JOURNAL OF VIROLOGY, Mar. 1987, p. 722–725
0022-538X/87/030722-04$02.00/0

Vol. 61, No. 3

Embryonic Infection with the Endogenous Avian Leukosis Virus Rous-Associated Virus-0 Alters Responses to Exogenous Avian Leukosis Virus Infection

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Received 15 September 1986/Accepted 4 November 1986

We inoculated susceptible chicken embryos with the endogenous avian leukosis virus Rous-associated virus-0 (RAV-0) on day 6 of incubation. At 1 week after hatching, RAV-0-infected and control chickens were inoculated with either RAV-1 or RAV-2, exogenous viruses belonging to subgroups A and B, respectively. The chickens injected with RAV-0 as embryos remained viremic with exogenous virus longer and either failed to develop type-specific humoral immunity to exogenous virus or developed it later than the control chickens not inoculated with RAV-0. The RAV-0-injected chickens also developed neoplasms at a much higher frequency than did the control chickens. We suggest that the lower immune responses of the RAV-0-injected chickens were due to an immunological tolerance to envelope group-specific glycoproteins shared among endogenous and exogenous viruses.

Avian leukosis viruses (ALV) can be acquired by exogenous infection of susceptible chickens. Infection occurring after the onset of immunocompetence generally leads to the development of neutralizing antibody specific for the subgroup (type specific) of the infecting virus and the subsequent elimination of viremia. By contrast, congenital transmission or experimental infection of embryos before immunological maturity generally leads to persistent viremia with the absence of neutralizing antibody (18). The lack of neutralizing antibody has been ascribed to the induction of a state of immunological tolerance to viral envelope antigen (17, 18).

Previous studies have shown that chickens expressing endogenous (subgroup E) envelope antigens encoded by *ev* genes as embryos do not produce neutralizing antibodies to subgroups A and B, the most common exogenous ALV found in commercial chickens, as frequently or to as high a titer as do chickens lacking such expression (6, 8). Immunological tolerance to cross-reactive determinants may likewise be responsible for the reduced immunological responiveness to exogenous ALV in chickens expressing *ev* gene-encoded envelope antigen. Such expression is one genetic factor that may increase the probability that horizontal infection will lead to congenital transmission from dam to offspring, thus thwarting efforts aimed at reducing ALV transmission in commercial breeding flocks maintained in an infected environment (4).

As an extension of our earlier studies on the influence of *ev* gene expression on response to exogenous ALV infection (6, 8), we report here the influence of embryonic infection with the endogenous ALV, Rous-associated virus-0 (RAV-0), on the responses of chickens lacking *ev* gene expression to subsequent infection with exogenous ALV.

MATERIALS AND METHODS

Experimental design. Line $15B_1$ males that were susceptible to ALV of subgroups A, B, and E and carried the unexpressed endogenous viral gene *ev*1 were mated to line 0 females that were susceptible to virus subgroups A and B but resistant to subgroup E and lacked detectable endogenous viral genes (1; L. B. Crittenden, unpublished data). Because genes for susceptibility to ALV infection are dominant, the hybrid embryos were susceptible to subgroups A, B, and E. The parental lines were maintained free of exogenous ALV infection. Embryos from this cross were used for two experiments of similar design. Approximately 50 embryos were inoculated in the yolk sacs with 10^4 infectious units of the endogenous virus RAV-0 on day 6 of incubation and were then returned to the incubator with a comparable number of control embryos. On day 19 of incubation, live embryos of both groups were placed in hatching units in separate plastic canopy isolators. At 1 week of age, the chickens from both groups were inoculated intraperitoneally with 10^4 infectious units of RAV-1. The second experiment was identical in design, except that all of the chickens were inoculated with RAV-2 at 1 week of age.

Blood was collected from all birds at 7, 11, and 17 weeks of age with syringes containing a small amount of chicken embryo extract to enhance clotting. The samples were allowed to clot and placed on ice within 0.5 h of collection. The sera were stored at less than −70°C. When the birds were 17 weeks of age, cloacal swabs were collected and stored at less than −70°C in vials containing 1 ml of cell culture medium containing 1,000 U of gentamicin. Necropsies for the gross diagnosis of neoplasms were performed on all birds that died prematurely and that were killed at 17 weeks of age. Tissue samples were taken for histopathological examination when the gross diagnosis was in doubt.

Virus stocks and assays. RAV-0 stock virus was derived from supernatant fluid from line 100B chicken embryo fibroblasts (CEF) known to carry *ev*1 and *ev*2, to be susceptible to subgroup E ALV, and to spontaneously release RAV-0 (2, 9). RAV-1 and RAV-2, which belong to subgroups A and B, respectively, have been previously described (24). Rous sarcoma virus pseudotypes were propagated from those described by Vogt and Ishizaki (24) and Vogt and Friis (23).

The ALV assays were modifications of those previously described (5). Briefly, secondary CEF or turkey embryo

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TABLE 1. RAV-0 and neutralizing antibody to subgroup E in the sera of 17-week-old $15B_1$ × line 0 chickens inoculated with RAV-0 as embryos and of controls

Inocula (embryo,[a] 1 week[b])	% of chickens (no. assayed) showing subgroup E:	
	Virus	Antibody
None, RAV-1	0[c] (11)	0 (32)
RAV-0, RAV-1	100[d] (16)	0 (21)
None, RAV-2	0[c] (39)	10 (39)
RAV-0, RAV-2	100[d] (31)	0 (31)

[a] Infectious units in the yolk sac on day 6 of incubation.
[b] Infectious units by the intra-abdominal route.
[c,d] Percentages are statistically different within 1-week inoculum groups if values have different superscripts. $P \leq 0.01$.

fibroblasts of the appropriate subgroup susceptibility were inoculated with 0.1 ml of sample plus 2 g of DEAE-dextran per ml of cell culture medium. After three medium changes and 9 days in culture, the trays of cell culture plates were frozen and thawed twice with a final concentration of 0.25% Tween 80 in the culture medium. The supernatant fluid was assayed for ALV group-specific antigen by an eznyme-linked immunosorbent assay (22). Preliminary studies had shown that 9 days of culture without passage was sufficient to reach an endpoint after titration for all subgroups of ALV (M. M. Newton, L. P. Provencher, and L. B. Crittenden, unpublished data). RAV-1 or RAV-2 in the presence of RAV-0 was detected by assay of CEF of line 0 chickens that were resistant to subgroup E. RAV-0 in the presence of RAV-2 was detected by assay of turkey embryo fibroblasts that were resistant to subgroup B but susceptible to subgroup E (7, 14). RAV-0 in the presence of RAV-1 could not be detected because CEF resistant to subgroup A but susceptible to subgroup E were not available. Only RAV-0 was assayed for in line $15B_1$ CEF.

Inactivated sera, diluted 1:5, were screened for neutralizing antibody against Rous sarcoma virus pseudotypes of subgroups A, B, and E by a focus reduction assay (16). Briefly, dilutions of serum and virus stocks were mixed in a 1:1 ratio and incubated for 30 min at 37°C. A total of 200 to 400 focus forming units of the mixture was assayed for focus formation. A 90% reduction in focus counts was considered positive.

Immunoprecipitation assays. Immune precipitates with chicken sera were produced by the methods of Halpern and Friis (13). Briefly, 5 μl of serum was added to [^{3}H]glucosamine-labeled detergent-disrupted purified virus. Subgroup A, B, and C viruses were produced by CEF infected with Pr-RSV of the appropriate subgroup. Subgroup E virus was produced by RSV(RAV-0)-infected quail embryo fibroblasts. The samples were adjusted to a final volume of 1.0 ml and incubated at 37°C for 1 h. An excess of rabbit anti-chicken immunoglobulin was added, and the samples were incubated for 15 min at 37°C and then overnight at 4°C. The precipitates were centrifuged, washed, and solubilized before liquid scintillation counting.

TABLE 2. Percentage of birds with exogenous ALV in their sera or shed into cloacal swabs

Inocula (embryo, 1 week)	% of chickens (no. assayed) with exogenous ALV in:			Cloacal swabs at 17 wk
	7 Wk	11 Wk	17 Wk	
None, RAV-1	0[a] (27)	0[a] (27)	0[a] (32)	0[a] (32)
RAV-0, RAV-1	66[b] (35)	35[b] (34)	24[b] (21)	67[b] (21)
None, RAV-2	3[a] (30)	0[a] (30)	0[a] (39)	5[a] (39)
RAV-0, RAV-2	100[b] (38)	100[b] (37)	100[b] (31)	100[b] (31)

[a,b] Percentages are statistically different within 1-week inoculum groups if values have different superscripts. $P \leq 0.01$.

TABLE 3. Percentage of birds with type-specific neutralizing antibody to exogenous ALV in their sera

Inocula (embryo, 1 week)	% of chickens (no. assayed) with antibody to exogenous ALV in serum		
	7 Wk	11 Wk	17 Wk
None, RAV-1	100[a] (27)	100[a] (27)	100[a] (32)
RAV-0, RAV-1	11[b] (35)	35[b] (34)	62[b] (21)
None, RAV-2	100[a] (30)	100[a] (30)	100[a] (29)
RAV-0, RAV-2	0[b] (38)	0[b] (37)	0[b] (31)

[a,b] Percentages are statistically different within 1-week inoculum groups if values have different superscripts. $P \leq 0.01$.

RESULTS

Subgroup E viremia and neutralizing antibody. Sera of 17-week-old chickens were assayed for subgroup E virus and neutralizing antibody to subgroup E (Table 1). Of 21 chickens inoculated with RAV-0 as embyos and then with RAV-1 at 1 week of age, 5 produced sera containing RAV-1 but could not be assayed for RAV-0 because subgroup E viruses could not be detected in the presence of RAV-1 in our assays (Table 2); however, the remaining 16 chickens were viremic with subgroup E virus. A sample of chickens from those that had been inoculated with RAV-0 as embryos and then inoculated with RAV-2 at 1 week of age were all viremic with subgroup E virus.

All of the chickens that were inoculated with RAV-0 as embryos failed to produce neutralizing antibody to subgroup E virus. With the exception of four RAV-2-infected chickens, the exogenous-virus-infected chickens not exposed to RAV-0 lacked subgroup E-specific antibody.

Exogenous ALV in sera and cloacae. Sera and cloacal

TABLE 4. Serum immunoprecipitation assays for group-specific envelope glycoprotein determinants against lysates of infected cells labeled with [^{3}H] glucosamine[a]

Inocula (embryo, 1 wk)[a]	Serum immunoprecipation (cpm)[b] (no. of sera assayed) against subgroup:			
	Pr-RSV-A	Pr-RSV-B	Pr-RSV-C	RSV (RAV-0)
None, RAV-1	220[c] (10)	358[c] (6)	1,080[c] (6)	2,527[c] (9)
RAV-0, RAV-1	121[d] (10)	187[d] (6)	184[d] (6)	137[d] (9)
None, RAV-2	358[c] (10)	3,507[c] (6)	614[c] (10)	2,248[c] (10)
RAV-0, RAV-2	162[d] (10)	210[d] (6)	195[d] (10)	251[d] (10)

[a] Samples of immunoprecipitates from sera of 17-week-old chickens were tested on acrylamide gels and showed 85- and 37-kilodalton bands.
[b] Mean of triplicate assays for each serum. Mean counts per minute of standard negative sera ranged form 50 to 150.
[c,d] Mean counts per minute are statistically different within 1-week inoculum groups if values have different superscripts. $P \leq 0.05$.

swabs were tested for the presence of exogenous ALV (Table 2). RAV-1-infected chickens which had been infected with RAV-0 as embryos showed a high frequency of viremia which decreased from 66% at 7 weeks of age to 24% at 17 weeks of age. Cloacal swabs from 67% of the chickens in this group showed the presence of exogenous ALV at 17 weeks of age. RAV-1-infected chickens that had not been exposed to RAV-0 showed no viremia during the course of the experiment and also failed to shed virus into their cloacae.

RAV-2-infected chickens that had been inoculated with RAV-0 as embryos were always viremic with exogenous ALV, and all shed such virus into their cloacae. RAV-2-infected chickens that had not been exposed to RAV-0 were not viremic at any time, with the exception of one animal which was viremic at 7 weeks of age. Only 5% of these chickens shed virus into their cloacae.

Neutralizing antibody to exogenous ALV. All chickens not exposed to RAV-0 as embryos developed neutralizing antibodies to exogenous ALV by 7 weeks of age. This response persisted throughout the experiment (Table 3). In contrast, RAV-1-infected chickens that had been infected with RAV-0 as embryos gradually developed antibodies to subgroup A virus, and by 17 weeks of age, two-thirds of these chickens had developed antibodies. No RAV-2-infected chickens that had been infected with RAV-0 as embryos developed neutralizing antibodies to subgroup B virus. No sera that neutralized subgroup A virus neutralized subgroup B virus, nor did sera that neutralized subgroup B virus neutralize subgroup A virus (data not shown).

Antibodies to group-specific glycoprotein determinants. The results of the immunoprecipitation assays of serum samples from each group of 17-week-old chickens are shown in Table 4. Each comparison of data from RAV-0-infected and noninfected birds for the RAV-1 and RAV-2 experiments was based on data collected on the same day. However, other comparisons are not valid because the experiments may not have been conducted on the same day. The sera from RAV-0-infected birds consistently gave much lower counts for each of the four virus subgroups assayed than did the sera from chickens never exposed to RAV-0.

Development of neoplasms. After both RAV-1 and RAV-2 infection, more chickens in RAV-0-infected groups than in groups never exposed to RAV-0 died with neoplasms or had grossly visible neoplasms at termination (Table 5). Most of the neoplasms were bursal lymphomas typical of lymphoid leukosis. In the groups infected with RAV-0 as embryos, many more birds died from metastases from the bursal lymphomas to other visceral organs than occurred in groups not infected with RAV-0.

TABLE 5. Percentages of mortality with neoplasms before 17 weeks of age, mortality and lesions at killing at 17 weeks of age, and extra-bursal metastases

Inocula (embryo, 1 wk)	No. of birds at 2 wk	% Mortality[a]		% Mortality and lesions[b]		% LL metastases
		LL	ON	LL	ON	
None, RAV-1	37	0[c]	0	43	0	6[c]
RAV-0, RAV-1	36	25[d]	8	67	8	71[d]
None, RAV-2	42	0[c]	2	19[c]	2	12[c]
RAV-0, RAV-2	37	11[d]	3	49[d]	5	67[b]

[a] Before week 17. LL, Lymphoid leukosis; ON, erythroblastosis and hemangioma.
[b] At week 17.
[c,d] Percentages are statistically different within 1-week inoculum groups if values have different superscripts. $P \leq 0.05$.

DISCUSSION

Our results clearly show that embryonic infection with RAV-0, an endogenous subgroup E virus, limits the subsequent development of type-specific humoral immunity to the subgroup A and B ALV RAV-1 and RAV-2, respectively. RAV-0-infected chickens remained viremic with the exogenous viruses longer and either failed or were slower to develop neutralizing antibodies to these viruses. The observation that embryonic exposure to RAV-0 reduced the ability of sera to recognize envelope glycoprotein group-specific components supports our previous suggestion that induced tolerance to RAV-0 limits immune responsiveness to exogenous ALV (6, 8). Chickens infected with RAV-0 developed neoplasms at a much higher rate after inoculation with RAV-1 or RAV-2 than was observed in our previous experiments with chickens inheriting *ev*2 and, thus, spontaneously producing RAV-0 (6, 8). These neoplasms were probably not induced directly by inoculation with RAV-0 because chickens from this cross, when inoculated with RAV-0 as embryos, died with neoplasms at a rate of less than 5% during a 42-week experimental period (Crittenden, unpublished data). Other studies have shown that RAV-0 does not induce high incidences of neoplasms (10). It is not clear what factors determined the increased metastasis of bursal lymphoma cells in chickens inoculated with RAV-0, but this increase may have reflected the greater persistence of viremia and, consequently, the earlier onset of lymphoid leukosis, or it may have meant that the migration of transformed B cells carrying exogenous envelope antigens was inhibited by circulating antibody or immune effector cells.

The inoculation of embryos with RAV-0 had a much greater effect on the development of neutralizing antibody and loss of viremia after RAV-2 infection than after RAV-1 infection (Tables 2 and 3). These results are consistent with the observed closer homology for envelope glycoproteins of subgroups B and E than for those of subgroups A and E (11).

The effect of embryonic infection with RAV-0 on response to RAV-1 and RAV-2 appears to be greater than that induced by the genetic introduction of *ev*2, the gene that codes for RAV-0 (6, 8). We propose at least two explanations for this observation. The experiments reported here were conducted with crosses of line $15B_1$ and line 0, while earlier experiments were conducted with line 15B chickens, a difference that may have influenced the results. In the context of our study, it is relevant that line 0 chickens had an earlier and more intense immune response to exogenous ALV than did line 15B birds lacking *ev*2 (8).

An alternate possibility is that *ev*2, a gene that is present and presumably coding for RAV-0 production in all somatic cells, also influences the distribution of envelope antigen in various cell types, perhaps through the phenomenon of viral interference (24). Such a difference in distribution of envelope antigen, particularly in lymphocyte populations, may influence the level of immune tolerance. Experiments comparing exogenous with endogenous infection of susceptible chickens having comparable genetic backgrounds may differentiate between these possibilities.

Limited immune response to horizontal infection with exogenous ALV induced by endogenous ALV infection of embryos or expression of *ev* genes (6, 8) could be detrimental to flocks of chickens maintained in an ALV-infected environment because of increased mortality and loss of productivity due to the persistence of ALV infection (12).

The sources of subgroup E virus envelope glycoprotein expression in embryos are as follows: (i) inheritance of *ev* genes that express envelope glycoproteins or encode complete endogenous virus (6, 8), (ii) congenital transmission from subgroup E-susceptible dams that carry *ev* genes that code for complete endogenous virus (20), and (iii) injection of subgroup E-susceptible embryos with live virus vaccines contaminated with infectious endogenous virus (19).

Recently, the endogenous viral gene *ev*21 has been shown to be closely linked to the sex-linked dominant slow-feathering gene (*K*) that is used to feather-sex a large proportion of commercial white-egg-producing chickens (3, 20). *ev*21 has been shown to code for the complete endogenous virus EV21. If this subgroup E endogenous virus is congenitally transmitted to fast-feathering female progeny that lack gene *ev*21, these birds could have a reduced immune response to exogenous virus infection. Such a mechanism could lead to the increased ALV infection rate and poor performance observed in fast-feathering female progeny of slow-feathering dams (15). Certain live poultry vaccines are highly effective when administered on day 18 or 19 of incubation (19). Contamination of these vaccines with infectious endogenous viruses could limit the immune response of vaccinated chickens to exogenous ALV infection. However, a detrimental effect would be expected only if the embryos were susceptible to subgroup E ALV infection and if infection of embryos on day 18 or 19 of incubation rather than on day 6 indeed limits immune response to exogenous ALV.

Since endogenous-virus-induced limitation of immune response to ALV is clearly specific for that class of viruses (6; Crittenden, unpublished data), the detrimental effects of endogenous-virus expression would not adversely influence chicken flocks that were free of exogenous ALV infection.

ACKNOWLEDGMENTS

We thank Leonard Provencher for expert technical assistance.

This research was supported in part by a grant from the United States-Israel (Binational) Agricultural Research and Development Fund.

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