Low Oncogenic Potential of Avian Endogenous RNA Tumor Virus Infection or Expression [1, 2]

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SUMMARY—Of chickens either spontaneously producing or exogenously infected in ovo with Rous-associated virus, type 0 (RAV-0), an endogenous virus of the chicken, only 1 died with lymphoid leukosis (LL), the most common neoplasm associated with the leukosis–sarcoma virus group. Because the chickens were not kept in strict isolation, it could not be assumed that the one LL was induced by RAV-0. In contrast, RAV-1-infected chickens from the same lines had a high incidence of LL and other neoplasms. Over 800 chickens of several inbred lines were maintained in plastic isolators free of exogenous avian leukosis–sarcoma virus infection for from 500 to nearly 1,000 days of age. No LL was observed, even though some lines are known to produce RAV-0 spontaneously or to express inherited gs antigen. Three neoplasms of unknown etiology were observed, but none generally associated with leukosis virus infection. We concluded that avian endogenous virus expression had little, if any, oncogenic potential, and that exogenous avian leukosis viruses were responsible for most naturally occurring neoplasms.—J Natl Cancer Inst 55: 685–689, 1975.

Rous-associated virus, type 0 (RAV-0) was recognized by Vogt and Friis (*1*) as an endogenous virus of the chicken, spontaneously released by some chick embryo cultures of Regional Poultry Research Laboratory (RPRL) line 7. RAV-0 had properties of a leukosis–sarcoma virus belonging to subgroup E. We have shown that the spontaneous release of RAV-0 from line 7, subline 2 (7_2), and from the closely related line 100 chick embryos is probably controlled by dominant genes of the host that are independent of the gene controlling the expression of gs antigen (*2, 3*).

Nucleic acid hybridization studies of cellular DNA with RAV-0 RNA indicate that the *RAV-0* genome is completely homologous to the cellular genome, whereas known exogenous sarcomagenic and lymphomagenic viruses of the leukosis–sarcoma group contain RNA sequences not homologous to cellular DNA or the RAV-0 RNA. Conversely, RAV-0 RNA contains some sequences not found in the exogenous viruses studied but appears to have only sequences found in normal cell DNA (*4–6*). If RAV-0 were oncogenic, one would conclude that normal cell DNA contains the genetic information for the development of neoplasms (the oncogene) as well as the potential for infectious virus production (the virogene) (*7*). If RAV-0 were nononcogenic, the cell either would lack oncogenic potential or the oncogene would reside in DNA sequences not homologous to RAV-0 RNA.

The purpose of this study was to evaluate the potential of endogenous and exogenous RAV-0 infection for the induction of lymphoid leukosis (LL), the most common spontaneous neoplasm of the chicken, associated with the leukosis–sarcoma group of viruses.

MATERIALS AND METHODS

Chickens.—Line 100 is maintained at Beltsville by backcrossing to line 7_2 (*8*). The cultured cells of both these lines spontaneously release RAV-0. Line 7 is resistant to exogenous infection with subgroup E virus, whereas line 100 is segregating for susceptibility (*8*). Half the chickens of line 100 were expected to carry the tvb^{s1} allele for susceptibility to subgroup B and E viruses associated with the R_1 erythrocyte isoantigen [(*9*); Crittenden LB, Motta JV: In preparation]. Fluids from susceptible cells of line 100 contain RAV-0 in a much higher titer than do those from resistant cells, and presumably the titer is also higher in the serum of susceptible chickens of this line (*2, 10*). Line 15 cells are highly susceptible to RSV [8] (RAV-0) and RAV-0, but this line has a low frequency of infection with RAV-0 (*2*). The lines and sublines maintained at RPRL have been described by Stone (*11*), and at least some are known to carry gs antigen and produce infectious RAV-0 (Okazaki W, Crittenden LB: Unpublished data). All were maintained in filtered-air–positive-pressure isolators throughout their lifetime and with one exception had no evidence of infection with leukosis–sarcoma viruses of subgroups A, B, C, or D.

Virus stocks.—RAV-1 was originally obtained as a purified seed stock from Peter K. Vogt (School of Medicine, University of Southern California) and was propagated by us in C/B cell cultures. The RAV-0 stock was supernatant from line 100 cells spontaneously producing RAV-0 and contained about 10^7 line 15 infectious units per ml. The RSV(RAV-0) stock was originally obtained from H. Hanafusa (Rockefeller University) as RSVβ(0) and was propagated in line 100 C/A cells which spontaneously release RAV-0. The RSV(RAV-1) (subgroup A) and RSV(RAV-2) (subgroup B) pseudotypes were originally obtained from Vogt and were propagated in C/B and C/A cells, respectively.

Cell culture assays.—The basic cell culture procedures were those described by Vogt (*12*) as modified (*13, 14*). Blood was collected from a wing vein in heparinized syringes and the plasma stored at −70° C for assay for lymphoid leukosis viruses (LLV). We detected virus infection in the Beltsville experiment by adding 0.2 ml plasma to 60-mm plates containing about 1.5×10^6 line 15 C/C tertiary cells plated in medium containing 2 μg/ml DEAE-dextran or Polybrene. After two passages and 14 or 15 days, cell fluids were collected and stored at −70° C for future assay. The cells were scraped off the plates and suspended in Veronal-buffered saline, and 20% (vol/vol) extracts were made by freezing and thawing three times. Complement fixation (CF) tests for gs antigen were conducted with a pig antiserum to purified avian myeloblastosis virus [(*10*); Purchase HG, Okazaki W: Personal communication]. Tests were made at 1/2

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[8] RSV = Rous sarcoma virus.

and 1/4 dilution, and a 3+ reaction in the absence of anticomplementary reactions was considered positive (*15*). Positive and negative controls were used in each assay. Any test in which the controls were not as expected was repeated. This assay was expected to detect LLV of subgroups A, B, D, and E. If the CF test was postive, supernatants were assayed for subgroups A, B, and D viruses by the phenotypic mixing (PM) test (*16*). Line 15 cells were infected with approximately 10^5 focus-forming units of RSV(RAV-0) in the presence of 2 μg/ml DEAE-dextran or Polybrene, and 0.2 ml of the test supernatant was added. After 4 days in culture, the cells were highly transformed, and 0.5 ml of supernatant was tested on Japanese quail cells susceptible to subgroup E and line 6 or SPAFAS[9] C/E cells. High focus counts on the C/E cells indicated that a virus other than subgroup E was present. If high focus counts were not observed on the quail cells, the assay was repeated, on the assumption that the line 15 test cells were not adequately infected with RSV(RAV-0). Assays for virus infection in the RPRL birds were made on similarly obtained plasmas or embryos by either the nonproducer (NP) (*14*) or the PM (*16*) test.

We detected serum neutralization activity by adding 0.1 ml of a 1/5 plasma dilution to 0.1 ml of a dilution of the appropriate RSV pseudotype expected to give 100–300 foci when assayed on susceptible cells. The mixture was incubated for 40 minutes at 37° C, and 0.1 ml of the mixture was tested on either line 15 C/C or SPAFAS C/E cells plated in medium containing 2 μg/ml DEAE-dextran or Polybrene. A 90% reduction in focus count was considered positive.

Design of Beltsville experiment (table 1).—Lot 1 was composed of line 100 × 7, which spontaneously released RAV-0 and therefore had an endogenous infection. Lot 2 was composed of line 15 chickens naturally infected at a low rate with RAV-0 but inoculated with RAV-0 as embryos. This line is highly susceptible to exogenous infection with RAV-0, and it was expected to become infected. Lots 4, 5, and 6 were hatched 3 weeks after the first hatch.

Inoculation of chickens.—For the Beltsville experiment, embryos were inoculated iv at day 12 of incubation with 0.05 ml virus dilution. The RAV-0 inoculum was undiluted cell fluid containing approximately 5×10^5 infectious units. The RAV-1 inoculum contained approximately 5×10^2 infectious units. The eggs were returned to the incubator to be hatched (*17*).

Management of chickens.—The chicks were individually wing banded, and the groups within each of the two hatches were randomly mixed. Sexes were separated at about 15 weeks, but birds in the second hatch, all inoculated with RAV-1, were never intermingled with those of the first hatch not inoculated with RAV-1. All birds that died between 2 and 54 weeks of age were necropsied, and the bursa of Fabricius was inspected for grossly visible nodules of lymphoid tumor cells. Parts of the sciatic and brachial nerves and bursa were taken from each chicken to facilitate differential diagnosis between Marek's disease (MD) and LL. Tissues were also taken from any organ that on gross examination appeared to have evidence of a neoplasm. Final diagnoses were based on histopathologic examination. All birds were given a standard series of vaccinations including immunization with herpesvirus of turkeys (HVT) for MD when they were 1 day old, Newcastle disease virus, fowl pox, and avian encephalomyelitis. The vaccines, except HVT, were commercial and all were known to be free of LLV of subgroups A, B, C, and D.

A description of the management of isolated chickens at RPRL was given by Stone (*11*). On initial isolation, all birds were screened for exogenous infection with LLV, subgroups A–D, by the NP test (*14*) for virus and the serum neutralization test for antibody. Only birds with negative tests were introduced into the reproduction units producing the next generation.

In subsequent generations, a 25% sample of birds was bled for antibody to subgroups A and B viruses when they were approximately 18 weeks of age and at the end of the reproductive period. All were negative except as noted in "Results."

All birds were necropsied and examined for gross lesions of LL or MD. Tissues for histopathologic examination were taken only to confirm questionable diagnoses.

Detection of the R_1 antigen.—Red blood cells were collected from the wing vein of the line 100 chickens about 15 weeks of age and tested by standard agglutination procedures with a specific R_1 antiserum obtained from W. E. Briles (Northern Illinois University) (*9, 18*).

RESULTS

Mortality and Infection in the Beltsville Experiment

Mortality with neoplasms (table 1).—Only 1 neoplasm occurred in hatch 1: a case of LL in a line 15 chicken of lot 2 that had been inoculated with RAV-0 alone. Many neoplasms developed in all lots of hatch 2, all of which had been inoculated with RAV-1. The largest category was LL followed by other neoplasms associated with viruses of the leukosis–sarcoma groups including sarcomas, nephroblastomas, hemangiomas, and erythroblastosis). MD also occurred in hatch 2, despite the fact that all birds were vaccinated with HVT. Mortality with nonneoplastic lesions was higher in hatch 2 than in hatch 1. All categories of mortality in hatch 2 were significantly higher than those in hatch 1 when tested by chi square (*19*).

Virus infection.—To interpret the mortality data, we needed to establish that lines 100 and 15 were actually infected with subgroup A and E viruses as planned. The results in table 2 are given by the R_1 status of lots 1 and 4, because the R_1-positive chickens of line 100 were expected to be highly susceptible to RAV-0 and were expected to have a higher titer of endogenous RAV-0 in their plasmas than were the R_1 negative chickens (*2*).

No subgroup A, B, or D virus was detected in hatch 1 at either 6 or 16 weeks. However, all lots had become infected with RAV-0 or a similar subgroup E virus. The difference in rate of subgroup E virus infection between the R_1-positive and -negative line 100 birds was less than that expected from previous experience (*2*). However, 13 of 20 were infected at 6 and 16 weeks (a fairly high rate). Inoculation of line 15 with RAV-0 was effective: All tested chickens of lot 2 had a subgroup E virus in their plasmas at 6 weeks but only 4 of 18 did at 16 weeks. Lot 3 birds became infected at a low rate; a slight increase by 16 weeks suggested contact transmission of the inoculated RAV-0.

Infection rates in hatch 2 were more difficult to inter-

[9] SPAFAS, Inc., Norwich, Conn. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

TABLE 1.—*Mortality with neoplasms after inoculation with, or exposure to, RAV-0, RAV-1, or both*

Hatch	Lot	Mating (♂ × ♀)	Exposure [a]		Number of chickens (2 wk)	Number dead with [b]						Percent dead with [b]			
			RAV-0	RAV-1		LL	ON+ LL	ON	MD	LL+ MD	Other	LL	ON	MD	Other
1	1	100 × 7	E	None	128	0	0	0	0	0	9	0.0	0.0	0.0	7.0
	2	15 × 15	I	None	42	1	0	0	0	0	6	2.4	0.0	0.0	14.3
	3	15 × 15	C	None	47	0	0	0	0	0	6	0.0	0.0	0.0	12.8
2	4	100 × 7	E	I	52	8	2	3	2	1	12	21.2	9.6	5.8	23.0
	5	15 × 15	I	I	12	4	0	1	0	0	3	33.3	8.3	0.0	25.0
	6	15 × 15	C	I	13	4	0	1	1	0	2	30.8	7.7	7.7	15.4

[a] E = endogenous (RAV-0 occurs naturally in line 100 × 7); I = inoculation; C = contact.
[b] ON = other neoplasms usually associated with the leukosis-sarcoma viruses; other = deaths with no recognizable neoplasms.

TABLE 2.—*Fraction of plasmas positive for virus (subgroups A and E) or antibody (subgroups A, B, or E) after inoculation with or exposure to RAV-0, RAV-1, or both*

Hatch	Lot	Mating (♂ × ♀)	R_1 antigen status	Exposure [a]		Virus (6 wk)		Virus (16 wk)		Antibody (16 wk)			Antibody (54 wk)
				RAV-0	RAV-1	A	E [b]	A	E [b]	A	B	E	A
1	1	100 × 7	+	E	None	0/6	5/6	0/6	4/6	0/6	0/6	1/6	3/8
	1	100 × 7	−	E	None	0/14	8/14	0/14	9/14	0/13	0/13	0/13	4/12
	2	15 × 15		I	None	0/20	20/20	0/18	4/18	0/19	0/19	17/19	2/40
	3	15 × 15		C	None	0/20	5/20	0/18	7/18	0/17	0/17	2/17	16/38
2	4	100 × 7	+	E	I	4/10	6/6	4/10	6/6	0/10	0/10	0/10	
	4	100 × 7	−	E	I	5/8	3/3	5/8	1/3	0/8	0/8	0/8	
	5	15 × 15		I	I	10/12	1/2	8/12	4/4	7/12	0/12	2/12	
	6	15 × 15		C	I	12/13	0/1	6/13	6/7	8/12	0/12	3/12	

[a] Abbreviations as in table 1.
[b] Proportion of plasmas containing subgroup E virus, with no subgroup A virus infection.

TABLE 3.—*Mortality from neoplasms in chickens maintained free of exogenous but not endogenous leukosis-sarcoma virus infection or expression*

Generation	Line	Number isolated	Number dead	Number with LL	Number with ON [a]	Age of chickens (days) at termination of expt.
1971–73	100	87	20	0	1 [b]	659
	15_1	20	5	0	0	548
	6_1	82	9	0	0	593
	7_2	86	24	0	1 [c]	645
	$15I_4$	44	8	0	0	547
	$15I_5$	43	5	0	0	530
	Total	362	71	0	2	
1972–73	100	86	17	0	0	651
	15_1	22	2	0	0	931
	6_1	22	2	0	0	891
	6_2	66	18	0	1 [d]	712
	7_2	87	18	0	0	993
	$15I_4$	44	7	0	0	843
	$15I_5$	44	9	0	0	858
	N	65	26	0	0	496
	P	66	19	1 [e]	0	503
	Total	502	118	1	1	

[a] Abbreviation as in table 1.
[b] Adenocarcinoma.
[c] Reticulum cell sarcoma.
[d] Leiomyoma.
[e] Birds in one isolator of this group had antibody to subgroup A virus.

pret than those in hatch 1, because we have not devised a good assay to determine whether subgroup A virus-infected plasma also contains a subgroup E virus. Therefore, the proportion of plasmas with subgroup E virus was based only on those plasmas lacking viruses of other subgroups. Even so, all lots had apparently become infected with both viruses.

Neutralizing antibodies.—All lots were assayed for neutralizing antibody to subgroup A, B, and E viruses at 16 weeks (table 2). The lack of subgroup A and B antibodies in hatch 1 confirmed the absence of infection with exogenous viruses of these subgroups at that age. One of 19 line 100 plasmas had anti-E activity; this finding suggested that most of these birds were tolerant to their own

endogenous virus. In contrast, a high proportion of line 15 birds in lot 2 had anti-E activity paralleling the reduction in subgroup E virus isolated in this lot at 16 weeks. The low proportion with antibody in lot 3 may reflect later infection by contact.

In hatch 2, the antibody data from lots 5 and 6 reflected infection by both subgroups, but line 100 (lot 4) did not develop antibody to either virus. Perhaps line 100 is generally more susceptible to induction of immunologic tolerance than line 15.

Because no evidence of infection with subgroup A virus was found in hatch 1 at the 16-week bleeding, this hatch was bled again at 54 weeks (table 2). If it remained free of evidence of subgroup A virus infection, we could conclude that the single death from LL in hatch 1 was induced by RAV-0. However, this group of birds had become infected with a subgroup A virus, as indicated by subgroup A virus-neutralizing activity. We noted a highly significant difference between lots 2 and 3 in the proportion with antibody at 54 weeks. A much smaller proportion of the RAV-0-inoculated line 15 birds had antibody than did the contact control group; this finding suggested an interaction between early infection with RAV-0 and development of antibody to subgroup A viruses later in life.

Mortality With Neoplasms in Isolated RPRL Chickens

Table 3 shows the mortality from neoplasms in two generations of chickens maintained in isolators at RPRL. All remained free of exogenous virus infection, except for line P chickens in one isolator. Some birds in this isolator had subgroup A antibody. We know that some birds from RPRL line 100 and line 7_2 spontaneously release RAV-0, and that the viscera of line 6 embryos are consistently positive for gs antigen in the absence of RAV-0 infection [(*2, 8*); Okazaki W, Crittenden LB: Unpublished data]. Despite known expression of endogenous virus, at least in some lines, no LL was detected that could not be accounted for by subgroup A virus infection. Three other neoplasms were observed in isolated chickens, but none generally thought to be associated with LLV infection.

DISCUSSION

The observations at Beltsville show that during the first year of life, chickens highly infected with RAV-0 either by its spontaneous production or by exogenous infection develop neoplasms at a much lower rate than do RAV-1-infected chickens. The lines of chickens tested are susceptible to the induction of LL, because they died at a high rate after infection with RAV-1. Line 100 apparently is especially susceptible to tumor development after infection, because a high proportion developed LL or other neoplasms associated with LLV infection even though half are genetically resistant to subgroup A virus infection, whereas line 15 chickens are all susceptible to subgroup A virus (*2, 8*).

The single LL observed in lot 2 could have been induced by RAV-0 in this highly infected group. However, it may also have been induced by a subgroup A virus infection occurring after 16 weeks, even though young chickens are much more susceptible to the viral induction of LL than are 16-week-old chickens. Also, the bird could have been infected congenitally with an undetected slow-growing virus.

These observations clearly cannot justify the conclusion that RAV-0 is nononcogenic, because of the one LL and because the chickens were observed through only a small fraction of their adult lifetime. However, no neoplasm observed can be unambiguously attributed to RAV-0. These data definitely show that the oncogenicity of RAV-0 is much less than that of RAV-1, a typical LLV. Therefore, RAV-0 cannot be considered a major problem to the poultry industry where chickens are generally kept no more than 2 years.

It might be argued that RAV-1 is an exceptionally oncogenic virus, because it was derived from RSV or because it was passaged many times in culture. However, recent field isolates of LLV induce essentially the same spectrum of neoplasms in the same length of time when compared with RAV-1 infection at similar infectious doses under the same experimental conditions (Neiman PE, Purchase HG, Okazaki W: In preparation; Okazaki W: Unpublished data). Earlier work also has shown clearly that recent field isolates consisting largely of subgroup A viruses induce high levels of LL and other leukosis–sarcoma virus-associated neoplasms (*20, 21*). Therefore, RAV-1 has about the same pathogenicity under experimental conditions as viruses that cause LL under commercial conditions and is, therefore, more representative of typical isolates of LLV than RAV-0 is.

Other observations support the idea that RAV-0 has little, if any, oncogenic potential. Purchase et al. (in preparation) have tested samples of subgroup C, D, and E virus and have found that they all induce LL, except for RAV-0 and RAV-60 which belong to subgroup E.

The lack of LL mortality in chickens maintained in isolation at RPRL strengthens the conclusion that neither endogenous expression of RAV-0 nor expression of gs antigen has a detectable effect on the occurrence of LL compared with that of exogenous infection with leukosis–sarcoma viruses. Previous experience showed that these same lines maintained outside of isolation are infected with exogenous virus and have a substantial incidence of LL (*22*). These results clearly agree with earlier observations that horizontal and congenital transmission of exogenous LLV largely belonging to subgroup A are responsible for most neoplasms associated with the leukosis–sarcoma viruses in commercial chicken flocks (*23, 24*).

The Beltsville data suggest that some interactions between RAV-0 and subgroup A virus infection occur. These interactions are most clearly seen in the results of the subgroup A antibody assay at 54 weeks. Inoculation of line 15 chicken embryos with RAV-0 reduces the incidence of subgroup A antibody after late infection with a subgroup A virus ($P \leq 0.01$). This reduced incidence could be due to decreased immunologic responsiveness, interference with infection, or some other mechanism. We have shown that these birds did not have a high frequency of subgroup A virus infection with immunologic tolerance, as might be expected if their immune response mechanisms were impaired. No subgroup A virus was found in 17 samples of plasma from subgroup A antibody-negative birds 54 weeks of age. Clearly these observations must be followed up under conditions of strict isolation so that other contaminating viruses are unlikely to affect the results.

An observation of peripheral interest was that RAV-1-infected birds had a significantly higher incidence of MD and deaths from causes unrelated to neoplasms than RAV-1-noninfected birds. These observations could be due to the dual involvement of the bursa of Fabricius as the target organ for the initial lesion of LL and as the central organ controlling humoral antibody produc-

tion (25). The fact that the lots of chickens compared were hatched at different times reduces the significance of this observation.

We conclude that spontaneously produced RAV-0 or gs antigen expression is of little importance in the induction of neoplasms as they occur in chickens during the first 2 years of life. The exogenous viruses similar to RAV-1 are much more important in the etiology of LL in chicken populations. If the chicken is considered as a model for human disease, one must consider the possibility that exogenous viruses have an important function in the lymphoid neoplasms observed in early life, whereas the endogenous viruses have no role or become a more important factor in much older animals. We have no evidence to support the latter suggestion, but the oncogenic potential of RAV-0 is clearly much lower than that of the exogenous avian viruses.

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