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► **To cite this version:**

Dana Průková, Zdeòka Vernerová, Tom Pílek, Volodymir Stepanets, Marie Indrová, et al.. Differences in pathogenicity among ALV strains of the same or different subgroup. *Avian Pathology*, 2007, 36 (01), pp.15-27. 10.1080/03079450601102921 . hal-00540061

HAL Id: hal-00540061

<https://hal.science/hal-00540061>

Submitted on 26 Nov 2010

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Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2006-0076.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	21-Sep-2006
Complete List of Authors:	Průková, Dana; Institute of Molecular Genetics, Viral and Cellular Genetics Vernerová, Zdeňka; Third Faculty of Medicine, Charles University, Department of Pathology Pilek, Tomáš; Institute of Molecular Genetics, Molecular and Cellular Immunology Stepanets, Volodymir; Institute of Molecular Genetics, Viral and Cellular Genetics Indrová, Marie; Institute of Molecular Genetics, Tumour Immunology Geryk, Josef; Institute of Molecular Genetics, Viral and Cellular Genetics Plachý, Jiří; Institute of Molecular Genetics, Viral and Cellular Genetics Hejnar, Jiří; Institute of Molecular Genetics, Viral and Cellular Genetics Svoboda, Jan; Institute of Molecular Genetics, Viral and Cellular Genetics
Keywords:	Avian leukosis virus (ALV), wasting disease, course of wasting disease, immunopathology of tissues from infected animals

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Cavp-2006-0076

Differences in pathogenicity among strains of the same or different of avian leukosis virus subgroups

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A short title: ALV-induced wasting disease in chickens

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Differences in pathogenicity among strains of the same or different of avian leukosis virus subgroups

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Abstract.

An efficient induction of the wasting disease in chickens by avian leukosis virus (ALV), particularly ALV subgroup C, requires $>10^2$ IU of virus inoculated in mid embryogenesis. The most conspicuous symptoms of the disease were induced by ALV subgroup C; however, significant differences in the occurrence of wasting disease were found among individual members of this subgroup. Almost comparable pathogenicity was exhibited by ALV subgroup D, whereas viruses of subgroups B and A proved to be moderately and almost non-pathogenic, respectively. Using antibodies to cellular antigens, tissue alterations were shown clearly in ALV-C-infected chickens. An essential feature was depletion of lymphocytes in the thymus, bursa and spleen. While the number of dendritic cells (DC) in the bursa was increased, their representation in the thymus and spleen was reduced. In the spleen, however, the reduction of DC concerned only an ellipsoid compartment, which in itself was also markedly reduced. An increased number of macrophages in the thymus and spleen corresponded with the observed general activation of the monocyte-macrophage system. In the spleen $CD4^+$ T-cells were reduced while $CD8^+$ T-cells were increased. In agreement with this finding was a failure of chickens to respond to *Brucella* antigen and inability of their splenocytes to respond to ConA, both of which pointed to the damage of immune reactivity.

Variation in the pathogenicity among individual ALV strains provides ground for depicting gene sequences playing an important role in ALV acute pathogenicity.

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Introduction.

Avian leukosis viruses (ALV) are the most common naturally occurring avian retroviruses associated with neoplasia and adverse effects on immunity in domestic chickens. They represent a distinct group of simple alpharetroviruses that are classified into ten subgroups (A to J) according to the properties of viral envelope glycoprotein underlying their immunological cross-reactivity, viral cross-interference and host range (Hunter, 1997). Some of them carry a viral oncogene responsible for rapid malignant conversion of target cells (acutely transforming viruses). Slowly transforming viruses, which do not possess an oncogene, are able to induce a broad spectrum of tumours after a long latency by a mechanism of insertional mutagenesis based on the provirus integration into the genome near a cellular protooncogene (Payne, 1992).

In the acute phase of infection, particularly in ALV-congenitally infected chickens, a non-neoplastic syndrome, the wasting disease, with the latency of 2–3 weeks after hatching with an incidence approaching nearly 100%, is induced. The main clinical sign of the wasting syndrome is progressive weight loss, which is accompanied by metabolic and hormonal disorders, depletion of lymphatic organs and immunosuppression, and in some cases also by osteopetrosis, anaemia and occasionally diarrhoea (Meyers et al., 1976; Smith and Eldic, 1978; Price and Smith, 1982; Rupp et al., 1982; Fadly et al., 1982; Carter and Smith, 1983; Carter et al., 1983; Carter and Smith, 1984; Landmann et al., 2002; reviewed in Svoboda et al., 2003). Carter and Smith (1984) pointed out a specific pathogenic effect of subgroup C viruses, characterized by stunting, hypothyroidism, hyperinsulinemia, alteration of lipid metabolism and lymphoblastoid infiltration of liver, thyroid and pancreas. However, a

detailed comparative study of pathomorphological changes in lymphoid tissues induced by ALVs of different subgroup specificity, has not been performed.

Under natural conditions ALVs spread in the flock by three modes: (1) horizontal transmission, when the virus is passed from bird to bird by direct contact or indirectly, (2) vertical or congenital transmission, when the virus is passed from hen to offspring by the egg albumen, and (3) genetic transmission, which refers to endogenous viral genome transmission from parents to offspring. The mode of transmission has a strong impact upon the pattern of infection: depending on the presence of viraemia, antibodies, or both, infected birds can be classified into four serological classes: (1) viraemia, no antibody, (2) no viraemia, with antibody, (3) viraemia with antibody, (4) no viraemia, no antibody (reviewed in Payne, 1992a). Recently, a novel infection profile was identified in ALV-J-infected broiler breeder flocks, which includes hens shedding viral antigens into the cloaca intermittently (Witter et al., 2000). The most prevalent in the field are exogenous viruses of subgroup A, to a lesser extent viruses of subgroup B, and the occurrence of subgroup C and D viruses was reported only rarely. However, epidemic outbreaks of newly generated viral recombinants occurred at the present time, causing significant economical losses (Payne, 1992b). Based on the knowledge of ALV biology and host response to the infection, eradication programs were developed for viruses of subgroups A and B and are successfully applied to commercial and experimental breeding flocks. For original eradication programs criteria proposed by Payne (1992a) were employed. They include selection of hens negative for viral antigens in the egg albumen and vaginal swabs, hatching of chicks in small groups, and testing for the presence of ALV in blood followed by breeding of ALV-free chicks in isolation. Additional approaches for selection of resistant chicken lines such as use of resistance to ALV, a recessive genetic trait, and PCR techniques are being introduced. As we have shown previously, a heterologous avian species, ducks are sensitive to wasting disease induction by

intraembryonic inoculation of ALV-C (Stepanets et al. 2003). However, the scarcity of diagnostic tools such as monoclonal antibodies to duck cellular antigens and the sensitivity of ducks to viruses of subgroup C only (Duff and Vogt, 1969, Nehyba et al. 1990) limit the exploitation of this model. On the other hand, such limitations did not hold for chickens, where a series of diagnostic antibodies as well as chicken strains sensitive to ALVs of all subgroups are available.

Using the chicken model we aimed at clarification of additional features, especially pathomorphological and immunological characteristics of the wasting disease induced by different strains of ALV-C. In order to achieve a proper comparison, we included additional ALV subgroups (A, B, D) as a control.

Material and Methods

Experimental design and animals. In order to prevent cross-contamination with ALV, newly hatched control and infected animals were kept separately. Because of this requirement, the experiments were spread over a period of two years and, during this period 12 experimental groups were set (Table 1). It should be noted that in each experimental group one control group was included. Data from individual experiments were pooled to obtain representative values. Chickens were inoculated intraembryonally via the chorioallantoic vein with 0.1 ml of virus suspension and/or culture medium or post-hatching with the same volume into the jugular vein.)

We defined wasting disease induced by ALV as the reduced ability of infected chickens to grow. The first symptoms of the wasting could be noted by gross physical examination as soon as one week after hatching; however, it turned into a fully developed disease a week later. As a quantitative parameter of the disease we selected the body mass of

chickens two weeks after hatching, when the body mass data of infected and control chickens were significantly different.

Brown Leghorn (BL) chickens of the phenotype C/E from the breeding colony of the Institute of Molecular Genetics were used in all experiments. They are free of avian leukosis viruses (ALV), chicken anaemia virus (CIAV), infectious bursal disease virus (IBDV) and other common poultry pathogens. The BL stock meets the SPF status except for reovirus infection since only reovirus-specific antibodies can be detected; however, no clinical symptoms of tenosynovitis or infectious stunting syndrome associated with avian reovirus infection has ever been found in the flock. No vaccination has been applied in our breeding colony of chickens. The animals are regularly examined by the state veterinarian.

Experimental chickens were reared on wire floored isolation units and fed commercial feed mixture „400 AGK K1“ (complete feed mixture for breeding young chickens of the first period; metabolizable energy, 12,031 MJ/kg: ZZN, Slaný, Czech Republic) and tap water ad libitum. All experiments were carried out in accordance with the Home Office of the Czech Republic Guidelines.

Virus strains. RAV-1, virus progeny of molecularly cloned RAV-1/OR (Felder et al., 1994) was kindly provided by Dr. G. Calothy, Institute Curie-Biologie, Orsay, France. Viruses RAV-2, RAV-7, RAV-49 and RAV-50 were kindly supplied by Dr. V. Nair, Institute of Animal Health, Compton, United Kingdom. The origin of *td* daPR-C representing the progeny of a molecular clone of a transformation-defective mutant of the Prague strain Rous sarcoma virus of subgroup C was described previously (Stepanets et al., 2003). *td* B77-C, a leukosis virus of subgroup C specificity, was kindly provided by Dr. K. Toyoshima, University of Osaka, Japan. (For virus strains see Table 2). Viruses were grown on chicken embryo fibroblast cell line DF-1 cell line (Himly et al., 1998) except for subgroup C viruses, which

were propagated on duck embryo fibroblasts (DEF, isolated from inbred Khaki Campbell ducks of the D/ABDE phenotype from the breeding colony of the Institute of Molecular Genetics, Stepanets et al., 2001). As measured by the 16Q test (see below), standard production of 10^{6-7} infectious units (IU) per ml was obtained with all virus strains.

Determination of the replicating virus in plasma The presence of infectious virus in chicken plasma was determined by the complementation test with 16Q cells (quail cells harboring the provirus of high-titer Bryan strain Rous sarcoma virus (BH-RSV) lacking the *env* gene) (Murphy, 1977), essentially as described previously (Stepanets et al., 2001). Briefly, chicken embryo fibroblasts (CEF) were incubated with 100 μ l of chicken plasma, subcultured twice and co-cultivated with 16Q cells for one week. Transforming pseudotypes of BH-RSV were detected by the focus assay.

Immunization of chickens with heat-inactivated *Brucella abortus*. Chickens were immunized with *Brucella abortus* essentially as described by Stepanets et al., (2003). Briefly, preimmune sera were collected two days before the immunization started at the age of 26 days. Chickens were inoculated with heat-inactivated *Brucella abortus*, strain Weybridge S99 twice a week for a period of one month. A single immunization dose represented 6×10^7 bacteria per 10 g of the body weight and was administered in 0.2 ml of phosphate-buffered saline (PBS) intravenously. Three days after the last immunization, chicken sera were collected and antibody titers determined using the agglutination method as described previously (Stepanets et al., 2003). The titers were expressed as reciprocals of the terminal serum dilution producing the positive reaction.

Isolation of splenocytes and mitogenesis assay. Spleens were harvested from 14-day-old chickens at autopsy. After cutting with scissors, single-cell suspensions were made by straining the pieces through a 70- μ m nylon mesh (Falcon, Becton Dickinson Labware, NJ, USA). Splenic lymphocytes were resuspended in RPMI 1640 (Sigma, St. Louis, USA) supplemented with 1% fetal bovine serum (FCS) (Gibco BRL Life Technologies, Germany), purified by centrifugation for 20 min at 150 g over a Ficoll-Paque (Amersham Pharmacia Biotech, Buckinghamshire, UK) gradient and the collected cells were washed twice in RPMI 1640 supplemented with 1% FCS. ^3H -TdR incorporation assay was performed as described earlier (Indrová et al., 1995, Sobota et al., 1997). Briefly, for priming of proliferative spleen cell responses, freshly isolated lymphocytes were cultivated in a concentration of $0.5 \times 10^6/\text{ml}$ in complete RPMI 1640 medium supplemented with 10% FCS, 0.5 ml of 200 mM L-glutamine (Sigma, St. Louis, USA), antibiotics and 10^{-5} M of mercaptoethanol (Sigma, St. Louis, USA) alone or supplemented with 1 $\mu\text{g}/\text{ml}$ of ConA (Pharmingen, San Diego, USA). Cells were resuspended in a total volume of 0.2 ml of culture medium and incubated minimally in quadruplicate in 96-well round bottom microtiter plates (Nunc, Roskilde, Denmark) for 48 hrs, at 37°C in a humidified atmosphere with 5% CO_2 . Eighteen hours before harvesting, the cells were labelled with 0.04 MBq ^3H -TdR per well and the uptake of ^3H -TdR was measured by LS 6000 Liquid Scintillation Counter (Beckman, Fullerton, CA, USA) and recorded as counts per minutes (cpm). Proliferative responses were determined as the stimulation index (SI) = average cpm value in samples incubated with mitogenic agent/average cpm value in samples incubated in medium alone.

Histology, histochemistry and immunohistochemistry. Animals were weighed and euthanased at 14th day after hatching. Their lymphatic organs were quickly removed, weighed and trimmed into two parts. One was fixed in buffered 4% paraformaldehyde, pH

7.2, and the other was frozen in liquid nitrogen vapor and stored at -80°C before use. Paraformaldehyde-fixed samples were embedded in paraffin, stained with hematoxylin and eosin and Gomori stain. For immunohistochemistry 4 % paraformaldehyde-fixed 6 μm cryostat sections were used. The antibodies used, their origin, working dilutions and cell types with which they react are listed in Table 3. The reaction product was visualized by incubation with En Vision detection kit (Dako, Cytomation, Denmark). Only the antibody against CD3 was visualized with LSAB+ detection kit (Dako, Cytomation, Denmark). Acid phosphatase activity was demonstrated by the simultaneous azo-coupling method with naphthol AS BI phosphate (Sigma) as a substrate and hexazotized pararosanilin as diazonium salt according to Lojda et al. (1976).

Western blot analysis. Two-week-old control and infected chickens (RAV-1 and *td* daPR-C) were sacrificed and thymus, bursa and spleen were collected, immediately frozen and stored at -80°C . Protein extracts were prepared using Triton lysis buffer (25 mM HEPES, 1% Triton, 2 mM EDTA, 0.1 M NaCl, 25 mM NaF, 1 mM Na_3VO_4) supplemented with leupeptin, pepstatin and antipain (Sigma-Aldrich, St. Louis, MI, USA) (1 $\mu\text{g}/\text{ml}$ of each) and PMSF (Sigma-Aldrich, St. Louis, MI, USA) (3 mg/ml). Total protein concentration was assessed and normalized by the standard Bradford assay. The amount of 30 μg of total protein was separated on 15% SDS PAGE, transferred onto the PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA), blocked with 5% non-fat milk in PBS-T buffer (PBS supplemented with 0.1% TWEEN, Sigma-Aldrich, St. Louis, MI, USA) and incubated for 60 min with anti-p27^{gag} and anti-alpha-tubulin TU-01. After washing the membranes were incubated with anti-mouse IgG – HRP (horse raddish peroxidase)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) for 60 min at room temperature, washed, and

immunoreactive proteins were detected with the chemiluminescence system ECLplus (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical data analysis. To test the null hypothesis – i.e. no difference exists in the above-mentioned parameters between chickens infected with leukosis viruses and chickens that belong to the control group - we used the Dunnett test as an extension to ANOVA (GLM), implemented in: <http://www.R-project.org>. Version 2.1.0 (R, 2005).

Results

Role of the age and the administered virus dose on ALV-C-induced wasting disease in chickens. ALVs have a large potential to induce a broad spectrum of neoplastic and non-neoplastic disorders, particularly when congenitally transmitted or intraembryonally inoculated, since this mode of infection provides optimal conditions for induction of persistent viraemia after hatching. Actively replicating virus reintegrates into the genome of target cells, which is a prerequisite for its accidental integration into the vicinity of a cellular protooncogene, leading finally to oncogene activation and tumour conversion of the cell. ALV-induced tumours could therefore be detected after a long latent period. In the meantime, however, the infected organism is continually supplied by a large quantity of viral antigens, which may disturb either directly or indirectly by so far unknown mechanisms different physiological functions. The outcome of this disturbance is acute non-neoplastic diseases such as wasting disease detected soon after hatching. To evaluate the potential of the highly pathogenic *td* daPR-C in wasting disease induction, we inoculated chickens at early stages of their development with 10^5 IU of virus (Table 4).

We observed a significant decrease ($p < 0.01$) of the body mass in all intraembryonally infected chickens at two weeks after hatching. In addition, the mass of thymus and bursa was

also decreased, while the mass of the spleen was increased ($p < 0.01$). The significance of the observed parameters was not influenced by the sex of experimental animals. Moderate changes, yet still significant ($p < 0.05$), were obtained when newly hatched chickens were inoculated. However, chickens inoculated later at 7th or 14th day of age and sacrificed on day 21 or 28, respectively, exhibited only a significant increase of the spleen mass ($p < 0.05$). Since different time periods of virus inoculation during embryogenesis (10th to 14th day) did not play a role in the induction of wasting disease, we selected mid embryogenesis (12th day) as an optimal time and used it in all further experiments.

To reveal possible virus-dose dependent induction of wasting disease we infected experimental animals with decimally diluted *td* daPR-C. As given in Table 5, the most pronounced changes were observed in the group inoculated with the highest dose of the virus (10^5 IU); however, virus diluted up to 10^2 IU still exerted significant changes in all monitored parameters. This data agreed with the occurrence of viraemia detected actually in all animals infected with a highly diluted virus (10^2 IU). In the group of birds inoculated with 10^1 IU, 85% of animals were viremic, displaying significantly increased spleen mass; however, no wasting and only moderate impairment of the thymus were found. Interestingly, also in the group receiving 10^0 IU some viremic animals were present (18%), but no wasting was detected. Viraemia at two weeks of age was not tested; however, the absence of any wasting or alteration of lymphoid organs at this time (Table 5) would also indicate the absence of efficiently replicating virus in these animals. Since it is known that the course of infection is variable (Payne, 1992a, Witter et al., 2000), we cannot exclude the possibility that chickens inoculated with highly diluted virus and tested negatively on the 2nd day after hatching might shed virus later in life.

Wasting disease induced by intraembryonic inoculation of different ALV strains. To estimate the potential of individual virus strains in wasting disease induction, we decided to

employ undiluted virus stocks (10^5 - 10^6 IU/0.1ml) for infection of chicken embryos. As shown in Table 6, RAV-1 of subgroup A proved to be quite non-pathogenic. Pathogenicity of RAV-2 of subgroup B was clearly demonstrated since significant changes could be observed in the thymus and bursa, which, however, were not accompanied by wasting and spleen alteration. On the contrary, viruses of subgroup C (RAV-7, *td* daPR-C and *td* B77-C) and RAV-50 of subgroup D turned out to be highly pathogenic, producing significant changes of both the body and lymphatic organ mass. The only exception was RAV-49 (subgroup C), producing more moderate, yet still significant decrease of the body mass. As it has been already well documented (Carter and Smith, 1983, 1984, Carter et al., 1983), RAV-7 appeared to be most virulent among the studied viruses, since both body and organ mass were strikingly decreased in infected chickens. This conclusion is also supported by the highest post-hatching mortality found in RAV-7-infected chickens. It should be noted that in comparison to control animals no markedly decreased hatchability was found in infected chickens. Only in some pathogenic viruses (RAV-50, RAV-49 and *td* daPR-C), an increased embryonic mortality could be registered (Table 6).

All viruses replicated well *in vivo* (Table 6). Only moderate differences in the level of viraemia were observed between individual viral strains. Surprisingly, RAV-1 and RAV-2, both of which did not induce wasting disease, replicated even better (10^6 – 10^7 IU/ml) than viruses of subgroups C and D (10^5 IU/ml), which exhibit a higher pathogenicity. This finding was in accord with the higher and lower amount of the viral Gag protein detected by the Western blotting in lymphatic tissues of chickens infected with RAV-1 and *td* daPR-C, respectively (not shown).

Morphological picture of wasting disease in ALV-infected chickens. In all but control and RAV-1-infected animals examined at two weeks after hatching (see Table 6) a

uniform pathological finding was characterized by involution of the thymus and bursa. In some cases, mainly in subgroup C-infected chickens, the thymic lobes were represented only by a narrow strip of lymphoid tissue hardly discernible from the surrounding connective tissue. Strikingly increased spleens were regularly found in all chickens inoculated with both, subgroup C and D viruses. No other pathological changes were identified even in chickens that died before killed for examination. Only some RAV-1-infected animals developed peritonitis accompanied by ascites at the time of sacrifice.

As severe alterations of thyroid, pancreas and liver by ALVs have already been recognized and described in detail, including disturbances in blood chemistry (Carter and Smith, 1983, 1984, Carter et al., 1983), we concentrated on lymphoid tissue alterations induced by ALVs of different subgroup specificity and above all by viruses of subgroup C.

The gross morphological changes of lymphatic organs corresponded well with tissue alterations revealed by light microscopy (Table 7). The thymic cortex of ALV-C-infected chickens was totally disintegrated with pronounced lymphatic depletion, accentuated cortical epithelial cells and activated monocyte-macrophage system, as demonstrated on the sample from *td* daPR-C-infected chicken (Fig. 1a, b). In animals infected with RAV-7 and *td* B77-C the lobular architecture of thymuses vanished. The majority of cells in the cortex was stained positively for the T-cell-specific antigen CD3. Depletion of lymphocytes was not accompanied by marked differences in representation of CD4⁺ and CD8⁺ T lymphocytes. Only a slight increase (up to 50% in comparison with 30% in controls) of thymic TCR2⁺ T-lymphocytes was noted in RAV-7, RAV-49 and *td* daPR-C-infected chickens. A slight reduction of follicular dendritic cells and paucity of Hassal's corpuscles was revealed in the thymic medulla of all ALV C-infected chickens.

In bursa of Fabricius (BF) of ALV-C-infected chickens, a markedly decreased number of lymphoid follicles having a reduced amount of cortical lymphocytes was partially

compensated by interstitial fibrous changes. The most impaired architecture of lymphatic follicles was produced by RAV-7 and *td* B77-C. The connective tissue around follicular residues contained a slightly increased amount of T lymphocytes and the monocyto-macrophage system appeared to be activated. Follicular dendritic cells were moderately increased in follicular centers of chickens infected with *td* daPR-C and RAV-49.

Morphological changes in the spleen were observed only in ALV-C and ALV-D-infected chickens and were present predominantly in the white pulp. The red pulp was only slightly deteriorated. B-cell areas (the peri-ellipsoid lymphocyte sheaths - PELS and the germinal centers) were reduced or in the case of RAV-49 and *td* B77-C-infected chickens nearly absent (Fig. 1c, d). T-cell areas, which together with B cells participate in the formation of periarteriolar lymphocyte sheaths (PALS), were also slightly reduced (Fig. 1e, f). The majority of T cells had the CD8⁺ and TCR2⁺ phenotype. In the red pulp CD8⁺ T cells predominated, too. Splenic ellipsoid-associated reticulum cells (EARC; they form a sheath of spindle-shaped cells around penicilliform capillaries) identified by CVI 68.2 antibody were markedly reduced or even disintegrated in chickens infected with RAV-7 and *td* daPR-C (Fig. 1g, h). Spleens of all infected chickens contained increased numbers of macrophages arranged mostly around the ellipsoid (Fig. 1i, j). Slight fibrous changes of the reticular tissue might have contributed to the observed increased splenic mass in ALV-C and D-infected chickens.

Lymphatic tissues were stained positively for viral p27^{gag} antigen in all infected chickens; however, a weaker signal was observed in the thymus and bursa in ALV-C and D-infected chickens. At the cellular level p27^{gag} was regularly revealed in macrophages of all tissues examined, thymic epithelium, follicular dendritic cells, splenic ellipsoid cells and in some B lymphocytes.

Impairment of the immune system in chickens with ALV-induced wasting disease.

Morphological alteration of lymphoid organs of diseased chickens, indicated a heavily impaired immune system. To evaluate the level of immunosuppression we performed a blastogenic assay with ConA-stimulated spleen lymphocytes of two-week-old chickens. Infection with *td* daPR-C clearly inhibited blastogenic response of splenocytes stimulated with 10 µg of the lectin. On the contrary, only moderate inhibition was recorded in splenocytes from RAV-1-infected chickens (Table 8). Similarly, a difference between both viruses was scored when responsiveness to the bacterial antigen was tested in one-month-old chickens. After repeated administration of heat-inactivated *Brucella abortus*, about 70% of *td* daPR-C-infected chickens did not produce any antibodies and only a minority of them was able to mount a potent humoral response to the antigen (Table 9). In contrast, RAV-1-infected chickens responded to the antigen as efficiently as control animals, with the exception of a small fraction of birds (13%) producing a lower titre (40-80) of antibodies.

Discussion. Retroviruses belong to infectious agents that can efficiently establish persistent infection, the outcome of which is frequently wasting disease and immunosuppression leading to the general devastation and finally to the death of the host. Pathogenesis of the wasting disease appears to be associated with both, hormonal imbalance manifested by a reduced thyroxine level (Carter and Smith, 1984) and apparently a direct toxic effect of the virus. Cytotoxicity has been well documented in ALVs of subgroup B (ALV-B) and D (ALV-D) employing for cellular entry a receptor (tvb) related to the tumor necrosis factor receptor (Brojatsch et al. 2000). Also ALVs of subgroup C (ALV-C), whose the cellular receptor has been identified recently (Elleder et al. 2005), are well known as potent inducers of the wasting disease (Carter et al., 1983; Carter and Smith, 1983; Carter and Smith, 1984) and anemia, the severity of which, however, appears to be strain-dependent (Smith and Schmidt, 1982;

Karakoz et al., 1980; Stepanets et al., 2003). The function of the Tvc protein, a new member of the butyrophilin family, is not known. However, the documented cythopathogenicity of ALV-C for DF-1 cells (Himly et al., 1998) indicates, that some death-promoting activity of the tvc receptor might be stimulated upon binding of the retrovirus. Obviously, the signalling pathway would be different from those one activated by ALV-B and -D viruses. It should be noted that in addition to the genetic outfit of the virus and tissue tropism determined by a functional receptor, other tissue-specific cellular factors supporting efficient retroviral expression (Svoboda, 1998, Sorin and Kalpana, 2006) decide upon the pathogenicity of the particular viral strain.

In the present study we have attempted to define the potential of several ALV-C virus strains to induce wasting disease within the first two weeks after hatching and to compare it to other ALVs of subgroups A, B, and D.

For efficient wasting disease induction, the embryonic stage of the host plays a critical role (Smith and Ivanyi, 1980). As shown in Table 5 the lowest virus dose administered intraembryonally into chicken embryos and exerting the pathological activity approaches the value of 1 – 10 IU. This observation is in accordance with data obtained in another experimental system, juvenile pigtail macaques infected with highly pathogenic simian immunodeficiency virus variant SIVsmmPBj14, where 1 TCID₅₀ (tissue culture infecting dose) produced acutely lethal enteropathic syndrome (O'Neill et al., 1999).

Clearly, the threshold amount of inoculated virus is a necessary, but not sufficient, prerequisite for induction of the acute wasting disease. We can specially imagine that the virus in terminal dilutions produces a low degree of viraemia over the observation period, which is not sufficient for triggering wasting disease. Similarly, chicken endogenous virus RAV-0 equipped with a deficient enhancer and not efficiently replicating shows no signs of pathogenicity (Robinson et al., 1980; Cullen et al., 1983).

In our case, a high virus dose was used for inoculation and all tested ALVs replicated well *in vivo* (Table 6). Hence, they should readily produce wasting disease. Surprisingly, the opposite is true since RAV-1 and RAV-2, reaching the highest titres *in vivo*, exhibit no or moderate pathogenicity, respectively, and viruses of subgroups C and D, which replicate less efficiently, are highly pathogenic. Apparently, viral gene products participate in the acute pathogenicity of ALVs and, of them, the Env protein appears to be the main candidate. An abundant knowledge has been accumulated so far on the role of the Env protein in retrovirus pathogenicity (reviewed in Svoboda et al. 2003).

When comparing the acute pathogenicity of ALV viruses of A, B, C and D subgroup specificity we have encountered notable differences among individual viral strains. In agreement with published data (Carter and Smith, 1984; Stepanets et al., 2003), the most distinctive changes were produced by viruses of subgroup C, among them only RAV-49 appeared less efficient in wasting disease induction (Table 6). A moderate pathogenicity not accompanied by wasting was scored in RAV-2-infected chickens, in which involution only of the thymus and bursa were found. The moderate pathogenicity of RAV-2 was surprising, as other viral strains employing the same receptor associated with cytotoxicity, MAV-2(O) of subgroup B (Hirota et al., 1980; Smith and Ivanyi, 1980) and RAV-50 of subgroup D (this study), were proven to be highly pathogenic *in vivo*. The data on RAV-2 and RAV-49 document that within both subgroups, B and C, differences in pathogenicity exist between individual viral strains and indicate that both, wasting and the lymphoid tissue alteration may be induced independently. This conclusion is also supported by recently published data on ALV-J pathogenicity for *in ovo* infected chickens, in which significant growth suppression was not accompanied by alteration of immune responsiveness (Landman et al., 2002). Endogenous avian retroviral sequences (EAV) that participated in ALV-J formation (Bai et al., 1995) represent a potential source of genetic material for the occurrence of recombinants

accommodating new pathogenic properties different from those of non-recombinant viral strains. Whether and how the differences in pathogenicity are related to certain mutations in the viral genome, especially in the *env* gene is being investigated.

The most conspicuous histopathological changes were found in chickens infected with subgroup C and D, and less distinctively with subgroup B viruses. It should be stressed that accidental infection of chickens with immunosuppressive agents such as CIAV and IBDV seems to be unlikely since no antiviral antibodies could be detected in our breeding flock of BL chickens. The pathological lesions found in ALV-infected chickens differed clearly from those induced by CIAV or IBDV infection (von Bülow and Schacht, 1997, Lukert and Saif, 1997). We also never observed clinical symptoms of arthritis induced by reovirus infection, which can be accompanied by other disease conditions, e.g. growth retardation, bursal and thymic atrophy (Rosenberger and Olson, 1997). Alterations revealed in the thymus tissue of these animals, accompanied in some cases even with collapsed lobular architecture and slight fibrosis, resembled precocious puberty (Seemayer et al., 1984) or thymic epithelial injury found in both children and adult patients suffering acquired immunodeficiency syndrome (Ye et al., 2004; Hazra and Mackal, 2005). Detection of p27^{gag} protein indicated replication of the virus in thymic epithelial cells. Expressed retroviral proteins may injure functions of this cellular compartment, which plays an important role in promotion and maturation of thymocytes through the action of secreted humoral substances or through the direct contact with thymocytes (Lobach and Haynes, 1987). An observed increase of TCR2⁺ T cells in the thymus might represent a consequence of such functional thymic disturbance, the detailed mechanism of which remains, however, unclear.

Impairment of bursal tissue in animals infected with subgroup C viruses resembled the changes induced by intraembryonic infection with MAV-2(0), subgroup B virus (Smith and Ivanyi, 1980; Hirota et al., 1980). In accord with altered bursal morphology was a finding of

severe impairment of humoral immunity in *td* daPR-C-infected chickens that failed in mounting an antibody response to *Brucella abortus* antigen. Surprisingly, a small fraction of animals were, however, able to respond to the antigen in the same manner as controls (Table 9). Since outbred chickens were used, probably host factors would influence their resistance to immunosuppressive activity of the virus. MHC-dependent variations in immune response to viral antigens and the rate of progression of Rous sarcoma virus-induced tumours in congenic chicken lines have been well established (Plachý et al., 1994, Hoffman et al., 2003). Two weeks after hatching RAV-1-infected chickens have apparently preserved intact humoral immunity.

The most remarkable changes in the spleen were encountered again in chickens infected with subgroup C and D viruses. Namely ALV-C viruses produced an impairment of PELS (considered for a functional equivalent of the mammalian marginal zone, Jeurissen et al., 1992) and PALS regions, which is characterized by a marked reduction of B- and T cells with increased CD8⁺ and depleted CD4⁺ lymphocytes. These changes resembled both damage of the marginal zone and shifts in CD8⁺ and CD4⁺ T-lymphocyte representation in the spleen of HIV-infected patients (Wilkins et al., 2003). The observed disruption of a sheath of ellipsoidal cells is apparently in accord with the presence of live virus in the spleen as similar results were found by Jeurissen et al. (1989) in chickens infected with Marek's disease virus. However, intact ellipsoids in RAV-1- and RAV-2- infected chickens would indicate some selectivity of this cellular compartment in responding to different viral antigens.

The severe alteration of lymphoid tissues agreed well with the inefficiency of splenic lymphocytes isolated from *td* daPR-C-infected chickens in responding properly to ConA (Table 6). Subgroup C viruses thus appear as immunosuppressive as MAV-2(O) (Smith and Van Eldik, 1978; Price and Smith, 1982). Contrary to that, splenic lymphocytes of RAV-1-

infected chickens responded to stimulation by ConA, which is in accord with published data (Rupp et al., 1982; Fadly et al., 1982).

The affinity of ALVs for lymphoid tissues resulting in severe morphological alteration of the thymus, bursa and spleen followed by subversion of immune regulatory functions appears to be a unifying aspect of ALV acute pathogenicity. However, in tissue sections from infected animals irrespective of the viral strain, only some B lymphocytes stained positively for the viral Gag protein, indicating active viral replication. **Instead, other lymphoid cellular compartments such as macrophages, thymic epithelium, follicular dendritic cells and splenic ellipsoid cells were largely infected.** In the pathogenesis of ALV-induced immune damage the direct toxic effect of viruses, namely those of subgroup B and D, has been invoked. However, the direct toxicity may not be a principal cause of lymphoid tissue depletion; more likely lymphocytes are attacked in an indirect way, as it has been shown in another retroviral infection (Alimonti et al., 2003).

The striking differences in acute pathogenicity of individual strains of ALVs offer a unique experimental system for analysis of viral genome regions playing a significant role in establishing wasting disease. Despite a large body of our knowledge about adverse effects of retroviral infections, the mechanism responsible for wasting disease in infected hosts has not yet been fully clarified. In this respect, comparative studies of the wasting disease and immunosuppression employing different experimental models appear valuable, because they could bring new insights into the pathogenesis of acute retrovirus-induced diseases.

Acknowledgements

The authors are very thankful to Dr. V. Nair, (Viral Oncogenesis Group, Institute for Animal Health, Compton, United Kingdom), to Dr. G. Calothy (Institute Curie-Biologie, Orsay, France) and to Dr. K. Toyoshima (University of Osaka, Japan) for providing avian leukosis

viral strains. Also, providing the *B. abortus* antigen and determination of agglutination titers in chicken sera by Dr. Plačkov, P. (Bioveta Ivanovice na Hané, Czech Republic) is greatly acknowledged. The authors wish to thank Mikušová, L., Takáčová, Š. and Kolářová, M., for excellent technical assistance. This work was supported by Grant No. 523/04/0489 (Grant Agency of the Czech Republic) and in part by project no. AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic.

Figure legends

Fig. 1 Morphological picture of lymphatic organs in ALV-C-intraembryonally infected chickens^a

^a Chickens were sacrificed two weeks after hatching, their lymphatic tissues fixed and subjected to immunohistochemical analysis.

a, b. Activated monocyte-macrophage system in the thymus of control (a) and *td daPR-C*-infected chicken (b). Note the reduced size of thymic lobes of the infected subject. Kul-1, bar = 100 μ m. **c, d.** Spleen of control (c) and *td B77-C*-infected chicken (d). Marked reduction of B lymphocytes in PELS of infected birds. Ellipsoid (asterisks). HIS-C1, bar = 100 μ m. **e, f.** Spleen of control (e) and *td daPR-C*-infected chicken (f) with T-cell depletion in PALS. Antibody against CD 3, bar = 100 μ m. **g, h.** Marked disintegration of splenic ellipsoid-associated reticulum cells in RAV-7-infected chickens (h) in comparison to control animals (g). CVI 68.2, bar = 100 μ m. **i, j.** Spleen. Activation of the monocyte-macrophagic system around ellipsoid (asterisks) in *td daPR-C*-infected chicken (j). Control subjects (i). Kul-1, bar = 100 μ m.

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Table 1. *Design of twelve experiments performed*

Experiment	Design	Data referred-to
1	Chicken embryos and young chickens inoculated with 10^5 IU of <i>td</i> daPR-C on 10th, 12th, and 14th day of incubation or 1st, 7th and 14th day post hatching. Determination of body and organ mass two weeks after hatching (intraembryonic infection) or on 14th, 21st and 28th day of age (post-hatching infection).	Table 2
2 and 3	Chicken embryos inoculated in mid embryogenesis with different doses of <i>td</i> daPR-C (10^5 to 10^{-2} IU). Determination of body and organ mass two weeks after hatching.	Table 3
4 to 10	Chicken embryos inoculated in mid embryogenesis with viruses of different subgroup specificity (A-D). Determination of body and organ mass and immunohistochemistry of lymphoid tissues two weeks after hatching.	Table 4
11	Proliferative response to ConA stimulation of splenocytes isolated from two-week-old chickens inoculated in mid embryogenesis with RAV-1 or <i>td</i> daPR-C.	Table 6
12	Humoral immune response to <i>Brucella abortus</i> antigen of chickens inoculated in mid embryogenesis with RAV-1 or <i>td</i> daPR-C and repeatedly immunized in the period 2-8 weeks after hatching.	Table 7

Table 2. *List of viruses used*

Virus strain	Subgroup specificity	Virus dose used in the experiments ^a	Origin of virus strains
RAV-1	A	10 ⁶	Felder et al., 1994
RAV-2	B	10 ⁶	Nair, V., Institute for Animal Health, Compton, United Kingdom
RAV-7	C	10 ⁶	Nair, V., Institute for Animal Health, Compton, United Kingdom
RAV-49	C	10 ⁶	Nair, V., Institute for Animal Health, Compton, United Kingdom
<i>td daPR-C</i>	C	10 ⁵	Geryk et al., 1979, Kashuba et al., 1993
<i>td B77-C</i>	C	10 ⁶	Toyoshima et al., 1970
RAV-50	D	10 ⁶	Nair, V., Institute for Animal Health, Compton, United Kingdom

^a Indicated virus doses (in 0.1 ml) were used for inoculation of animals in all experiments. Only *td daPR-C* was serially diluted up to 10⁻² IU (see Table 5) when testing the relation of virus pathogenicity to the injected virus dose.

Table 3. List of primary antibodies used

Antibody	Type	Source	Dilution	Recognized molecule and/or cells
11-39	MAB against Ch ^a , IgG ₁ , ascites	Dr.O. Vainio, University of Turku, Turku, Finland (Luthala et al., 1995)	1:200	CD8 ⁺ T-cell subpopulation
2-6	MAB against Ch, IgG ₁ , ascites	Dr.O. Vainio, University of Turku, Turku, Finland (Luthala et al., 1993)	1:200	CD4 ⁺ T-cell subpopulation
MAB6	MAB against Ch, IgG ₁ , ascites	Dr. J. Čihák, University of Munich, Munich, Germany (Cihak, J. et al., 1988)	1:300	TCR2 ⁺ T-cell subpopulation
Kul 01	MAB against Ch, ascites	Dr. J. Mast, Veterinary and Agrochemical Research Centre, Brussels, Belgium. (Mast et al., 1998)	1:200	monocytes, macrophages, interdigitating cells
HY 21.1	MAB against Avian leukemia/sarcoma viruses, IgG _{2a} , culture supernatant	Dr. E. Humphries, West Virginia University, WV, U.S.A (unpublished)	1:1000	epitope on the retroviral p27 ^{gag}
CK AE1/AE3	MAB against H ^b , IgG ₁ , ascites	DakoCytomation, Denmark A/S, Denmark	1:50	thymic epithelial cells
HIS-C1	MAB against Ch, IgG ₁	Cedi-Diagnostics B.V., Lelystad, The Netherlands	1:10	Bu-1/B cells
CVI-68.2	MAB against Ch, IgG ₁	Cedi-Diagnostics B.V., Lelystad, The Netherlands	1:10	ellipsoid-associated reticulum cells
CD 3	Rb against H ^c	DakoCytomation, Denmark A/S, Denmark	1 : 200	T cells
CVI-74.3	MAB against Ch, IgG ₁	Cedi-Diagnostics B.V., Lelystad, The Netherlands	1:10	follicular dendritic cells
TU-01	MAB against pig alpha-tubulin, IgG1	EXBIO Praha, Prague, Czech Republic	1:2000	epitope on the N-terminal structural domain of alpha-tubulin

^a MAB against Ch, monoclonal antibody directed against chicken antigen; ^b MAB against H, monoclonal antibody directed against human antigen; ^c Rb against H, polyclonal rabbit antibody directed against human antigen.

Table 4. Body mass and relative mass of lymphoid organs in chickens infected in different periods of embryogenesis and post-hatching with *td daPR-C*^a

Age of inoculated animals ^d	No. of chickens tested ^e	Body mass ^c (g)			Relative organ mass ^b								
		non-infected	infected	S ^f	Thymus			Bursa			Spleen		
		non-infected	infected	S ^f	non-infected	infected	S	non-infected	infected	S	non-infected	infected	S
10-day-old embryos	18/18	96.5 ± 11.1	76.7 ± 15.6	**	265 ± 10	160 ± 72	**	230 ± 36	139 ± 65	**	110 ± 79	140 ± 75	ns ^g
12-day-old embryos	7/12	110.5 ± 4.6	82.9 ± 21.1	**	400 ± 95	177 ± 61	**	268 ± 52	118 ± 20	**	97 ± 15	221 ± 60	**
14-day-old embryos	13/16	110.3 ± 11.7	88.0 ± 16.2	**	340 ± 11	182 ± 89	**	257 ± 56	152 ± 39	**	110 ± 28	195 ± 28	**
1-day-old chickens	23/29	111.4 ± 16.7	101.1 ± 12.0	*	368 ± 86	312 ± 63	*	250 ± 52	252 ± 54	ns	105 ± 25	207 ± 45	**
7-day-old chickens	10/9	140.4 ± 27.6	136.9 ± 24.6	ns	307 ± 60	335 ± 68	ns	269 ± 41	292 ± 70	ns	121 ± 23	226 ± 42	**
14-day-old chickens	10/11	327.3 ± 21.7	333.4 ± 25.6	ns	266 ± 59	270 ± 48	ns	236 ± 48	252 ± 31	ns	114 ± 27	179 ± 58	**

^a Chickens were inoculated intravenously in different periods of embryogenesis into a chorioallantoic vein or into jugular vein after hatching with medium (non-infected) or *td daPR-C*. Intraembryonically infected chickens were sacrificed two weeks after hatching, newly hatched chickens two weeks after administration of the virus (ie. on 14th, 21st and 28th day of age). At the time of sacrifice the mass of the body and lymphoid organs was determined. ^b Mean ± SD; Relative organ mass: (organ mass in mg/body mass in g) x 100. ^c Mean ± SD. ^d Hatchability: 10-day-old embryos non-infected 61%, infected 38%; 12-day-old embryos non-infected 77%, infected 71%; 14-day-old-embryos non-infected 100%, infected 94%. All chickens inoculated post-hatching are from one batch the hatchability of which was 98%. ^e Numerator, no. of non-infected chickens; denominator,

no. of infected chickens. ^fThe estimates for control and infected chickens marked ** are significantly different (Dunnett test; $p < 0.01$), those marked * are slightly significantly different (Dunnett test; $p < 0.05$). ^g ns, not significant.

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Table 5. Body mass and relative mass of lymphoid organs in chickens infected in mid embryogenesis with serial 10- fold dilutions of *td daPR-C*^a

Experiment	Virus dose (IU)	No. of chickens tested ^d	Viraemia ^b (viraemic/ tested)	Body mass (g) ^e	S ^f	Relative organ mass ^c					
						Thymus	S	Bursa	S	Spleen	S
I.	Non-infected	11	na ^g	84.7 ± 8.4	na	348 ± 68	na	245 ± 51	na	79 ± 11	na
	10 ⁵	7	nt ^h	57.5 ± 15.8	**	149 ± 80	**	137 ± 45	**	190 ± 38	**
	10 ⁴	13	nt	61.4 ± 10.1	**	101 ± 38	**	134 ± 22	**	187 ± 22	**
	10 ³	15	nt	74.4 ± 7.4	**	142 ± 37	**	161 ± 41	**	219 ± 61	**
	10 ²	11	nt	72.2±11.9	**	152 ± 33	**	151 ± 19	**	164 ± 29	**
II.	Non-infected	12	nt	106.9 ± 14.2	na	352 ± 69	na	264 ± 64	na	113 ± 15	na
	10 ²	11	11/11	76.4 ± 8.6	**	184 ± 80	**	192 ± 45	**	193 ± 27	**
	10 ¹	13	11/13	91.9 ± 11.7	ns ^h	284 ± 86	*	232 ± 58	ns	190 ± 61	**
	10 ⁰	11	2/11	89.0 ± 11.1	ns	363 ± 105	ns	275 ± 68	ns	125 ± 67	ns
	10 ⁻¹	12	0/12	117.3 ± 25.3	ns	396 ± 95	ns	288 ± 38	ns	120 ± 24	ns
	10 ⁻²	13	0/13	117.7 ± 11.1	ns	390 ± 61	ns	266 ± 85	ns	109 ± 21	ns

^a Chickens were inoculated intravenously in mid embryogenesis with medium (non-infected) or decimally diluted *td daPR-C*. Two weeks after hatching the animals were sacrificed and the mass of the body and lymphatic organs was determined. ^b Infectious virus present in the sample of blood plasma collected on the second day after hatching was detected by 16Q test (see Material and Methods). ^c Mean ± SD; relative organ mass see legend to Table 4. ^d Hatchability: non-infected animals 94%, 10⁵ IU *td daPR-C* 78%, in all other groups infected with diluted virus it ranged from 85 to 100% without any relation to the inoculated virus dose. ^e Mean ± SD. ^f The estimates for control and infected chickens marked ** are significantly different (Dunnett test; p<0.01), those marked * are slightly significantly different (Dunnett test; p<0.05). ^g na, not applicable. ^h not tested

Table 6. Body mass and relative mass of lymphoid organs in two-week-old chickens infected in mid embryogenesis with avian leukosis viruses of different subgroup specificity^a

Virus strain	No. of chickens tested	Hatchability/Post-hatching death ^c (%)	Body mass (g) ^e	Relative organ mass ^b							Viraemia ^d (IU/ml) [median(min /max) (n)]
				S ^f	Thymus	S	Bursa	S	Spleen	S	
Non-infected	177	89/0	100.5 ± 20.8	na ^g	334 ± 86	na	257 ± 70	na	114 ± 40	na	na
RAV-1	71	86/0	101.9 ± 17.5	ns ^h	328 ± 73	ns	247 ± 61	ns	124 ± 32	ns	1.0 x 10 ⁷ (10 ⁵ -10 ⁷) (4)
RAV-2	32	87/3	93.7 ± 26.1	ns	241 ± 80	**	199 ± 42	**	119 ± 37	ns	5.5 x 10 ⁶ (10 ⁶ -10 ⁷) (4)
RAV-7	41	87/11	64.4 ± 13.7	**	132 ± 48	**	99 ± 44	**	166 ± 51	**	1.0 x 10 ⁵ (10 ⁵ -10 ⁶) (6)
RAV-49	29	75/0	91.6 ± 14.6	*	171 ± 56	**	133 ± 41	**	186 ± 51	**	5.5 x 10 ⁵ (10 ⁵ -10 ⁶) (4)
<i>td</i> daPR-C	114	81/2	73.4 ± 16.5	**	156 ± 59	**	121 ± 36	**	192 ± 54	**	5.5 x 10 ⁵ (10 ⁵ -10 ⁶) (4)
<i>td</i> B77-C	67	89/0	70.5 ± 14.1	**	155 ± 67	**	119 ± 47	**	156 ± 52	**	1.0 x 10 ⁵ (10 ⁴ -10 ⁶) (6)
RAV-50	25	70/4	81.2 ± 24.0	**	168 ± 48	**	157 ± 40	**	164 ± 53	**	5.5 x 10 ⁵ (10 ⁵ -10 ⁶) (4)

^a Chickens were inoculated intravenously in mid embryogenesis with medium (non-infected) or avian leukosis viruses of subgroups A to D. Two weeks after hatching the animals were sacrificed and the mass of the body and lymphatic organs was determined. ^b See legend to Table 2. ^c Animals died within two weeks after hatching. ^d The titer of infectious virus present in the blood plasma collected two weeks after hatching was determined by 16Q test (see Material and Methods). ^e Mean ± SD. ^f S, see legend to Table 4. ^g na, not applicable. ^h ns, not significant.

Table 7: Summary of morphological findings in lymphatic organs of ALV-infected chickens^a

Tissue	Cellular compartment	Virus strain						
		RAV-1 (n=6) ^b	RAV-2 (n=6)	RAV-7 (n=8)	RAV-49 (n=6)	<i>td</i> daPR- C (n=11)	<i>td</i> B77-C (n=6)	RAV- 50 (n=7)
Thymus	Cortex	0 ^c	- ^d	---	--	---	----	--
	Thymic reticular cells ^e	0	0	+++ ^f	++	+++	++++	+
	Macrophages ^g	+	+	+++	++	++++	+	++
	T cells (TCR2 ⁺) ^h	0	0	++	++	++	+	+
	Follicular dendritic cells ⁱ	0	0	-	-	--	--	0
Bursa	Lymphoid ^j follicles	0	-	----	--	---	----	-
	Follicular dendritic cells ⁱ	0	0	+	+++	++	+	+
Spleen	PELS ^k (B cells)	0	0	---	----	---	----	0
	PALS ^l (T cells – CD3 ⁺)	0	0	--	-	--	--	0
	PALS (T cells - CD8 ⁺) ^m	0	+	++	0	+	++	++
	PALS (T cells - CD4 ⁺) ⁿ	0	-	--	0	-	--	-
	T cells (TCR2 ⁺) ^h (red pulp)	0	0	-	-	-	-	0
	Macrophages ^g	+	+	+++	++++	+++	++	++
	EARC ^o	0	0	----	-	---	---	--
	Follicular dendritic cells ⁱ (in ellipsoid)	0	+	0	0	+	+	++

^aTissue samples for immunohistochemical analysis were collected from at least two independent experiments. ^b Number of animals examined by immunohistochemistry. ^c No alteration. ^d Decreased number of cells or size of the cellular compartment: -, slightly, --, intermediately, ---, considerably, ----, strongly. ^e CK AE1/AE3 antibody. ^f Increased number of cells or size of the cellular compartment: +, slightly, ++, intermediately, +++, considerably, +++++, strongly. ^g Kul-1 antibody. ^h MAb6 antibody. ⁱ CVI-74.3 antibody. ^j B-cells detected by HIS-C1 antibody. ^k PELS, peri-ellipsoid lymphocyte sheaths, B cells detected by HIS-C1 antibody. ^l PALS, periarteriolar lymphocyte sheaths, T cells detected by CD3 antibody. ^m CD8⁺ T cells detected by 11-39 antibody. ⁿ CD4⁺ T cells detected by 2-6 antibody. ^o EARC, ellipsoid-associated reticulum cells detected by CVI 68.2 antibody.

Table 8. Proliferative spleen cell responses of control and virus-infected chickens^a

Spleen cell donor	Hatchability (%)	3 ^H -thymidine incorporation x 10 ³ [mean ± SD (n)] ^b		SI	S ^c
		Non-stimulated	Con A-stimulated		
Non-infected	90	0,39 ± 0,22 (8)	3,73 ± 2,21 (8)	9,6	**
<i>td</i> daPR-C	75	0,27 ± 0,09 (8)	0,36 ± 0,15 (9)	1,3	ns
RAV-1	87	0,60 ± 0,17 (5)	2,26 ± 0,89 (5)	3,8	*

^a Chickens were inoculated intravenously in mid embryogenesis with a culture medium (non-infected) and *td* daPR-C or RAV-1. Two weeks after hatching the animals were sacrificed and spleens were used to prepare of cell suspensions. ^b Incorporation of ³H-TdR in non-stimulated and Con-A-stimulated cells was determined and the stimulation index (SI) was calculated. ^c S, see legend to Table 4 .

Table 9. Antibody response of Brown Leghorn chickens intraembryonally infected with avian leukosis viruses to *Brucella abortus* antigen^a

Inoculated virus (No. of animals tested)	Hatchability (%)	No. of animals producing antibody				
		20 ^b	40 - 80	160 - 320	640 - 1280	2560
Non-infected (22)	87	0	0	15	6	1
<i>td</i> daPR-C (17)	74	12	0	0	4	1
RAV-1 (8)	78	0	1	4	2	1

^a Chickens were inoculated intravenously in mid embryogenesis with a culture medium (non-infected), *td* daPR-C and RAV-1. Bacterial antigen was administered into jugular vein twice a week in a period from 4 to 8 weeks of age. ^b The titer of antibodies represents the reciprocals of the terminal dilution of sera producing the positive reaction. (Note that titer of antibodies corresponding to 20 signifies almost a lack of immune response.)

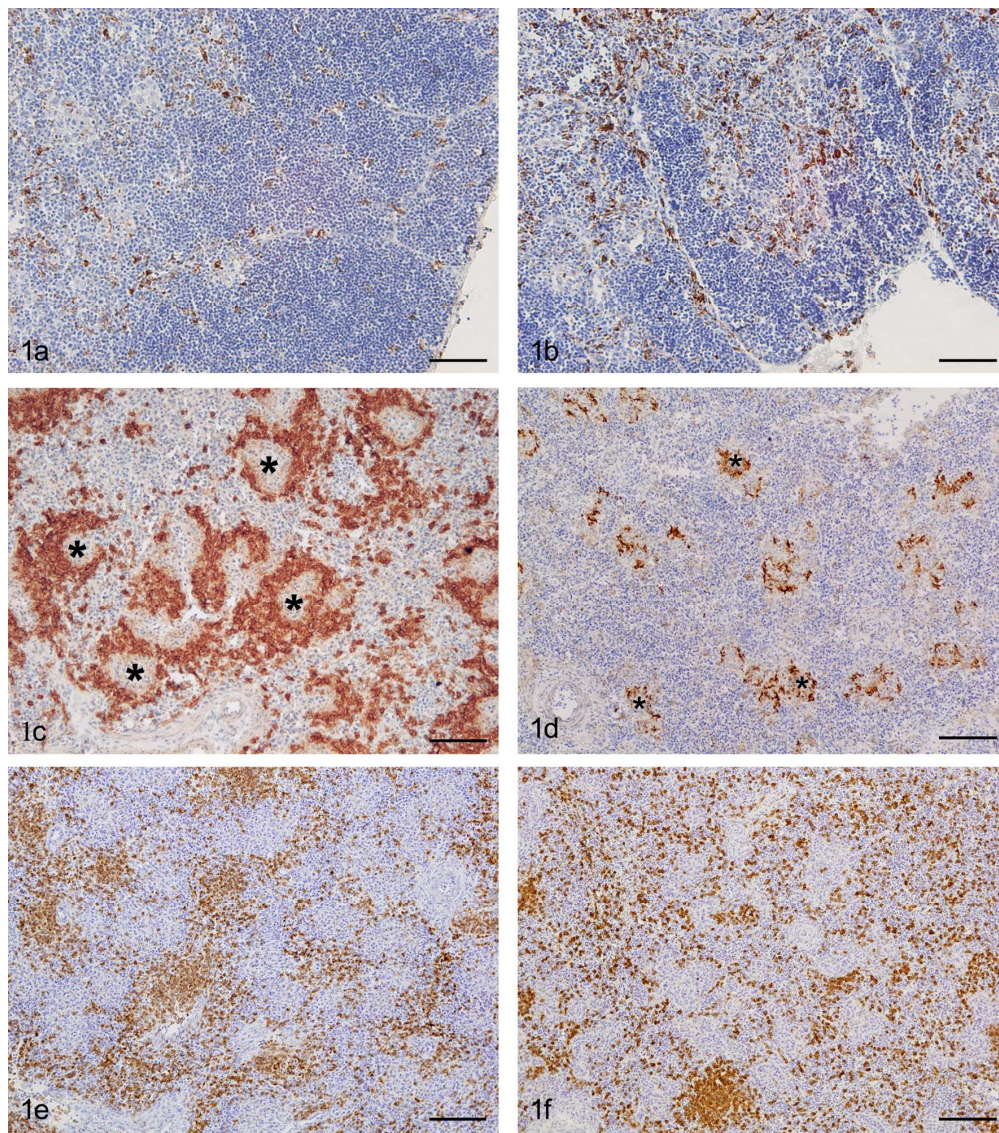


Fig. 1a - f

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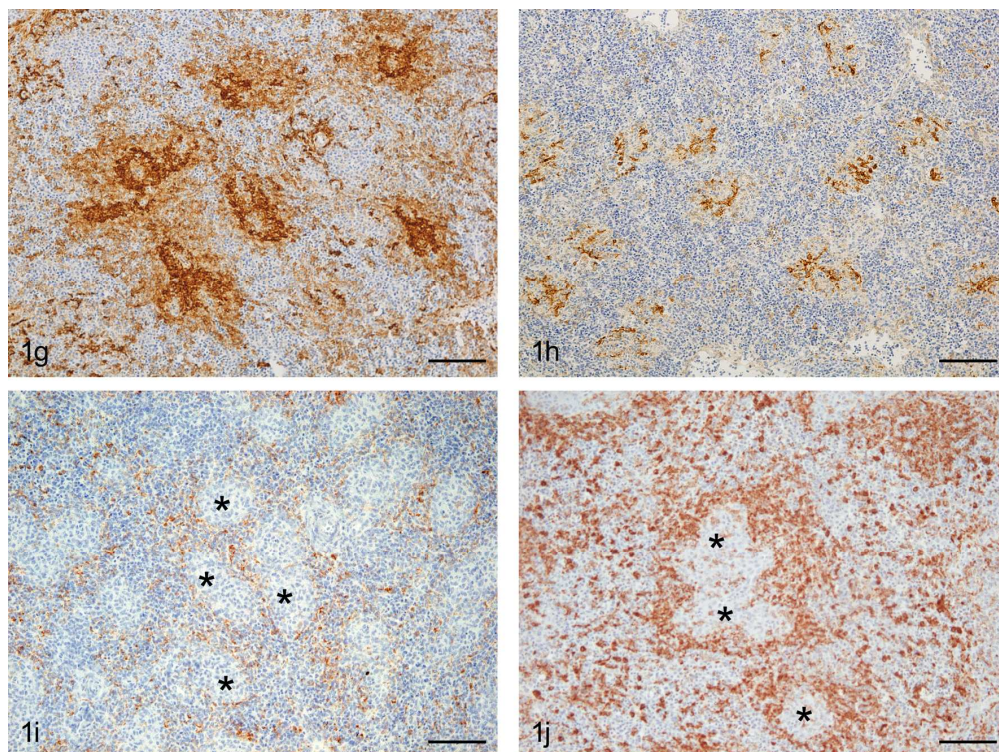


Fig. 1g - j

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