Avian Leukosis Virus Infection: Analysis of Viremia and DNA Integration in Susceptible and Resistant Chicken Lines

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Avian leukosis viruses induce lymphoid leukosis, a lymphoma which develops within the bursa of Fabricius several months after virus infection. Chickens from the Hyline SC and FP lines are, respectively, susceptible and resistant to avian leukosis virus-induced lymphoid leukosis. We examined plasma and cellular DNA obtained from avian leukosis virus-infected chickens for the presence of viremia and integrated viral sequences to determine whether the extent of virus infection is comparable in individuals of both lines. A less than twofold difference in the frequency of viremia was detected between chickens of the two different lines. Although the analysis of plasma samples, which were obtained at different times postinfection, demonstrated that the duration of viremia was comparable in both susceptible and resistant chickens, the onset of the viremia observed in susceptible chickens generally preceded by 1 week that observed in resistant chickens. Moreover, integrated viral sequences were detected in approximately 90% of the SC and 40% of the FP chickens. The appearance of infectious virus in the plasma was, in general, associated with the presence of integrated viral sequences in both the bursal cells and the erythrocytes obtained from the same chicken. The presence of both the viremia and the integrated viral DNA sequences was transient, suggesting a mechanism for the elimination of virus-infected cells in both susceptible and resistant chickens. Furthermore, at 5 weeks postinfection no integrated exogenous viral sequences were detected in splenic lymphocytes obtained from either chicken line, regardless of whether these chickens were viremic or had integrated viral sequences detectable in other tissues. Our results indicate that extensive avian leukosis virus replication occurs in approximately 50% of the FP and 100% of the SC chickens. Although it appears that the viral infection spreads more quickly in the SC chickens, our results afford no obvious explanation of the resistance to the development of lymphoma exhibited by FP chickens.

Infection of susceptible chickens with avian leukosis virus (ALV) induces bursal lymphomas after a long latent period (20). These tumors develop within the follicles of the bursa of Fabricius, a specialized organ required for normal B-lymphocyte differentiation (6), and are clonal in nature. They subsequently metastasize into the viscera, resulting in death of the chicken (15, 16, 18). The tumor cells are of the B-lymphocyte lineage, expressing immunoglobulin M (IgM), but not IgA or IgG, isotypes (7). Analysis of DNA obtained from a majority of these tumors has revealed the presence of viral sequences integrated into the host cell genome in the vicinity of the cellular oncogene *c-myc*, the cellular homolog of the transforming gene found in the acute transforming retrovirus MC29 (15, 18, 24, 25). By acting as an insertional mutagen in these tumors, ALV enhances the transcriptional activity of this genetic locus. ALV-induced tumorigenesis appears to be a multistage process (17). In addition to *c-myc*, a recently described oncogene, *B-lym*, detected by transfection of DNA from bursal tumors into recipient NIH 3T3 mouse cells, may also be involved in the formation of this tumor (4, 5, 11). At present, neither the role these activated oncogenes play in tumor development nor the temporal order in which they participate has been determined.

Whereas most lines of chickens are susceptible to the development of lymphoid leukosis (LL), studies in this laboratory have demonstrated Hyline SC and FP chickens to be susceptible and resistant, respectively, to the development of these ALV-induced lymphomas (T. W. Baba and E. H. Humphries, *Virology*, in press). Approximately 50% of the ALV-infected Hyline SC chickens developed LL

within 24 weeks after virus infection. These tumors were found to have viral sequences integrated adjacent to *c-myc*, which is consistent with the observations described above. In contrast, no tumors were observed in the Hyline FP chicken line. Fibroblasts prepared from embryos of both lines have receptors for Rous-associated virus-1 (RAV-1) and are susceptible to viral infection *in vitro*. In view of the fact that virus infection and subsequent integration into the host genome appear to play a significant role in the activation of at least one oncogene, we performed several experiments to examine whether the spread of the virus in these susceptible and resistant chickens is comparable. In the present study, the extent of virus infection was assessed by two independent criteria. Plasma samples were obtained from RAV-1-infected chickens at different times after experimental infection and were analyzed for the presence of infectious virus. In addition, the extent of viral integration into the DNA obtained from both target and nontarget tissues was compared. Our results indicate that the entire population of SC chickens and approximately one-half of the FP chickens transiently exhibit circulating infectious virus and integrated viral DNA sequences in target and nontarget tissues.

MATERIALS AND METHODS

Viruses and cells. The procedures used in this laboratory for culturing chicken embryo fibroblasts have been previously described (14). RAV-1 was kindly provided by L. Crittenden. A cloned virus stock, prepared by endpoint dilution, was used throughout this study. Interference analysis demonstrated that the virus was characterized by subgroup A envelope glycoproteins (28). Infectious virus present in the

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plasma obtained from RAV-1-infected chickens was titrated by endpoint dilution onto chicken embryo fibroblast cultures. Dilutions of plasma, prepared in medium, were used to infect cultures of chicken embryo fibroblasts. Five 17-mm plates were infected with each dilution of virus. Cells were grown to confluency, trypsinized, and transferred to 50-mm plates. Upon reaching confluency, these cultures were split and transferred to 50-mm plates. Medium harvested from confluent overnight cultures was assayed for the presence of pelletable reverse transcriptase as previously described (14).

Virus infection and animal care. Fertile eggs, obtained from Hyline International, Dallas Center, Iowa, were incubated in a humidified incubator at 39°C until they hatched. The SC line (B^2B^2) is maintained as an F_1 hybrid. The FP line ($B^{15}B^{21}$) is maintained as a cross between the dam of one line ($B^{21}B^{21}$) and the sire of an F_1 line ($B^{15}B^{15}$) prepared from two additional lines. Despite the genetic background of these two genetically unrelated lines, no heterogeneity was observed either in the expression of the endogenous loci that are segregating in these birds or in their susceptibility to the different subgroups of the avian leukosis-sarcoma virus complex. Their observed phenotypes were FP (C/E, $gs^+ chf^+$) and SC (C/O, $gs^- chf^-$). We did not test these lines for susceptibility to tumor development by avian retroviruses other than RAV-1.

RAV-1 (2×10^5 IU), obtained from a single source of biologically cloned virus, was injected intravenously into SC and FP chicks 1 to 2 days after hatching. The chickens were housed in isolation cubicles in the Animal Resource Center, University of Texas Health Science Center at Dallas. To follow bursal development, bursas obtained at different times after virus infection were fixed in 10% neutral buffered Formalin and weighed on an analytical balance.

Preparation of plasma, erythrocytes, splenic lymphocytes, and bursal cells. Plasma, collected by heart puncture at different times after virus infection, was mixed with an equal volume of sterile Alsever solution, separated from cellular constituents by centrifugation at $200 \times g$ for 10 min at 4°C, and stored at -70°C for subsequent viral analysis. The buffy coat was removed from the cell pellet and discarded. The cells were resuspended in cold phosphate-buffered isotonic saline and centrifuged as described above. The remaining buffy coat was removed and discarded, yielding an erythrocyte suspension containing less than 1% leukocyte contamination, as visualized by light microscopy. These erythrocytes were lysed as described below for the preparation of DNA.

Spleens, obtained at 5 weeks postinfection, were minced with scissors. The cells were resuspended in isotonic medium and pipetted several times with a large orifice pipette. These cell suspensions were filtered through two layers of gauze and layered onto cushions of Isolymp (density, 1.077 g/cm³; Gallard-Schlesinger). Centrifugation at $2,000 \times g$ was performed at room temperature for 15 min. The cells that were harvested from the interface were resuspended in medium, pelleted, and used for the preparation of DNA as described below. Preparations consisting of less than 0.1% erythrocyte contamination, as assessed by light microscopy, were routinely obtained.

Bursas obtained at autopsy were minced with scissors. The cells were resuspended in isotonic medium, pipetted several times with a large orifice pipette, filtered through two layers of gauze, and subsequently pelleted at $200 \times g$ for 10 min at 4°C. Supernatants were discarded, and the cell pellets were used for the isolation of DNA.

Preparation of cellular DNA. Cellular DNA was prepared as previously described (14). Briefly, bursal cells, erythrocytes, or splenic lymphocytes were washed twice with isotonic saline, lysed with 0.5% sodium dodecyl sulfate-100 µg of proteinase K per ml in 10 mM Tris-hydrochloride (pH 8.0)-10 mM NaCl-10 mM EDTA. After digestion overnight at 38°C, the DNA preparation was extracted with phenol-chloroform, precipitated with ethanol, and redissolved in 10 mM Tris-hydrochloride (pH 8.0)-10 mM NaCl-10 mM EDTA. After digestion with RNase A and subsequent digestion with proteinase K, the DNA preparation was again extracted with phenol-chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-hydrochloride (pH 8.0)-10 mM NaCl-1 mM EDTA. Spectrophotometry was used to assess purity and concentration.

Restriction endonuclease analysis, DNA electrophoresis, and nucleic acid hybridization. Restriction endonuclease analysis, DNA electrophoresis, and nucleic acid hybridization have been described in detail elsewhere (14). *EcoRI* and *SalI* restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). For electrophoretic analysis, 1% agarose gels (Sea Kem; Marine Colloids, Rockland, Maine) in 60 mM Tris-acetate (pH 8.0)-60 mM NaCl-6 mM EDTA at approximately 1 V/cm were used. Agarose gels were prepared for DNA transfer to BA85 nitrocellulose filter paper (Schleicher & Schuell Co., Keene, N.H.).

DNA which was bound to nitrocellulose filter paper was analyzed for the presence of virus-specific DNA sequences by hybridization to a ³²P-labeled DNA probe, which was labeled by nick translation with [α -³²P]deoxycytidine triphosphate to a specific activity of approximately 2×10^8 cpm/µg (22). The probe was boiled in 0.3 M NaOH for 10 min before hybridization. Filters were prehybridized for 2 h at 68°C in $6 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1× Denhardt solution-0.005 M EDTA-0.5% sodium dodecyl sulfate-20 µg of denatured calf thymus DNA per ml. Hybridization with the virus-specific ³²P-labeled DNA probe was carried out under the conditions of the prehybridization and with 5 to 10 ng of the specific DNA probe per ml for 10 h or more.

Detection of RAV-1-specific DNA sequences. A molecular clone of the Schmidt-Ruppin strain of avian sarcoma virus, pSRA-2, was obtained from J. M. Bishop (8). The 1,300-base-pair *BamHI* fragment was subcloned into the *BamHI* site of pBR322. This clone, pBB-12, was used to detect the 5' half of RAV-1 and is referred to as the 5'-viral probe. A 2.1-kilobase-pair (kb) viral DNA fragment, which corresponds to viral *gag* sequences, has been shown to be generated by *EcoRI* cleavage of the integrated RAV-1 DNA provirus (10, 13). This fragment is not observed after *EcoRI* cleavage of any of the endogenous loci and is specific for the presence of exogenous viral DNA. The amount of integrated RAV-1 DNA sequences present in a preparation of cellular DNA was determined visually by estimating the amount of the 2.1-kb viral DNA fragment present from the intensity of hybridization of the pBB-12 viral ³²P-labeled DNA probe. The 8.7-kb 5' junction fragment derived from *ev-1*, which also hybridized to the pBB-12 DNA probe, served as an internal control for sequences present at a frequency of 1 copy per haploid genome. The amount of 2.1-kb DNA fragment present was estimated as +3 (approximately 1 copy per haploid genome), +2 (approximately 0.5 copy per haploid genome), and +1 (approximately 0.1 copy per haploid genome). A hybridization intensity of less than 0.1 copy per haploid genome was considered negative.

FIG. 1. Comparison of Hyline FP and SC bursa mass. Chickens were injected intravenously with 2×10^5 IU of RAV-1 1 day after hatching. The animals were sacrificed at different times, and their bursas were fixed in 10% neutral buffered Formalin. The mass of each bursa was determined with an analytical balance. Each point represents the mean of 10 to 20 bursas.

RESULTS

Bursal development in susceptible and resistant chickens. The target organ for LL is the bursa of Fabricius. Chemical bursectomy prevents LL, and animals which are surgically bursectomized up to 12 weeks of age have a significantly reduced incidence of disease (19, 21). Because the development of LL appears to require a relatively rare virus-cell interaction (17), it is possible that the size of the bursa influences the incidence of the disease. Resistant RPRL line 6₃ chickens have been observed to have significantly smaller bursas and consequently fewer bursal cells than susceptible chickens (unpublished data). Therefore, we compared the bursas from RAV-1-infected Hyline SC and FP chickens to ascertain whether the development of this organ is similar in both types of animals. Bursas from RAV-1-infected chickens were fixed in 10% neutral buffered Formalin and weighed. No significant difference in bursal mass was observed in these lines when chickens up to 10 weeks of age were examined (Fig. 1). Additionally, no difference in the cell size distribution of the bursal population or the percentage of lymphocytes expressing cell surface IgM, as assessed by flow cytometry, was observed in these lines (data not shown). Using these experimental approaches, therefore, we detected no difference in the target tissue of these chickens.

Viremia in susceptible and resistant chickens. One of the goals of this study was to determine to what extent the chickens of the two different lines exhibited viremia. Therefore, the presence of infectious virus in the plasma of SC and FP chickens was assessed by obtaining plasma at different times after virus infection. The plasma samples were assayed for the presence of infectious virus as described above. A compilation of data from several experiments is presented in Table 1. We observed a significant difference in the percentage of SC and FP chickens which were viremic at 1 to 2.5 weeks after virus infection. One week after infection, there were more than five times as many viremic SC chickens as there were viremic FP chickens. However, the percentage of viremic SC chickens was observed to decrease

after 2.5 weeks, whereas that of the FP chickens increased, so that by 4 weeks after infection, no difference in the incidence of viremia was observed between these two lines. The titer of virus detected in the plasma of viremic birds was comparable at both 2 and 4 weeks after infection in both FP and SC chickens. Twenty-four weeks after virus infection, viremia was not detected in any Hyline SC chickens, including those animals which had developed widespread tumor metastasis.

Since the data presented above were obtained by examining plasma obtained from individual chickens at a single time point rather than sequentially as a function of time, these data must be interpreted as the percentage of chickens exhibiting viremia at a specific time after infection. The data indirectly suggest that viremia is transiently exhibited by both susceptible and resistant chicken lines. Furthermore, it appeared that FP chickens, which were not viremic 1 week after infection, subsequently became viremic. Moreover, both resistant and susceptible chickens which exhibited viremia appeared to have eliminated the virus after 5 weeks of infection. Consequently, an alternative approach was used to determine the frequency and the kinetics of viremia occurring in these animals. The development of viremia in these chickens was evaluated directly by obtaining plasma from several consecutive bleedings of individual animals (Table 2). These data are consistent with those presented in Table 1, which directly demonstrate the transient nature of viremia in both resistant and susceptible chickens. In each of two experiments in which cumulative data were acquired, approximately 90% of the susceptible line of chickens (SC) exhibited evidence of viremia during some part of the experimental period, compared with 53% of the chickens in the resistant line (FP). Although the duration of viremia in most of the chickens in these two lines was approximately 4 weeks, the onset of viremia in Hyline SC chickens was much more rapid than in Hyline FP chickens. Viremia was still present in many chickens in the resistant line 5 weeks after infection at a time when, for the most part, no virus was detected in the susceptible chickens. Therefore, compared

TABLE 1. Analysis of viremia in Hyline SC and FP chickens^a

Wk post-infection	Viremia in FP chickens ^b (%)	Titer (TCID/ml) ^c	Viremia in SC chickens ^b (%)	Titer (TCID/ml)
1	7/41 (17)		27/30 (90)	
2	6/20 (30)	4×10^3 – 4×10^5	20/20 (100)	4×10^3 – 4×10^5
2.5	19/40 (48)		30/33 (91)	
4	5/12 (42)	4×10^3 – 4×10^5	5/12 (42)	4×10^3 – 4×10^5
5	10/32 (31)		2/21 (10)	
10	0/9 (0)		0/8 (0)	
16	1/10 (10)	4×10^3 ^d	0/10 (0) ^e	
24	1/20 (5)	4×10^2	0/10 (0)	

^a One-day-old chicks were infected intravenously with 2×10^5 IU of RAV-1. Plasma was obtained at different times after infection and assayed for the presence of infectious virus as described in the text. The data are taken from multiple experiments.

^b Values indicate the number of chickens with greater than 4×10^2 IU/ml of plasma per number of chickens tested.

^c Six plasma samples, collected from SC and FP chickens at 2 weeks after infection, and five plasma samples, collected at 4 weeks after infection, were titrated for the presence of infectious virus. TCID, Tissue culture infective dose.

^d One chicken, sequentially bled, was viremic at both 16 and 24 weeks after infection.

^e Bursal lymphomas were found in 7 of the 10 Hyline SC chickens examined sequentially 16 and 24 weeks after infection.

TABLE 2. Analysis of viremia in individual FP and SC chickens examined sequentially during a 10-week period^a

Wk post-infection	Chicken line ^b			
	FP		SC	
	Expt 1 (%)	Expt 2 (%)	Expt 1 (%)	Expt 2 (%)
1	3/23 (13)	ND ^c	11/13 (85)	ND
2.5	11/23 (48)	4/9 (44)	12/13 (92)	7/8 (88)
Cumulative	11/23 (48)	4/9 (44)	12/13 (92)	7/8 (88)
5	8/23 (35)	2/9 (22)	2/13 (15)	0/8 (0)
Cumulative	12/23 (52)	5/9 (56)	12/13 (92)	7/8 (88)
10	ND	0/4 (0)	ND	0/3 (0)
Cumulative		5/9 (56)		7/8 (88)

^a One-day-old chicks were infected intravenously with 2×10^5 IU of RAV-1. The chickens were bled sequentially at several different times, and the plasma samples were assayed for infectious virus as described in the text.

^b Values indicate the number of chickens with greater than 4×10^2 IU/ml of plasma per number of chickens tested.

^c ND, Not determined.

with Hyline FP chickens, analysis of the kinetics of infection of the Hyline SC chickens revealed a more rapid onset of viremia, with approximately twice as many chickens exhibiting viremia. In both lines, however, the viremia was transient, lasting approximately 4 weeks and exhibiting virus titers between 4×10^3 and 4×10^5 IU/ml of plasma.

Analysis of integrated viral DNA. The replication of retroviruses involves the formation of a DNA replicative intermediate that is capable of integrating in multiple sites within the host cell genome, leading to the formation of the provirus (27). To compare further the virus infection occurring in SC and FP chickens, we analyzed the extent of viral integration present in the DNA of several tissues obtained at different times after virus infection. Since the chickens used in this study also have endogenous virus (*ev*) sequences, a DNA restriction fragment which is characteristic for the exogenous virus RAV-1 must be used to distinguish RAV-1 from *ev* sequences. By quantifying the intensity of this DNA fragment, revealed by nucleic acid hybridization analysis, the extent of viral integration can be determined.

The restriction endonuclease *Eco*RI cuts the provirus four times, producing three internal fragments. Since the two outermost recognition sites are absent in the *ev* sequences present in the chickens used in this study, *Eco*RI digestion of RAV-1 produces two internal fragments not produced by *Eco*RI digestion of *ev* sequences (10, 13). We used the internal 2.1-kb DNA fragment derived from the 5' *gag* portion of the provirus as an indicator of RAV-1 infection.

DNA was prepared from erythrocytes and bursal cell suspensions obtained from uninfected and RAV-1-infected Hyline SC and FP chickens at several times after virus infection. The DNA was digested with *Eco*RI; subjected to agarose gel electrophoresis, Southern DNA transfer, and nucleic acid hybridization with the ³²P-labeled 5'-virus-specific probe; and examined for the presence of the 2.1-kb *Eco*RI-specific DNA fragment derived from RAV-1 (Fig. 2). The presence of the 2.1-kb DNA fragment can be seen in bursa DNA (Fig. 2A, lanes 4, 5, and 6) and in erythrocyte DNA (Fig. 2B, lanes 2, 3, 4, 5, and 6). The data presented in Fig. 2 are representative of those obtained from several experiments, the results of which are displayed in Table 3. These data, which reveal the transient nature of viral integra-

tion occurring in the tissues of SC and FP chickens, resemble those from the analysis of viremia presented above. As early as 1 week after infection, 87% of the Hyline SC chickens exhibited evidence of viral integration in the DNA obtained from erythrocytes and bursal cells. A similar incidence of integration was observed at 2.5 weeks. However, 5 weeks after virus infection, only 52% of the animals displayed integrated viral sequences, and by 7.5 weeks after virus infection, no integrated virus was detected in bursal tissue of the SC chickens. Furthermore, no integrated viral sequences were found in DNA preparations obtained from the erythrocytes of 31 Hyline SC chickens examined from

FIG. 2. Detection of the *Eco*RI-specific 2.1-kb DNA fragment indicative of RAV-1 infection. DNA samples, prepared from bursal cells (A) or erythrocytes (B) obtained 2 weeks after virus infection, were digested by *Eco*RI endonuclease and analyzed by electrophoresis, Southern DNA transfer, and hybridization to the 5'-viral DNA probe. Ten micrograms of DNA obtained from three infected FP chickens (lanes 1 to 3) and three infected SC chickens (lanes 4 to 6) were analyzed. DNA from RAV-1-infected FP (FP+) or SC (SC+) chicken embryo fibroblasts was used as a positive control. The 2.1-kb RAV-1 DNA fragment and the 8.7-kb *Eco*RI-specific junction fragment of *ev*-1 are indicated to the left of the gels.

TABLE 3. Presence of the RAV-1-specific *Eco*RI 2.1-kb DNA fragment in bursal and erythrocyte DNA obtained from infected FP and SC chickens^a

Wk post-infection	Expt no.	Chicken line and DNA source ^b			
		FP		SC	
		Bursa (%)	Erythrocyte (%)	Bursa (%)	Erythrocyte (%)
1	1	1/15 (7)	3/15 (20)	13/15 (87)	13/15 (87)
2.5	2	0/5	ND ^c	3/5	ND
	3	1/3	1/3	3/3	3/3
	4	2/5	2/5	3/4	4/5
	Total	3/13 (23)	3/8 (38)	9/12 (75)	7/8 (88)
5	1	3/10	5/10	4/10	8/10
	2	1/5	2/5	1/6	3/6
	3	1/5	1/5	1/5	3/5
	4	0/6	0/6	1/6	0/6
	Total	5/26 (19)	8/26 (31)	7/27 (26)	14/27 (52)
7.5	2	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
10	2	1/5 (20)	1/5 (20)	0/5 (0)	0/5 (0)
24	5	NA ^d	0/12 (0)	NA	0/21 (0)

^a One-day-old chicks were infected intravenously with 2×10^5 IU of RAV-1. DNA was prepared from erythrocytes and bursal cells and analyzed for the presence of the 2.1-kb *Eco*RI DNA fragment specific for RAV-1 infection. Chickens were considered positive or negative for presentation of these data as described in the text.

^b Values indicate the number of chickens positive for virus per number of animals tested.

^c ND, Not determined.

^d NA, Not available. Bursal tissue, which normally begins involution in the chicken at 14 to 16 weeks of age, was absent from these animals.

7.5 through 24 weeks after virus infection, including those chickens found to have widespread metastatic lymphomas.

In contrast to the observations made in SC chickens, only 20% of the RAV-1-infected Hyline FP chickens exhibited evidence of viral integration 1 week after infection. However, by 2.5 and 5 weeks after infection, 38 and 31%, respectively, of the Hyline FP chickens exhibited viral integration. At 7.5 through 24 weeks, only 1 of 22 chickens examined exhibited viral integration in the DNA obtained from erythrocytes or bursal cells. These results differ significantly from those of Fung et al. (10), who compared RAV-1 viral integration in two other chicken lines that are resistant and susceptible to bursal lymphoma development and that are discussed below.

Loss of integrated viral sequences. The results presented in Table 3 revealed that viral sequences were eliminated from the cellular DNA prepared from either bursal cells or erythrocytes from both chicken lines. The absence of viremia 8 weeks and later after infection was consistent with the loss of integrated viral DNA sequences, demonstrating that the course of RAV-1 infection observed in these animals was distinct from that observed in other chicken lines (10, 23). Analysis of the data presented in Tables 3 through 5 revealed

lyzed for the presence of the 2.1-kb *Eco*RI-specific DNA fragment as described in the legend to Fig. 2. All three samples were prepared 5 weeks after virus infection from the corresponding FP (lanes 1 to 3) or SC (lanes 4 to 6) chicken. The FP+ and SC+ control lanes are as designated in the legend to Fig. 2. The 2.1-kb RAV-1 DNA fragment and the 8.7-kb *Eco*RI-specific junction fragment of *ev-1* are indicated to the left of the gels.

FIG. 3. Absence of the *Eco*RI-specific 2.1-kb DNA fragment from splenic DNA of RAV-1-infected chickens. DNA from bursal cells (A), erythrocytes (B), and splenic lymphocytes (C) was ana-

TABLE 4. Viremia and integrated viral DNA in tissues from individual Hyline SC chickens^a

Bird no.	Presence of viremia at the following times after infection (wk) ^b :			Presence of integrated viral DNA sequences at 5 weeks after infection in the following ^c :		
	1	2.5	5	Bursa	Erythrocyte	Spleen
1	+	+	0	0	+1	0
2	+	+	0	0	+1	0
3	+	+	0	0	+1	0
4	+	+	0	+1	+1	0
5	+	+	0	0	0	0
6	+	+	+	+3	+2	0
7	+	+	0	0	0	0
8	+	+	0	+1	+1	0
9	+	+	0	0	+1	0
10	+	+	+	+1	+1	0
11	-	+	0	0	+2	-
12	-	0	0	0	0	-
13	-	+	0	0	0	-
14	-	+	0	+2	+2	-
15	-	+	0	0	+1	-

^a Individual birds, injected intravenously with RAV-1, were bled sequentially at 1, 2.5, and 5 weeks after infection, and the plasma was assayed for the presence of infectious virus. At 5 weeks after infection, DNA was prepared from bursal cells, erythrocytes, and splenic lymphocytes. DNA samples were examined for the presence of the 2.1-kb *EcoRI* DNA fragment specific for RAV-1 infection.

^b +, Positive; 0, negative; -, not done.

^c +3, +2, and +1 indicate, respectively, approximately 1, 0.5, and 0.1 copy per haploid genome. 0, Negative; -, not done.

additional information concerning the course of viral infections occurring in these chickens.

Examination of RAV-1-infected Hyline SC and FP chickens 5 weeks after infection, at a time when the number of animals exhibiting viral integration was declining, revealed approximately two times as many birds that contained viral sequences integrated in their erythrocyte DNA than in their bursal cell DNA, suggesting that the viral marker was eliminated more rapidly from the bursa. This could result from the deletion of viral sequences from the DNA of these cells, the existence of a surveillance mechanism capable of killing the virus-infected cells within the bursa, or a lethal effect exerted by the virus during B-cell differentiation. Alternatively, since during normal B-lymphocyte differentiation cells from within the bursa migrate to bursa-dependent areas within the spleen, maturation, which is accompanied by a change in venue, could account for the loss of the viral DNA marker from the bursa. If the latter explanation were correct, we would expect to find integrated viral sequences in the DNA prepared from splenic lymphocytes.

To test this hypothesis, DNA was prepared from Ficoll-Hypaque density gradient-purified splenic lymphocytes 5 weeks after RAV-1 infection and was examined for the presence of the characteristic *EcoRI* 2.1-kb exogenous virus DNA fragment. No viral sequences were detected in any of the 10 Hyline SC splenic lymphocyte DNA preparations examined, regardless of whether viral sequences were present in the bursal cell or erythrocyte DNA obtained from the same chicken (Fig. 3 and Tables 4 and 5). Additionally, only 1 of the 10 Hyline FP splenic samples exhibited a very low level (+1) of viral integration. Moreover, the absence of detectable viral integration in these splenic lymphocyte preparations, obtained primarily from chickens which had viral DNA sequences integrated in their erythrocytes, attests to the purity of these cell suspensions. In addition, since

integrated viral sequences generally were not detected in splenic lymphocytes obtained from chickens which were concurrently viremic, we suggest that these cells may be resistant to infection or upon infection become eliminated from the spleen.

A further difference is illustrated between the SC and FP lines (Tables 4 and 5). The SC chickens responded to the infection as a uniform population. They exhibited viremia at 1 and 2.5 weeks. By 5 weeks after infection, the circulating virus was almost eliminated. In contrast, the FP chickens responded as two distinct populations. Approximately one-half showed no evidence of infection. The other half was characterized by viremia and the presence of integrated viral sequences. Additionally, this group was more heterogeneous with respect to the presence of viremia and integrated viral DNA than the SC chickens. The factor(s) that influences the response of the FP chickens to RAV-1 infection and that contributes to the resistance to lymphoma development is unknown.

DISCUSSION

The principal aim of this study was to analyze the extent of virus infection in chickens which are resistant or susceptible to the development of ALV-induced bursal lymphomas so that we could determine whether spread of the virus was comparable in both chicken lines. Two independent indicators of virus infection which were analyzed at several different times after experimental infection were used to achieve this objective. Plasma from RAV-1-infected chickens was assayed for the presence of infectious virus, whereas cellular DNA, prepared from different tissues, was examined for the presence of integrated exogenous viral DNA sequences. Although the former method is more sensitive, the latter method permits the definitive identification of infected cells.

TABLE 5. Viremia and integrated viral DNA in tissues from individual Hyline FP chickens^a

Bird no.	Presence of viremia at the following times after infection (wk) ^b :			Presence of integrated viral DNA sequences at 5 weeks after infection in the following ^c :		
	1	2.5	5	Bursa	Erythrocyte	Spleen
1	+	+	+	+2	+1	0
2	0	0	0	0	0	0
3	0	0	+	0	0	0
4	0	+	+	+3	+2	0
5	0	+	0	0	+1	0
6	+	+	+	+2	+3	+1
7	0	+	+	0	+1	0
8	0	0	0	0	0	0
9	0	+	0	0	0	0
10	0	0	0	0	0	0
11	-	0	0	0	0	-
12	-	+	+	+2	+2	-
13	-	0	+	0	0	-
14	-	+	0	0	0	-
15	-	0	0	0	0	-

^a Individual birds, injected intravenously with RAV-1, were bled sequentially at 1, 2.5, and 5 weeks after infection, and the plasma was assayed for the presence of infectious virus. At 5 weeks after infection, DNA was prepared from bursal cells, erythrocytes, and splenic lymphocytes. DNA samples were examined for the presence of the 2.1-kb *EcoRI* DNA fragment specific for RAV-1 infection.

^b +, Positive; 0, negative; -, not done.

^c +3, +2, and +1 indicate, respectively, approximately 1, 0.5, and 0.1 copy per haploid genome. 0, Negative; -, not done.

Comparison of plasma from susceptible and resistant chickens revealed a less than twofold difference in the number of animals which became viremic. Although the duration of viremia and the titer of virus in the plasma of these two lines of chickens were comparable, the onset of viremia was delayed approximately 1 week in the resistant animals. This result may reflect the ability of SC chicks to replicate the virus more efficiently compared with FP chicks. Alternatively, we cannot exclude the possible existence of neutralizing substances, such as maternal antibody, in the plasma of resistant birds, which would effectively limit the inoculating dose. If present, however, this neutralization does not adequately protect these animals from subsequently developing viremia, as several FP chicks which were not viremic at 1 week became viremic 1.5 weeks later. Furthermore, 5 weeks after infection, the incidence of viremia among the FP birds was virtually unchanged, whereas the incidence among the SC birds was reduced tenfold. However, virus does not persist in the plasma of either host, as we detected viremia in only 1 of the 47 chickens that we examined at 10 through 24 weeks postinfection. These results directly demonstrate the transient nature of the viremia that occurs in these animals. This observation differs significantly from that of Robinson et al. (23), who analyzed viremia in K₂₈ chickens, a line susceptible to lymphoma development. They reported that most chickens had high titers of virus several months postinfection.

It should be noted that the results of the present study indicate that the FP chickens are heterogeneous and, based on their response to infection with RAV-1, consist of two populations. Approximately 50% of the birds exhibit no detectable markers of viral infection. The other half demonstrates levels of circulating infectious virus and integrated viral DNA similar to those observed in the SC chickens. The mechanisms of resistance in these two populations are most probably different.

We have shown that the endogenous virus (*ev*) loci are different in FP and SC chickens. Although neither line produces infectious virus, FP chickens possess the *ev*-3 locus which confers a *chf*⁺ *gs*⁺ phenotype upon these animals, whereas the SC animals are *chf*⁻ *gs*⁻. Recently, the kinetics of development and extent of the humoral immune response to RAV-1 infection were shown to be quite different in *ev*-3⁺ and *ev*-3⁻ birds (12). *ev*-3⁺ birds responded later, had lower antibody titers, and subsequently demonstrated a more prolonged viremia than *ev*-3⁻ birds. Furthermore, *ev*-3⁺ chickens demonstrated evidence of immunological tolerance to RAV-1. At present, we do not know what effect this genetic locus, which is present in our resistant line, exerts upon viral infection in these animals. Moreover, we do not know what role, if any, active immunity plays in resolving RAV-1 infection in these lines.

The target organ for ALV-induced lymphomas is the bursa of Fabricius (20). We analyzed the extent of RAV-1 integration present in the DNA of bursal cells obtained from resistant and susceptible chicken lines at different times postinfection. In accordance with the data obtained from the analysis of viremia in susceptible and resistant animals, viral integration in RAV-1-infected SC chickens was more rapid in onset than that observed in FP chickens. However, the amount and the persistence of integrated viral sequences present in individual animals from both lines were comparable. These viral sequences were not detected later than 5 weeks postinfection, except in end-stage tumors as previously noted (Baba and Humphries, in press). When compared with the results of Fung et al. (10), our findings are strikingly

different. Analysis of viral integration in bursal tissues obtained from chickens selected for resistance (RPRL line 6₃) and susceptibility (RPRL cross 7₁ × 15₁₅) to lymphoma development revealed the 2.1-kb *Eco*RI viral marker in six of six susceptible chickens and five of five resistant chickens examined. Furthermore, each of the RPRL animals demonstrated viral integration in the bursa 8 weeks postinfection at a time when our chickens were no longer viremic and exhibited a negligible incidence of viral integration in any of the tissues studied. It would be of interest to determine whether RPRL chickens eliminate integrated viral sequences at some later time.

Comparable levels of viral integration were observed in the DNA obtained from various tissues, including erythrocytes and bursal cells, at 1 and 2.5 weeks postinfection. However, by 5 weeks after infection, several SC and FP chickens had detectable viral sequences in their erythrocyte DNA but not in their bursal DNA. Several explanations could account for this observation. It is conceivable that concomitant with cellular differentiation at a time when complex DNA rearrangements are occurring within the cell (26), viral sequences are deleted from the host genome. Alternatively, bursal lymphocytes, upon subsequent differentiation, may migrate from this central lymphoid organ to peripheral lymphoid organs such as the spleen (2, 3). When we examined DNA from splenic lymphocytes obtained at 5 weeks postinfection for the presence of the *Eco*RI 2.1-kb viral DNA marker, none was detected. The absence of detectable viral sequences in these cells was curious. Infected bursal lymphocytes, which display cell surface viral antigens, may become accessible to effector mechanisms responsible for host immune surveillance. This may allow these cells to be eliminated once they migrate from what may be an immunologically privileged site to the periphery. Since no viral DNA sequences were detected in splenic lymphocytes, even when obtained from animals that were concurrently viremic, these cells may be uniquely resistant to viral integration. Alternatively, the expression of viral antigens on their cell surface membrane may lead to their efficient elimination. However, since our analysis of splenic cells was limited to 5 weeks postinfection, we do not know whether these cells contain integrated viral DNA at other times after infection. Erythrocytes, which probably do not express viral surface glycoproteins, could persist until they undergo normal physiological turnover.

Although ALV-induced lymphomas express IgM, the target cell in which tumorigenesis is initiated may not express this phenotype. Human chronic myelocytic leukemia is characterized by an overabundance of myelocytes and more mature granulocytic cells. The clonally derived neoplastic cell in this malignancy, however, has been shown to be a hematopoietic stem cell (9). Moreover, pre-B-cell neoplasms induced by Abelson murine leukemia virus continue to differentiate during in vitro culture (1). We saw no gross differences in the development of the bursa of Fabricius in susceptible and resistant animals. We would be unable, however, to detect subtle differences by the techniques used.

It has been suggested that the development of focal follicular hyperplasia is important in the tumorigenic process (6, 17). The significance and the molecular basis of this intriguing lesion that occurs within the target organ for lymphoma development is not known. It is possible that hyperplasia results from a rare virus-cell interaction that occurs most frequently in a very immature bursal environment. RAV-1 infection of FP chicks did not produce viremia

or integrated viral DNA in a significant number of animals until 2.5 weeks after infection. Delayed viral integration into bursal DNA may reduce the incidence of hyperplasia to a frequency which is insufficient to initiate tumorigenesis. Consequently, hyperplasia and the concomitant development of LL would occur only within the bursas of susceptible animals.

Lymphomagenesis is believed to be a multistep process, which is an hypothesis that is consistent with the long latent period that characterizes this malignancy (17). Therefore, the probability that a lymphoma will develop *in vivo* is predicated upon the frequency or rate of occurrence of each requisite step in this process. At least two genetic abnormalities appear to play a role in ALV-induced tumorigenesis. The enhanced expression of *c-myc* is effected by viral integration in the vicinity of this locus. Our data are consistent with the fact that this is an early event, as most of the viral integration occurred in susceptible and resistant animals within the first 5 weeks after virus infection. Another gene (*B-lym*), detectable by transfection of tumor DNA into NIH 3T3 mouse cells, also appears to be involved in the generation of these tumors. We do not know how or when this gene participates during tumorigenesis. Furthermore, whether additional steps or events are needed which allow these cells to evade host immune surveillance mechanisms has not been determined.

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