Ducks: a new experimental host system for studying persistent infection with avian leukaemia retroviruses

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Long-term persistence of the avian leukosis virus (ALV), the transformation-defective mutant of Prague strain Rous sarcoma virus subgroup C (*td* PR-C) was established in heterologous duck hosts after infection in mid-embryogenesis. Transient viraemia was observed for about 4 weeks after hatching and was lost in most of the infected ducks by about 6 months. Loss of viraemia was accompanied by the increasing synthesis of virus-neutralizing antibodies. In spite of strong virus-neutralizing antibodies, virus was detected by the cocultivation assay in duck tissues throughout the observation period up to 5 years. In the viraemic phase of infection, we found integrated proviruses in various tissues, preferentially in stomach muscle tissue and in the thymus. The long-term persistence of virus was frequently accompanied by liver necrosis and neoplastic diseases. Injection of *td* PR-C virus into early embryos resulted in more pronounced infection accompanied by an increased copy number of viral DNA per cell, high mortality and remarkable atrophy of thymus tissue in infected ducklings.

Introduction

The persistence of retroviruses has become an important issue, particularly with regard to possible eradication of retroviral infections in domestic animals and with regard to the control of human retroviruses, particularly human immunodeficiency virus (HIV).

In the chicken experimental model system, the pioneering studies of Rubin (Rubin, 1962; Rubin *et al.*, 1962), demonstrated that chickens infected with avian leukosis virus (ALV) before or at hatching became persistently viraemic in the absence of virus-neutralizing antibodies. It has been proposed that ALV-associated viraemia is mediated by complete immunological tolerance, i.e. by a permanent state of unresponsiveness of the humoral and cellular components of immunity to ALV (Rubin, 1962; Meyers *et al.*, 1976; Qualtiere & Meyers, 1979; Loan & Whiteaker, 1986). However, Rubin (1962) has also described a different course of infection. Infection of chickens with ALV several days after hatching induced transient viraemia, the termination of which was accompanied by the formation of virus-neutralizing antibodies; Rubin called these chicks immune birds. Later it was found that infectious virus could be detected in immune birds using tissue culture assays as long as 2 years after onset of virus-neutralizing antibody formation (De Boer *et al.*, 1981). It was demonstrated that such persistent infection of chickens with ALV results in a high frequency of malignancies, and is also associated with pathological phenomena which are non-oncogenic in nature (Spencer, 1984).

The study of persistent ALV infection in chickens is complicated, however, by the presence of endogenous ALVs in most laboratory lines of chickens. These endogenous ALVs may influence the immunological reactivity to exogenous ALVs (Halpern *et al.*, 1983), and new viral variants may occur by recombination between endogenous and exogenous (Vogt & Friis, 1971), which might in turn influence the persistence of inoculated virus in the host. Therefore, we employed another avian host for the study of ALV persistence: ducks of the semi-inbred line Khaki-Campbell, because it is known that endogenous ALVs or ALV-related sequences are not present in the duck genome (Frisby *et al.*, 1979; Chambers *et al.*, 1986). Ducks obviously are not natural hosts of exogenous ALVs (Purchase & Burmester, 1978), but they have been experimentally infected with avian sarcoma viruses (derived from ALVs) and used for virological studies (Purdy, 1932; Duran-Reynals, 1942). ALVs of subgroup C (ALV-C) multiply *in vitro* in Khaki-Campbell duck cells to the same titres as in cells of natural hosts, the chickens (Shimikage *et al.*, 1979; Geryk *et al.*, 1980) and infectious retrovirus can be demonstrated in blood cells of Khaki-Campbell ducks 1 year after *in ovo* infection with ALV-C (Svoboda *et al.*, 1980).

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The basic strategy of this study has been the *in ovo* inoculation of ALV-C into duck embryos of different ages and subsequent analysis of the distribution of various forms of the virus, including infectious virus in serum, infectious virus rescuable from cells, and the DNA provirus. Furthermore, formation of virus-neutralizing antibodies by the host and the pathological changes associated vith virus infection were examined.

Methods

Animals and viruses. The animals used in all experiments were Khaki-Campbell ducks of a semi-inbred line (Vilhelmová *et al.*, 1972). The hatch rate of this line is about 20%. Prague strain Rous sarcoma virus subgroup C (PR-C) was kindly provided by Dr E. H. Bernstein (University Laboratories, Highland Park, N.J., U.S.A.). The transformation-defective mutant of PR-C was isolated by Vogt (1971); this mutant is referred to as *td* PR-C throughout this paper.

Injections of td *PR-C into duck embryos.* Ducks were inoculated with *td* PR-C *in ovo* on days 1, 2, 14 and 16 of incubation. On days 1 or 2 of incubation duck embryos were injected with 20 µl of viral suspension [10^4 infectious units (i.u.)] into the subgerminal cavity. These injections were done through a small aperture (diameter 1 mm) in the egg shell. Localization of the embryo was estimated and accuracy of inoculation was controlled by candling. On days 14 or 16 of incubation duck embryos were injected with 100 µl of virus (10^5 i.u.) into the chorioallantoic vein. During incubation embryos were inspected by candling and the dead ones were dissected.

Virus titration and cell culture methods. The titre of *td* PR-C in duck serum was determined by the complementation test with 16Q cells [quail cells harbouring the provirus of high-titre Bryan strain Rous sarcoma virus (BH-RSV) lacking the *env* gene (Murphy, 1977)], according to Geryk *et al.* (1980) and expressed as infectious units. Briefly, serial dilutions of duck serum were used for the infection of chicken embryo fibroblasts (CEFs) and after three passages CEFs were cocultivated with 16Q cells for 1 week. Formation of the transforming pseudotype of BH-RSV was measured by the focus assay. Ducks with the minimal titre of 1 i.u. per 0·2 ml of serum were considered viraemic.

The presence of virus in blood cells and in other tissues was also determined by this complementation test. Four ml of heparinized blood were washed three times with phosphate-buffered saline and cells of buffy coat together with approximately 10^8 erythrocytes were cocultivated with CEF for 1 week and then with 16Q cells for another week. Fragments from approximately 100 mg of various other tissues were cocultivated in a similar manner.

Virus-neutralizing antibodies in duck serum were measured by the neutralization test according to Hložánek & Svoboda (1972). Briefly, heat-inactivated duck serum was incubated with 100 f.f.u. of PR-C at 37 °C for 1 h and then the fraction of surviving PR-C was measured by the focus assay. Classification of neutralizing capacity of sera was done according to Rubin (1962). Because the fraction of surviving PR-C after incubation with sera from 10 control, uninfected ducks was in the range 1·2 to 0·3, the value 0·29 was accepted as the limit for the presence of weak neutralizing antibodies [see footnote in Table 1(‡)].

Preparation of tissue DNA, restriction endonuclease analysis, DNA electrophoresis, and nucleic acid hybridization. These procedures were performed essentially as previously described (Svoboda *et al.*, 1983). Southern blots of *Eco*RI- or *Bgl*II-digested duck DNA were hybridized to pATV-8 or *gag* 2 cloned DNA. The plasmid pATV-8, represents the whole genome of PR-C (Guntaka & Mitsialis, 1980) and *gag* 2 is a 1389 bp *Bam*HI *gag* gene fragment subcloned from pATV-8.

Determination of the extent of integrated and unintegrated proviral copies in preparations of tissue DNA. The extent of total proviral DNA in duck tissues was determined visually on autoradiograms by comparing the intensity of a 2·4 kb *Eco*RI fragment with the same fragment in cells with one proviral copy per cell genome (H-20, Svoboda *et al.*, 1983) and in cells with approximately 20 proviral copies per cell genome (XC, Svoboda, 1960; Varmus *et al.*, 1973) hybridized to *gag* 2 probes essentially as described by Baba and Humphries (1984). To estimate less than 1 proviral copy per cell, we included $\log_2$ serial dilutions of H-20 DNA which were hybridized to the *gag* 2 probe. Unintegrated proviral copies were distinguished from integrated ones according to diagnostic restriction enzyme fragments.

Results

Infection by inoculation of td *PR-C in mid-embryogenesis*

The infection of duck embryos on day 14 (group D14) or day 16 of incubation (group D16) resulted in a viraemic phase and a post-viraemic phase. Onset of the latter phase was usually observed approximately 4 weeks post-hatching. The rate of decrease of viraemic birds was clearly more pronounced in group D16 (Fig. 1). Some birds in group D14 remained viraemic for more than 20 weeks after hatching and usually died within 1 year of life.

(i) *The course of viraemia and virus-neutralizing antibodies*

In the D14 group of ducks, the degree of viraemia and the titres of virus-neutralizing antibodies were measured. As shown in Table 1, viraemia in the absence of virus-

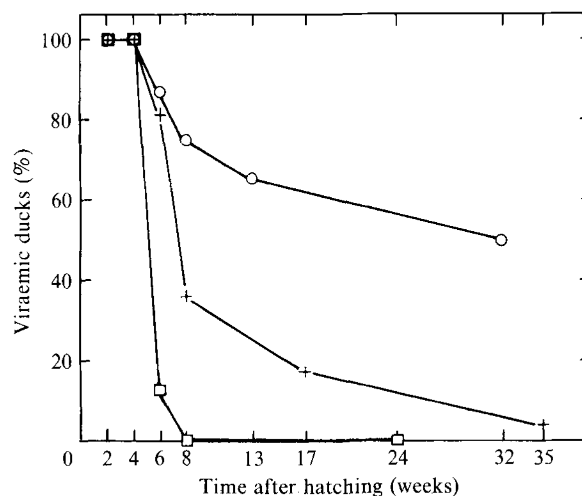

Fig. 1. Viraemia in ducks inoculated with *td* PR-C at different periods of embryonic development. Viraemia was estimated in 28 ducks of the D16 group (□), in 32 ducks of the D14 group (+) and in 10 ducks of the D2 group (○). The percentage of viraemic birds in the D2 group was calculated from the number of survivors.

neutralizing antibodies was most pronounced during the first 4 weeks after hatching; however, a maximum of 10^4 i.u./ml of virus was detected in the serum. In the course of the next 4 weeks, the viraemia decreased, accompanied by the appearance of low titres of virus-neutralizing antibodies. At the same time, the non-viraemic birds synthesized comparable amounts of neutralizing antibodies. Between 8 and 20 weeks after hatching, all birds produced strong neutralizing antibodies, even those which maintained a low-grade viraemia for more than 20 weeks.

(ii) *Proviral DNA in different tissues*

To characterize the quantity of *td* PR-C provirus in DNA from different organs of viraemic birds, the copy number of viral DNA was estimated on the basis of radioactive signal intensity obtained after the hybridization of the *Eco*RI fragment to the *gag* 2 probe (Fig. 2). In

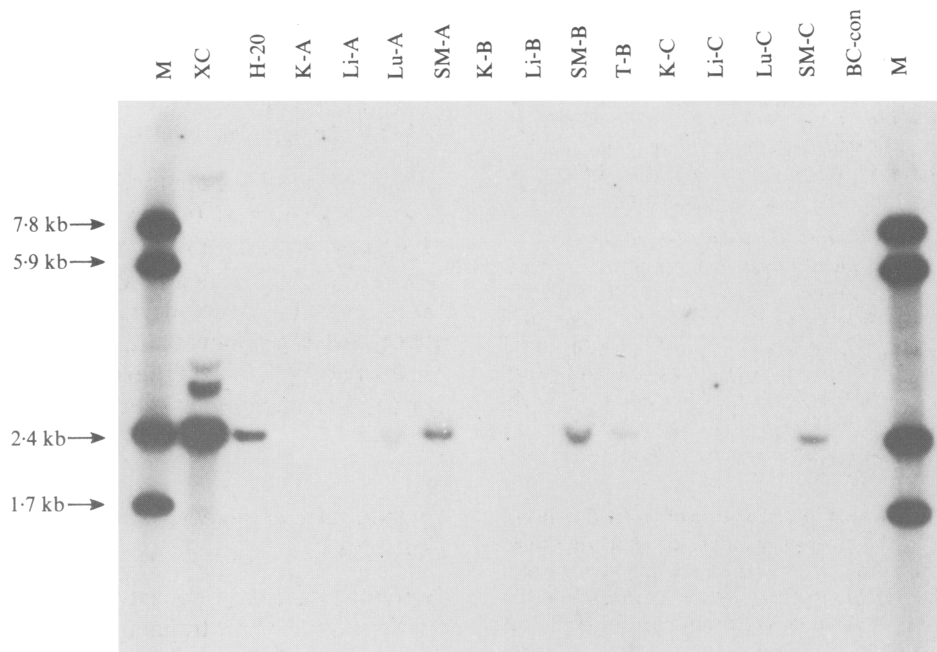

Fig. 2. Determination of the quantity of *td* PR-C in DNA from different organs of D14 ducks. After hybridization of *Eco*RI digests of DNA from duck organs to the *gag* 2 probe, 2·4 kb *Eco*RI long terminal region (LTR)-*gag* fragments of *td* PR-C are visible. Lanes: K, kidney; Li, liver; Lu, lungs; SM, stomach muscles; T, thymus. K-A represents kidney of animal A. Animals A, B, C are ducks nos. 3, 1 and 4, respectively, from Table 2. A, duck at 4 weeks after hatching; B, duck at 2 weeks after hatching; C, duck at 6 weeks after hatching. Control DNAs: M, M_r standards: a mixture (1:1) of *Eco*RI digests and *Eco*RI plus *Bgl*II double digests of plasmid pATV-8; BC-con blood cells control is red blood cells from a normal, uninfected duck; XC cells and H-20 cells are shown. The concentration of H-20 DNA was twice that of other DNAs on this blot.

Table 1. *Viraemia and neutralizing antibodies in D14 ducks*

Time post-hatching (weeks)	Viraemic ducks (%)*	Titre of *td* PR-C in duck serum (i.u./ml)	Virus-neutralizing antibodies in duck serum†,‡	
			Viraemic	Non-viraemic
4	100	10^1–10^4	0·52 ± 0·28	ND§
6	78	<5–10^4	0·12 ± 0·12	0·26
8	44	<5–10^1	0·11 ± 0·01	0·05 ± 0·03
12–17	22	<5–10^2	0·02 ± 0·04	0·00 ± 0·09

* The percentage of viraemic ducks is expressed as a percentage of 50 ducks tested.

† Ten of the 50 ducks tested for viraemia were assayed for neutralizing antibodies.

‡ Virus-neutralizing capacity of serum was expressed as the mean value of a fraction of surviving PR-C ±SD in the virus-neutralizing test. This value in ranges <0·1, 0·1 to 0·29 and >0·29 was classified as strong, weak and no virus-neutralizing antibodies, respectively.

§ ND, Not determined (no non-viraemic ducks were present).

the viraemic phase of infection, the DNA provirus was detected in amounts of 1/16 to 1/32 copy per cell in most organs of D14 ducks (Table 2). Substantially larger amounts of DNA provirus were observed only in stomach muscle tissue and in the thymus (one eighth to one copy per cell). The provirus was not found in the brain. Detected proviral DNA mostly represented the integrated form of the provirus. In stomach muscle tissue, the ratio of non-integrated : integrated forms was about 1 : 3, as determined by the sizes of DNA fragments obtained after digestion with *Bgl*II and hybridization to the pATV-8 probe (data not shown).

At the beginning of the post-viraemic period DNA proviruses had almost disappeared, decreasing below detectable levels (1/64 copy per cell) in all the organs examined (Table 2).

(iii) *Persistence of* td *PR-C in the post-viraemic phase*

Despite the loss of proviral DNA detectable by Southern blot hybridization and the presence of strong virus-neutralizing antibodies, the virus persisted into the post-viraemic phase. Virus was detected in blood cells of all D14 ducks by the cocultivation assay, in most throughout the 3 year observation period (Table 3). Only rarely was virus not found in blood cells, but it was detected upon later testing of blood from the same animal. Our results indicate that only a small fraction of D14 blood cells

Table 2. *Amount of proviral DNA in different organs of D14 ducks*

Duck no.	Time post-hatching (weeks)	Viraemia (i.u./ml)	Number of DNA provirus copies per cell*													
			E†	L†	T†	B†	S†	BM†	Li†	K†	Lu†	Br†	G†	SM†	BMT†	HM†
1	2	10^3	0	–	1/8	0	1/16	1/32	1/32	1/16	1/32	0	0	1	1/32	1/32
2	2	–	1/16	–	1/8	1/32	1/32	–	1/32	1/32	1/16	0	–	1	1/16	1/16
3	4	10^4	1/32	–	1	1/32	0	1/32	1/32	1/32	1/8	0	1/16	1	0	0
4	6	10^3	–	–	1/8	–	1/16	1/32	0	1/16	1/16	0	1/16	1	1/32	1/32
5	6	–	–	–	–	0	1/32	–	–	1/16	–	0	–	1/4	1/16	1/32
6	8	10^2	–	–	1/8	1/32	1/16	1/32	0	1/16	1/16	0	–	1/4	1/16	–
7	8	10^2	–	–	0	–	–	–	0	–	–	–	–	–	–	–
8	12	10^1	–	–	1/4	1/32	1/16	0	0	1/32	1/16	0	1/16	1/8	–	1/16
9	12	0	–	–	0	0	0	–	0	0	0	0	0	–	1/32	0
10	26	–	1/16	0	1/16	–	–	–	1/16	1/16	–	–	–	–	–	–
11	26	0	0	0	1/16	–	0	–	0	0	0	0	0	–	1/32	0
12	90	0	0	0	–	–	0	–	–	0	0	–	–	0	–	0
13	140	0	0	0	–	–	0	–	0	–	0	–	–	0	0	0

* Log_2 serial dilutions of H-20 DNA were used as standards to estimate the proviral copy number per cell.

† E, erythrocyte fraction of blood cells; L, leukocyte fraction of blood cells; T, thymus; B, bursa of Fabricius; S, spleen; BM, bone marrow; Li, liver; K, kidney; Lu, lung, Br, brain; G, gonads; SM, stomach muscle tissue; BMT, breast muscle tissue; HM, heart muscle tissue.

Table 3. *Long-term persistence of virus rescuable from blood cells of duck groups D14 and D16 in the presence of strong virus-neutralizing antibodies*

Age at testing (months)	D14 group		D16 group	
	Ducks with virus in blood cells (%)*	Virus neutralizing in serum†,‡	Ducks with virus in blood cells (%)*	Virus neutralizing antibodies in serum†,‡
6–12	100(39)	0·03 ± 0·03	58(24)	0·00 ± 0·00
12–18	100(21)	0·06 ± 0·31	57(19)	ND‖
18–24	92§(14)	ND		
24–30	100(17)	0·00 ± 0·00		
30–36	100 (8)	ND		

* The number of ducks tested is given in parentheses.

† Of the ducks tested for virus persistence in blood cells, five to seven birds were assayed for neutralizing antibodies.

‡ For explanation of numbers expressing virus-neutralizing capacity of sera, see legend in Table 1 (‡).

§ The 'negative' duck was positive when tested at 24 to 30 months post-hatching.

‖ ND, Not determined.

possess virus because at least 10^5 white blood cells or 10^6 red blood cells have to be cocultivated with CEFs to detect virus (Table 4). Persisting virus was detected by the cocultivation assay in a number of other tissues of D14 ducks, in addition to blood cells (Table 5). In D16 ducks, virus was rescued from blood cells of only half of the birds even when the same ducks were tested repeatedly at 3 month intervals (Table 3). Four ducks without virus in their blood were assayed for virus presence in different organs (Table 5). Surprisingly, virus was detected in all four of these ducks, most frequently in the thymus and/or breast muscle tissue. The persistence of *td* PR-C in both D14 and D16 ducks was highly stable. In 60 ducks followed for 1 to 3 years, not a single case of complete virus elimination was observed.

The permanent presence of strong subgroup C virus-neutralizing antibodies in all persistently infected ducks, both D14 and D16 groups (Table 3), is in agreement with the persistent nature of infection and confirms that virus detected by the cocultivation assay retained subgroup C specificity. This specificity was verified in two isolates rescued from blood cells of one 5-year-old and one 4-year-old D14 duck by the virus-neutralizing test with PR-C-specific neutralizing antibodies (data not shown). Furthermore, we tested the replication competence of persisting virus obtained by cocultivation of CEFs with blood cells of five D14 ducks (3 to 5 years old). All produced virus which efficiently complemented *env*⁻ BH-RSV. The titre of a measured viral isolate reached 10^6 i.u./ml, which corresponds to normal titres of *td* PR-C.

(iv) *Pathological changes associated with persistence of* td *PR-C virus in D14 and D16 ducks*

In the viraemic phase of infection no marked pathological changes were found. Hatchability of the inoculated ducks did not differ from that of non-inoculated controls. No pronounced mortality of D14 and D16 ducks in the first year of life was found except for very rare cases of long-term viraemic D14 ducks. There was no indication of humoral immunity suppression in D14 ducks, because 11 ducks at 7 weeks of age (seven of them were still viraemic) synthesized comparable titres of antibodies

Table 4. *Content of persisting virus in different fractions of blood cells in D14 ducks*

Duck no.	Age (months)	Lowest number of blood cells necessary for detection of virus by the cocultivation test: Erythrocytes	Leukocytes
1	6	10^7	10^5
2	18	10^6 to 10^8	10^5
3	18	10^6	10^5
4	29	10^7 to 10^8	10^7

Table 5. *Presence of persisting virus in different organs*

	D14 group						D16 group			
Duck no.	1	2	3	4	5	6	7	8	9	10
Time post-hatching (weeks)	6	6	20	32	35	35	18	18	19	19
Organs tested*										
Blood cells	+	+	+	+	+	−	−	−	−	−
Thymus	+	+	NT	NT	NT	NT	NT	+	+	+
Spleen	+	+	+	+	+	+	+	NT	−	−
Liver	+	+	−	NT	NT	−	NT	NT	−	NT
Kidney	+	+	−	NT	NT	+	NT	+	NT	NT
Lung	NT	+	+	+	+	−	−	−	−	−
Heart	NT	+	+	+	NT	−	−	NT	+	−
Stomach muscles	NT	+	+	+	+	+	−	−	−	−
Breast muscles	+	+	NT	+	+	+	−	+	+	+
Brain	+	+	+	+	NT	NT	−	−	−	−

* Organ fragments were cocultivated with CEF and 16Q cells (containing *env*⁻ BH-RSV), and production of BH-RSV pseudotype was measured (see Methods). Results of cocultivation assay: (+), virus was detected; (−), no virus detected; NT, organ was not tested.

against antigens of *Brucella abortus* as did the eight uninfected controls.

Pathological changes were noted in the post-viraemic phase of infection in ducks older than 1 year. Of the 17 D14 and D16 ducks 1 to 3 years old, 12 showed a severe sclerotic damage of the liver associated with intense amyloid formation, four developed cholangioma, and there was one case each of differentiated hepatoma, hemangioma and round-cell sarcoma.

Infection by inoculation with td *PR-C in early embryogenesis*

When *td* PR-C virus was introduced on day 1 of incubation into the subgerminal cavity of duck embryos (D1, group), all the birds died before the end of the first week after hatching (Fig. 3). Remarkable reduction of thymus tissue represented the only gross alteration found at autopsy. Slower manifestations of virus pathogenicity were observed in birds inoculated with virus on day 2 of incubation (D2 group) (data not shown). Of the 77 infected embryos, 10 ducks survived for 2 weeks after hatching. Of these, eight died within 8 months after hatching. Among D2 ducks there was a high proportion of birds with persistent viraemia (Fig. 1). In two ducks with viraemia which persisted for more than 20 weeks, virus-neutralizing antibodies were detected between 10 and 20 weeks post-hatching.

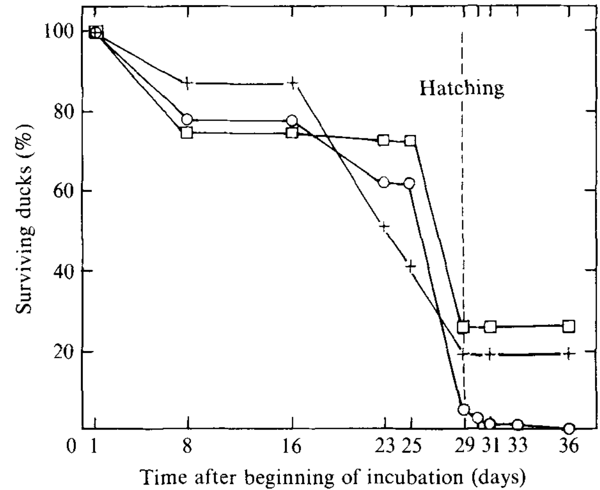

Fig. 3. Life-span of ducks inoculated on the first day of incubation with *td* PR-C. One hundred and two ducks were inoculated with *td* PR-C (○), 31 control, non-inoculated ducks (+) and 45 mock-inoculated ducks (□).

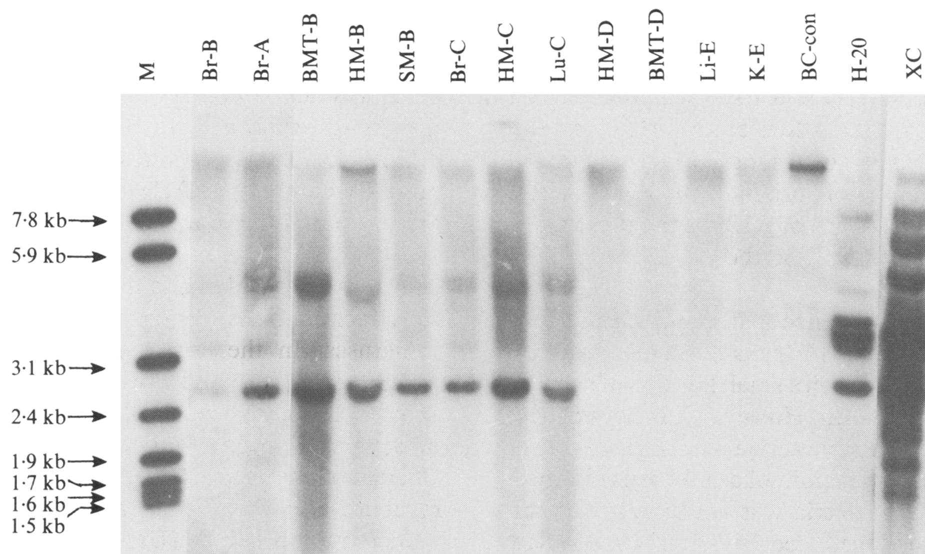

Fig. 4. Determination of the quantity of integrated and unintegrated forms of *td* PR-C provirus in DNA from different organs of D1, D2 and D14 ducks. After hybridization of *Bgl*II digests of DNA from duck organs to the pATV-8 probe, the following fragments are visible: a 2·6 kb Δ*gag*–Δ*pol* fragment common for integrated and unintegrated forms of *td* PR-C, a 1·75 kb LTR–Δ*gag* fragment and a 3·35 kb Δ*pol*–*env*–LTR fragment of unintegrated linear form of *td* PR-C provirus; a 4·8 kb Δ*pol*–*env*–LTR–Δ*gag* fragment of unintegrated circular form of *td* PR-C provirus with one LTR; a 5·1 kb Δ*pol*–*env*–LTR–LTR–Δ*gag* fragment of unintegrated circular form of *td* PR-C provirus with two LTRs. The uppermost fragment (>20 kb) is derived from c-*src*. Lanes: Br, brain; BMT, breast muscle tissue; HM, heart muscles; SM, stomach muscles; Lu, lungs; Li, liver; K, kidney. A, D1 duck at 1 day after hatching; B and C, two D2 ducks at 1 day after hatching; D, D14 duck at 8 weeks after hatching; E, D14 duck at 2 weeks after hatching. Control DNAs, see Fig. 2 legend.

In four randomly selected ducks from group D1 and three ducks from group D2, the number of integrated and non-integrated DNA proviral copies was determined between day 1 and the fifth week after hatching. All the ducks tested showed a high content of total DNA provirus (from one fourth of a copy to eight copies per cell) in all organs examined (Fig. 4). The highest content of DNA provirus was regularly found in breast muscle tissue (four to eight copies per cell). In contrast to group D14, stomach muscle tissue (one to four copies per cell) did not predominate in DNA provirus content, whereas a considerable amount of DNA provirus was found in the brain (one sixteenth to one copy per cell). In addition to integrated DNA provirus, the circular and linear non-integrated forms were detected in all of the organs tested from D1 and D2 ducks. The ratio of integrated : non-integrated forms was 2 : 1 to 4 : 1, respectively (Fig. 4).

Discussion

The results document for the first time long-term persistence of ALV (*td* PR-C) in ducks infected during embryogenesis. In contrast to chickens, ALV produced only a limited viraemia followed by a post-viraemic period in which virus-neutralizing antibodies were produced. Therefore the immunological tolerance to ALV, postulated by Rubin (1962) as the mechanism of virus persistence in chickens, does not appear to apply in the duck host.

Even after the onset of antibody formation, virus persisted in all birds in different tissues throughout a 3 year period of testing (for a few cases tested, up to 5 years). Persisting virus retained subgroup specificity and replication competence of original *td* PR-C virus. Similar long-term persistence of ALV in the presence of neutralizing antibodies was described by De Boer *et al.* (1981) in chickens.

The type of virus persistence is not fully clear. Some data obtained in a few old D14 ducks tested (Tables 2 and 4) suggest that virus persists in a small fraction of somatic cells in an intracellular form. However it is not possible to exclude reappearance of viraemia in some D14 and/or D16 ducks in the post-viraemic phase, because in most cases the presence of viraemia was not tested concomitantly with the blood cell cocultivation virus assay. Nevertheless, we propose that after elimination of post-hatching viraemia, all D14 and D16 ducks go through a phase in which virus expression is in some way blocked. Such lack of expression might be a consequence of infection of resting cells (Fritsch & Temin, 1977), the influence of *cis*-acting regulatory signals (Cooper & Temin, 1976; Akroyd *et al.*, 1987), or methylation (Katz *et al.*, 1983; Dyson *et al.*, 1985). Decreased expression of viral products should allow the infected cells to escape the immune surveillance directed against cells antigenically modified by ALV proteins. As the high titres of virus-neutralizing antibodies are produced in all animals, we have to invoke that either the low expression of provirus is enough for eliciting formation of such antibodies or that non-expressed proviruses become, with low efficiency, activated to produce infectious virus. Such activation might be a result of the action of some inducible cellular trans-activators on the viral genome, as exemplified in HIV (see Cullen & Greene, 1989). There is also a possibility that some ALV-infected duck cells are 'hidden' from the immune surveillance and produce either infectious virus or might spread the infection by cell-to-cell contact after being released in the circulation (Narayan *et al.*, 1982; Peluso *et al.*, 1985).

The immunoselective pressure against ALV was manifested in most of the ducks studied some time after the fourth week after hatching. Increased production of virus-neutralizing antibodies probably resulted in the elimination of circulating virus and the start of a post-viraemic phase. Infectious extracellular viral particles disappeared and the DNA provirus content in most tissues dropped below the levels detectable by Southern blotting. A similar situation has been described by Baba & Humphries (1984, 1986) in chickens infected with ALV after hatching; Welt *et al.* (1977) noted a decrease in viral protein content in most tissues of the chicken in conjunction with the loss of viraemia.

In the post-viraemic phase, virus was detected by the cocultivation assay in most tissues of D14 ducks. The virus was not spread by virus-containing blood cells in the organs examined, as in D16 ducks which had no detectable virus in their blood cells. Virus was rescued most frequently from skeletal muscle tissue and from the thymus only in D16 ducks.

Similarly, proviral DNA persisted for the longest period in both tissues and thymus, and stomach muscles of viraemic birds contained the highest number of proviruses. In the organs of D1 and D2 ducks, the integration of provirus was more pronounced, usually above one copy per cell. The increased provirus integration found in muscles might be related to the efficient transcription of ALV and Rous sarcoma virus in myoblasts (Flamant *et al.*, 1987; Howlett *et al.*, 1987).

Increased proviral DNA in the organs of ducks infected at earlier embryonic stages may result from an extended period of virus replication prior to antibody formation. Under such conditions the chance of infection is increased because proliferating progenitor cells rather than differentiated cells are exposed to the virus. Resistance of some differentiated cells to ALV (Gazzolo *et al.*, 1975) and lack of provirus integration in non-dividing cells has been clearly documented (Humphries

& Temin, 1974; Humphries *et al.*, 1981). In agreement with this possibility is the finding of proviral copies in the brain of D1 and not D14 ducks.

The long-term persistence of virus in ducks was accompanied by a few types of pathological changes. Most frequent was pronounced sclerosis of the liver with intense amyloid formation which may relate to the chronic stimulation of antiviral immunity (Karlson *et al.*, 1987). Association of the majority of malignancies, cholangiomas and hepatomas, with the liver is in agreement with the finding of Rigdon & Leibovitz (1970) that most of the spontaneous tumours in ducks are of hepatic origin. According to preliminary results, one sarcoma obtained did not contain acutely transforming virus but harboured about one copy of *td* PR-C provirus per cell. Whether or not the site of provirus integration is relevant to tumour formation is being investigated.

Despite the fact that *td* PR-C does not produce a c.p.e. on chicken fibroblasts, it causes severe anaemia in viraemic chickens (Karakoz *et al.*, 1980). In D1 ducks, we consistently found thymus atrophy as the only macroscopic alteration suggesting that *td* PR-C can affect also the lymphoid cell lineage. In such birds, non-integrated viral DNA was detected, up to four copies per cell, which is two orders of magnitude lower than the titres of non-integrated copies correlated with the c.p.e. produced by ALV (Weller & Temin, 1981). Decreased hatchability of ducks inoculated intraembryonally with murine xenotropic virus was also observed by Levy *et al.* (1982) but was not further characterized.

Our findings indicate that after embryonic infection subgroup C ALVs persist for a long time in a fraction of cells of the adult duck host. The duck host has certain advantages over chickens for the study of ALV persistence. It is free of ALV-related endogenous retroviral genomes which might modify the persisting virus or change the immunological response to it. In ducks, it is possible to modify the extent of viral persistence and the degree of virus pathogenicity, depending on the embryonic stage used for virus inoculation. In fact, the duck model offers a reproducible system for the study of transition from viraemia to permanent virus persistence in a small fraction of cells. In this respect, there is considerable similarity with the persistence of lentiviruses where the virus, restricted in its expression *in vivo* can persist in a small fraction of cells even in the presence of virus-neutralizing antibodies (see reviews by Haase, 1986; Narayan & Clements, 1989). The exact cause of the low production of virus *in vivo* as compared to *in vitro* and the mechanisms which keep the persisting virus in a silent but still activable state remain to be solved. From this point of view, the model of ducks infected intraembryonally with subgroup C ALV should be suitable for addressing these questions.

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References

AKROYD, J., FINCHAM, V. J., GREEN, A. R., LEVANTIS, P., SEARLE, S. & WYKE, J. A. (1987). Transcription of Rous sarcoma proviruses in rat cells is determined by chromosomal position effects that fluctuate and can operate over long distances. *Oncogene* **1**, 347–354.

BABA, T. W. & HUMPHRIES, E. H. (1984). Avian leukosis virus infection: analysis of viremia and DNA integration in susceptible and resistant chicken lines. *Journal of Virology* **51**, 123–130.

BABA, T. W. & HUMPHRIES, E. H. (1986). Selective integration of avian leukosis virus in different hematopoietic tissues. *Virology* **155**, 557–566.

CHAMBERS, J. A., CYWINSKI, A., CHEN, P. -J. & TAYLOR, J. M. (1986). Characterization of Rous sarcoma virus-related sequences in the Japanese quail. *Journal of Virology* **59**, 354–362.

COOPER, G. M. & TEMIN, H. M. (1976). Lack of infectivity of the endogenous avian leukosis virus-related genes in the DNA of uninfected chicken cells. *Journal of Virology* **17**, 422–430.

CULLEN, B. R. & GREENE, W. C. (1989). Regulatory pathways governing HIV-1 replication. *Cell* **58**, 423–426.

DE BOER, G. F., MAAS, H. J. L., VAN VLOTEN, J. & GROENENDAL, J. E. (1981). Horizontal transmission of lymphoid leukosis virus. Influence of age, maternal antibodies and degree of contact exposure. *Avian Pathology* **10**, 343–358.

DURAN-REYNALS, F. (1942). The reciprocal infection of ducks and chickens with tumor-inducing viruses. *Cancer Research* **2**, 343–369.

DYSON, P. J., COOK, P. R., SEARLE, S. & WYKE, J. A. (1985). The chromatin structure of Rous sarcoma proviruses is changed by factors that act in *trans* in cell hybrids. *EMBO Journal* **4**, 413–420.

FLAMANT, F., LE GUELLEC, D., VERDIER, G. & NIGON, V. M. (1987). Tissue specificity of retrovirus expression in inoculated avian embryos revealed by *in situ* hybridization to whole-body section. *Virology* **160**, 301–304.

FRISBY, D. P., WEISS, R. A., ROUSSEL, M. & STÉHELIN, D. (1979). The distribution of endogenous chicken retrovirus sequences in the DNA of galliform birds does not coincide with avian phylogenetic relationships. *Cell* **17**, 623–634.

FRITSCH, E. F. & TEMIN, H. M. (1977). Inhibition of viral DNA synthesis in stationary chicken embryo fibroblasts infected with avian retroviruses. *Journal of Virology* **24**, 461–469.

GAZZOLO, L., MOSCOVICI, M. G. & MOSCOVICI, C. (1975). Susceptibility and resistance of chicken macrophages to avian RNA tumor viruses. *Virology* **67**, 553–565.

GERYK, J., MAZO, A., SVOBODA, J. & HLOŽÁNEK, I. (1980). Replication of transformation-defective mutants of the Prague strain of Rous sarcoma virus and isolation of *td* mutant from duck-adapted PR-RSV-C. *Folia biologica (Praha)* **26**, 34–41.

GUNTAKA, R. V. & MITSIALIS, S. A. (1980). Cloning of avian tumor virus DNA fragments in plasmid pBR322: evidence for efficient transcription in *E. coli* from a virus-coded promoter. *Gene* **12**, 113–121.

HAASE, A. T. (1986). Pathogenesis of lentivirus infections. *Nature, London* **322**, 130–136.

HALPERN, M. S., EWERT, D. L., FLORES, L. J. & CRITTENDEN, L. B. (1983). The influence of the *ev* 3 locus on the inducibility of serum antibody reactivity for envelope glycoprotein group-specific determinants. *Virology* **128**, 502–504.

HLOŽÁNEK, I. & SVOBODA, J. (1972). Characterization of viruses obtained after cell fusion or transfection of chicken cells with DNA from virogenic mammalian Rous sarcoma cells. *Journal of General Virology* **17**, 55–59.

HOWLETT, A. R., CULLEN, B., HERTLE, M. & BISSELL, M. J. (1987). Tissue tropism and temporal expression of Rous sarcoma virus in embryonic avian limb *in ovo*. *Oncogene Research* **1**, 255–267.

HUMPHRIES, E. H. & TEMIN, H. M. (1974). Requirement for cell division for initiation of transcription of Rous sarcoma virus RNA. *Journal of Virology* **14**, 531–546.

HUMPHRIES, E. H., GLOVER, C. & REICHMANN, M. E. (1981). Rous sarcoma virus infection of synchronized cells establishes provirus integration during S-phase DNA synthesis prior to cellular division. *Proceedings of the National Academy of Sciences, U.S.A.* **78**, 2601–2605.

KARAKOZ, I., GERYK, J. & SVOBODA, J. (1980). *In vivo* effect of three transformation-defective mutants of subgroup C avian sarcoma viruses. *Folia biologica (Praha)* **26**, 62–69.

KARLSON, P., GEROK, W. & GROSS, W. (1987). Connective tissue. In *Pathobiochemistry*, pp. 342–358. Edited by P. Karlson, W. Gerok and W. Gross. Prague: Academia (In Czech).

KATZ, R. A., MITSIALIS, S. A. & GUNTAKA, R. V. (1983). Studies on the methylation of avian sarcoma proviruses in permissive and non-permissive cells. *Journal of General Virology* **64**, 429–435.

LEVY, J. A., OLESZKO, O., DIMPFL, J., LAU, D., RIGDON, R. H., JONES, J. & AVERY, R. (1982). Murine xenotropic type C viruses. IV. Replication and pathogenesis in ducks. *Journal of General Virology* **61**, 65–74.

LOAN, R. W. & WHITEAKER, R. S. (1986). Immune response to avian leukosis virus determined by the cell-mediated focus-reduction (CEMFOR) assay. *Journal of the National Cancer Institute* **77**, 757–762.

MEYERS, P., RITTS, G. D. & JOHNSON, D. R. (1976). Phytohemagglutinin-induced leukocyte blastogenesis in normal and avian leukosis virus-infected chickens. *Cellular Immunology* **27**, 140–146.

MURPHY, H. M. (1977). A new replication-defective variant of the Bryan high-titre strain Rous sarcoma virus. *Virology* **77**, 705–721.

NARAYAN, O. & CLEMENTS, J. E. (1989). Biology and pathogenesis of lentiviruses. *Journal of General Virology* **70**, 1617–1639.

NARAYAN, O., WOLINSKY, J. S., CLEMENTS, J. E., STRANDBERG, J. D., GRIFFIN, D. E. & CORK, L. C. (1982). Slow virus replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. *Journal of General Virology* **59**, 345–356.

PELUSO, R., HAASE, A., STOWRING, L., EDWARDS, M. & VENTURA, P. (1985). A Trojan horse mechanism for the spread of visna virus in monocytes. *Virology* **147**, 231–236.

PURCHASE, H. G. & BURMESTER, B. R. (1978). Leukosis/sarcoma group. In *Diseases of Poultry*, pp. 418–468. Edited by M. F. Hofstad, B. W. Calnek, W. M. Reid and H. W. Yoder. Ames, Iowa: Iowa State University Press.

PURDY, W. J. (1932). The propagation of the Rous sarcoma No. 1 in ducklings. *British Journal of Experimental Pathology* **13**, 473–479.

QUALTIERE, L. F. & MEYERS, P. (1979). A reexamination of humoral tolerance in chickens congenitally infected with an avian leukosis virus. *Journal of Immunology* **122**, 825–829.

RIGDON, R. H. & LEIBOVITZ, L. (1970). Spontaneous-occurring tumors in the duck: review of the literature and report of three cases. *Avian Diseases* **14**, 431–444.

RUBIN, H. (1962). Conditions for establishing immunological tolerance to a tumor virus. *Nature, London* **195**, 342–345.

RUBIN, H., FANSHIER, L., CORNELIUS, A. & HUGHES, W. F. (1962). Tolerance and immunity in chickens after congenital and contact infection with an avian leukosis virus. *Virology* **17**, 143–156.

SHIMAKAGE, M. I., KAMAHORA, T., HAKURA, A. & TOYOSHIMA, K. (1979). Selective replication of transformation-defective avian sarcoma virus mutants in duck embryo fibroblasts. *Journal of General Virology* **45**, 99–105.

SPENCER, J. L. (1984). Progress towards eradication of lymphoid leukosis viruses. *Avian Pathology* **13**, 599–619.

SVOBODA, J. (1960). Presence of chicken tumour virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma tissue. *Nature, London* **186**, 980–981.

SVOBODA, J., GERYK, J. & KARAKOZ, I. (1980). Long-term persistence of transformation-defective mutants of subgroup C in chickens and ducks inoculated early in life. *Archiv für Geschwulstforschung* **50**, 429–434.

SVOBODA, J., LHOTÁK, V., GERYK, J., SAULE, S., RAES, M. B. & STÉHELIN, D. (1983). Characterization of exogenous proviral sequences in hamster tumor cell lines transformed by Rous sarcoma virus rescued from XC cells. *Virology* **128**, 195–209.

VARMUS, H. E., BISHOP, J. M. & VOGT, P. K. (1973). Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. *Journal of Molecular Biology* **74**, 613–626.

VILHELMOVÁ, M., HÁLA, K., HAŠEK, M., KARAKOZ, I. & PLACHÝ, J. (1972). Inbreeding of Khaki-Campbell ducks. In *Proceedings of a conference on genetics and reproduction of farm animals*, pp. 163–165. Edited by J Matoušek & M. Valenta. Praha: Academia (In Czech).

VOGT, P. K. (1971). Spontaneous segregation of nontransforming viruses from cloned sarcoma viruses. *Virology* **46**, 939–946.

VOGT, P. K. & FRIIS, R. R. (1971). An avian leukosis virus related to RSV(O): properties and evidence for helper activity. *Virology* **43**, 223–234.

WELLER, S. K. & TEMIN, H. M. (1981). Cell killing by avian leukosis viruses. *Journal of Virology* **39**, 713–721.

WELT, S., PURCHASE, H. G. & THORBECKE, G. J. (1977). Viral protein synthesis by tissues from avian leukosis virus-infected chickens. I. Susceptible chickens infected after hatching. *Journal of Immunology* **119**, 1800–1805.

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