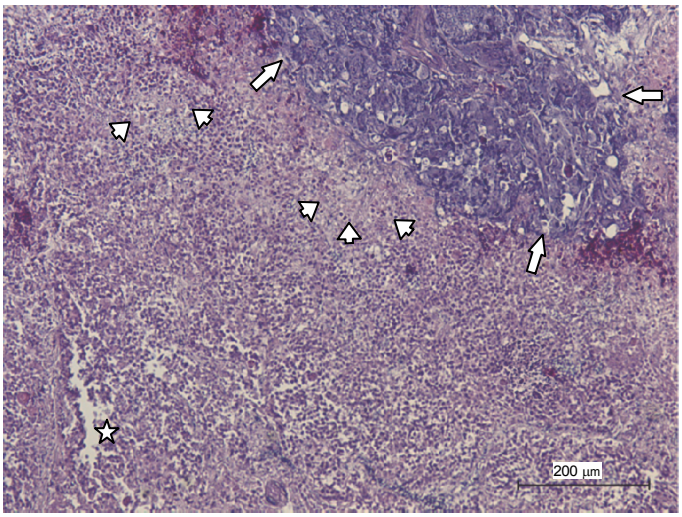

Fig. 2. Histopathological picture of the thymus of CAV/Mc29 inoculated chicken, developing tumour, 36 days post inoculation. A nodule of undifferentiated tumour cells with epithelial-like morphology and increased mitotic activity are noticed (arrow); depletion of lymphocytes replaced by stromal cells (arrowheads) and development of pseudo-ductal structures (star) and haemorrhages. H&E staining, scale bar 200 μm.

mour formations in the livers of all birds were observed, more precise histopathological investigation in some birds revealed carcinomatous neoplastic growth around blood vessels. Areas, depopulated from haemocytoblasts and accumulations of undifferentiated myelocytes were regularly seen in the bone marrow of chickens from group 3. A serious disorganisation of the thymic compartments with neoplastic growths of poorly differentiated epithelial-like cells, haemorrhages and necroses (type C thymoma) were observed in the thymuses of birds from the same group, which developed tumours (Fig. 2).

At the time of death PCR identified the presence of CAV DNA in the thymuses of five of the tested 6 chickens from group 3. In contrast, the pooled thymus samples of all chickens from group 2, as well as these from control group resulted PCR negative (Fig. 3).

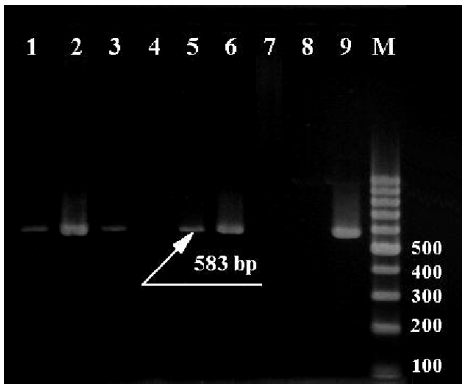

Fig. 3. Detection by PCR of CAV DNA in thymuses of experimentally infected birds. Lanes 1–6: group 3 chickens (CAV/Mc29); lane 7: group 2 chickens (Mc29) (pooled sample); lane 8: control non-infected chickens (pooled sample); lane 9: group 1 chicken (CAV infected); lane M: DNA ladder 100–1000 bp.

DISCUSSION

The present study was undertaken to investigate the effect of concurrent infection with CAV on the course of avian leukosis, induced by the myelocytomatosis virus Mc29. As criteria for such influence the differences in the incidence rates, clinical manifestations and mean time of death, gross pathology and histopathological changes between birds inoculated with both viruses compared with those inoculated only with Mc29 were accepted.

Our experiments did not demonstrate a direct effect of CAV on the incidence rate, mortality and the mean time of death in chickens in response to inoculation with Mc29. In fact, all birds, whenever inoculated with Mc29 alone, or in combination with CAV became diseased and died within 57 days. Incidence rates that have been described for experimental infection with Ms29 ranged from 48% to 73.7% (Nedyalkov, 1967), and have varied significantly depending on various factors including age of infection, way of inoculation and genetic composition of the breed (Nedyalkov, 1967; Mladenov *et al.,* 1980; Filchev *et al.,* 1993). The high percentage of diseased birds in our experiments could reflect the high susceptibility of the chicken strain 15I to Mc29, the intravenous way of inoculation and the fact that in our clinical setting we used one-day old chickens, proved to be most susceptible to infection with Mc29 than the older ones.

It is known that *in vivo* CAV replicates actively between the 7^{th} and 14^{th} d.p.i. when abundance of CAV antigen in the thymus and bone marrow of experimentally infected chickens could be demonstrated immunocytochemically (Smyth *et al.,* 2006). The serum antibodies generated at this period reach levels able to block active viral replication and immunohistochemical examination of the target organs after the 21 d.p.i. appears negative (Kuscu & Gurel, 2008). Conversely, although Mc29 is an acutely transforming virus, related clinical signs were not observed until 28^{th} d.p.i. These facts suggest that intense tumour growth started after the decline of the active CAV replication, so from pathogenetical point of view both infections occur at different times. This could be one of the reasons for the lack of CAV replication in cells transformed by the retrovirus. Another explanation could be the inability of Mc29-transformed cells to support replication of CAV. Mladenov *et al.* (1967) and Beug *et al.* (1979) showed that Mc29 transformed cells acquire macrophage-like properties, typical for the cells at relatively high level of maturation and only weakly express myeloblast cell surface antigen. In contrast, CAV replicates predominantly in less differentiated stem cells, or in actively dividing blast cells. Thus, the lack of suitable "substrate" of permissive cells could be another explanation for the lack of demonstrative effect of CAV infection on the course of Mc29-induced leukosis.

Mc29 has a broad oncogenic spectrum and in addition to the predominant myelocytomas has the potential to induce various types of carcinomas and sarcomas (Mladenov *et al.,* 1967; Beard *et al.,* 1975; 1976). Histopathological examination identified such neoplastic processes in the viscera (kidney and liver) both in the birds inoculated only with Mc29 and in these receiving both viruses. However, there were no indications of CAV replication in cells from these tumours (lack of apoptotic cells). This confirms the strict affinity of CAV to the lympho- and myeloblastoid cells and the inability of virus to replicate in epithelial cells, irrespective of their neoplastic transformation.

The most striking finding of this study was the larger number of dually infected birds that developed tumours in the thymus, compared to chickens infected only with Mc29. Along with the tumour lesions in other organs, apparently enlarged thymus lobes were observed in 50% of co-infected chickens. Similar data have shown that in myelocytomatosis induced by Mc29 the thymus was not, or was rarely involved (Nedyalkov, 1967; Mladenov *et al.,* 1980; Filchev *et al.,* 1993). In similar experiments, using the same source of Mc29 [LSCC-SF(Mc29)], Alexandrova (2009) observed thymus nodules in only 5.67% of inoculated birds. Interestingly, involvement of the thymus in neoplastic process at a higher degree has been described by the same author when experimental chickens were transplanted with Mc29-induced hepatoma cells of different origin [LSCC-Pr (Mc29)].

Our results, although based on a limited number of experimental birds suggest that CAV infection, even partially, could predispose Mc29 retrovirus inoculated chickens to develop tumours in thymus. The reasons and the mechanism by which CAV stimulates tumour development are not clear. Most probably this is due to the destruction of the local immune mechanisms and to the T cells depletion, induced by CAV. The resulting dysregulation in the production of some of cytokines, including interferon (IFN)-γ, IL-2 or tumour necrosis factor which is generally seen after 14^{th} d.p.i. (Adair, 2000; Ragland *et al.,* 2002) could also contribute to the explanation of this fact. We did not found essential difference in the mean survival time between birds that developed tumours in the thymus and those that didn't, hence, the primary cause of deaths appeared to be tumours developed in other locations.

Although, concerning the percentage of reacted birds and the mean survival time, significant differences between Mc29 and CAV/Mc29 inoculated groups were not found, our results suggest that infection with CAV probably affects the neoplastic process, at least modifying the topography of tumour development. The phenomenon observed by us applies only to the CAV-Mc29 model and assuming the different oncogenic potential of the avian retroviruses, it could not be extrapolated to other members of the leukosis-sarcoma complex. Thus, additional studies with other retroviruses, especially those causing predominantly erythroblastosis and lymphoid leucosis are necessary to determine the effects of co-infections with CAV.

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