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Immunocompetence of Chickens During Early and Tumorigenic Stages of Rous-Associated Virus-1 Infection

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A study was designed to determine the effects of congenital infection with the Rous-associated virus-1 (RAV-1) on the immune function of chickens during the early and late tumorigenic stages of infection. In another experiment, the effects of niridazole on the immune competence and the tumor incidence in chickens congenitally infected with RAV-1 were studied. Lymphocyte stimulation by phytohemagglutinin, the phytohemagglutinin skin test, the response to immunization with sheep erythrocytes and *Brucella abortus*, and histological evaluation of lymphoid organs were used to determine the immune competence in normal and infected chickens. Results indicated that both B- and T-cell immune functions during the early and late stages of RAV-1 infection were comparable to those of normal uninfected chickens. Administration of niridazole to congenitally infected chickens at 5 weeks of age for 7 or 21 days had no effect on the T-cell-mediated immunity; however, administration of the drug for 21 days eliminated lymphoma development. Unlike infection with other oncogenic viruses such as those causing Marek's disease and reticuloendotheliosis, infection with RAV-1 caused no detectable immunodepression during the early and late stages of infection.

Lymphoid leukosis (LL) is a B-cell lymphoma and is the major naturally occurring neoplasm associated with infection by a nondefective retrovirus of the avian leukosis virus (ALV)/sarcoma group (2, 17). Congenital transmission of ALV by dams to progeny chicks is the major source of infection that leads to lymphoma development (16). The disease is characterized by a long latent period that is seldom less than 14 weeks. Recently, it has been shown that such latency is a property of target B-cells and is unrelated to maturational events of the host physiology (7). Subgroup A viruses are the most common field isolates. Nonneoplastic lesions induced by these subgroup A viruses are uncommon (17). A number of reports have been published on the influence of ALV on the immune function of chickens (21); however, there is some controversy over whether humoral or cellular immune functions or both are affected during tumor development (16).

Earlier studies have shown that the most consistent effect of infection with two strains of ALV on the immunological competence of chickens was a small depression in humoral antibody to bovine serum albumin (18). Similar results were noted in chickens infected with a different strain of ALV (RPL-12) (15). In contrast, Dent et al. (4) reported no impairment of humoral response to a number of particulate and soluble antigens including bovine serum albumin in chickens infected with the RPL-12 strain of ALV. Recently, the myeloblastosis-associated virus-2, a member of subgroup B ALVs, has been shown to have a detrimental effect on the immune system of the chicken (23). However, unlike other isolates of ALV, myeloblastosis-associated virus-2 induces a cytopathic effect in cell cultures and a rapidly progressing acute osteopetrosis.

Preliminary studies from our laboratory indicated that inoculation of the Rous-associated virus-1 (RAV-1), a subgroup A ALV, into chickens that lack the endogenous virus gene expression caused an impairment of the humoral immune function (3). Such impairment was not observed after RAV-1 inoculation into chickens that express endogenous viral genes.

Available data on the effects of ALV infection on the cell-mediated immunity are also equivocal. Among chickens of two lines (White Leghorn and Brown Leghorn) infected with two strains of ALV, only the White Leghorn chickens had a significant reduction in the graft-versus-host reaction (18). Those authors also reported that rejection of the skin graft in ALV-infected White Leghorn chickens was significantly delayed only with one of the two strains of virus used. Recently, it has been shown that the cell-mediated immunity, as measured by

phytohemagglutinin (PHA)-P-induced cytotoxicity tests, was depressed only in chickens that had clinically visible leukosis, but not in those that appeared normal despite having gross tumors at necropsy (10). Furthermore, chickens congenitally infected with a subgroup A ALV were deficient in their blastogenic response to PHA, but only when suboptimal doses of PHA were used (13). Rup et al. (19) reported suppressed PHA responses in chickens infected with RAV-2, but not in those infected with RAV-1 or RAV-3. Apparently, the demonstration of immunodepression by ALV may depend upon several factors, such as the strain of virus used, the line of chickens, the stage of disease in which chickens are tested, and the procedures used to assess immunity (21). Reports in which the immune competence of chickens congenitally infected with ALV was evaluated by both in vitro and in vivo assays at different stages of the disease are scarce.

Interference with the normal development of the bursa-dependent lymphoid system by surgical bursectomy or by administering hormones or chemicals such as cyclophosphamide has been shown to eliminate or greatly reduce lymphoma development in chickens infected with ALV (17). This interference, however, usually results in severe defects in the ontogeny of the humoral immune response. Niridazole is an anti-inflammatory drug that has been used for reduction of granulomas due to schistosomal egg irritation in man (12). In chickens, niridazole has been shown to suppress only the cellular immune function (5, 26). Treatment of normal, 5-week-old chickens with niridazole caused suppression of the blastogenic response to PHA (26). Whether this suppression is transient or permanent was not determined. Further, the effects of this compound on the immune system and pathogenesis of disease in virus-infected chickens have not been studied.

In this study, we evaluated both humoral and cell-mediated immunity of chickens that were highly susceptible to ALV infection and tumors during the early viremic and late leukotic stages after congenital infection with a prototype strain (RAV-1) of ALV. We also examined the effects of niridazole (5, 12, 26) on the immune functions and rate of lymphoma development in chickens congenitally infected with RAV-1.

MATERIALS AND METHODS

Virus and virus assay. The ALV used in this study was the RAV-1. Propagation and titration of the virus were made in chicken embryo fibroblasts according to previously described methods (17). At hatching, meconia from day-old chicks were tested for virus by the phenotypic mixing test (14).

Chickens and chicken embryo inoculation. Chickens used in this study were crosses between males of the Regional Poultry Research Laboratory inbred line 15I subline 5 ($15I_5$) and females of the inbred line 7 subline 2 (7_2).The progeny chicks are susceptible to ALV and readily develop tumors (24). Embryos to be congenitally infected with RAV-1 were injected in the yolk sac on day 7 of incubation with 2×10^4 tissue culture infective doses. Congenitally infected and control chickens were housed separately at all times in filtered-air, positive-pressure, plastic canopy isolators.

Assay for humoral immunity. Congenitally infected and control chickens were intravenously inoculated at 7 weeks of age with 2.5×10^9 sheep erythrocytes (SE) and 2.5×10^9 *Brucella abortus* strain 119-3 (U.S. Department of Agriculture, Animal and Plant Health Service, Veterinary Service Laboratory, Ames, Iowa). Seven days later, antibody to SE was measured by a microhemagglutination test (27), and antibody for *B. abortus* was measured by the plate agglutination test.

In vitro assay of cellular immunity. A micromethod that uses whole blood for studying mitogen stimulation of chicken lymphocytes was used (11). Briefly, 10 μl of whole blood per culture was incubated at 41°C for 72 h in 0.2 ml of RPMI 1640 medium containing 100 μg of stock PHA-P. These cultures were pulse-labeled for 8 h with [5-^{125}I]iodo-2′-deoxyuridine. For the assay of radioactivity incorporated into the cells, whole blood was harvested in an automatic harvester on filter paper. The sample disk filters containing labeled cells were punched out as wet filters and placed in disposable tubes for gamma counting. Cellular immunity in niridazole-treated or untreated chickens was evaluated by measuring the response of [^{3}H]thymidine-treated peripheral blood lymphocytes to PHA-M stimulation.

In vivo assay of cellular immunity. The PHA skin response was used according to previously described procedures (9). Chickens were injected in the right wattles with 75 μg of PHA in 0.1 ml of phosphate-buffered saline (PBS). All chickens were also injected in the left wattles with 0.1 ml of PBS. The thickness of the wattles was measured by a micrometer at 24 h postinjection.

Pathology. Chickens that died or were sacrificed and all chickens that survived the experimental period of 22 weeks in experiments 1 and 2 and 16 weeks in experiment 3 were subjected to necropsy. LL was diagnosed on the basis of gross lesions or microscopic examination of tissue preparations stained with hematoxylin and eosin. In addition, sections of the bursa of Fabricius (BF), the thymus, and the spleen from congenitally infected and control chickens were examined for atrophic lesions at 1, 3, 5, and 7 weeks of age.

Experimental design. In experiments 1 and 2 whole blood was collected from 10 to 15 congenitally infected and control chickens at 1, 3, 5, 7, 12, 19, and 21 weeks of age for the PHA stimulation assay. Unless chickens died, the same group of chickens was bled at each time interval. The response of congenitally infected chickens to SE and *B. abortus* was compared with that of control chickens at 7 weeks of age. The PHA skin response of congenitally infected and control chickens was determined at 12 and 19 weeks of age in experiment 1 and at 12 and 21 weeks of age in experiment 2.

In experiment 3, congenitally infected chickens were divided into three groups at 5 weeks of age. One group received niridazole (Ciba-Geigy, Summit, N.J.)

TABLE 1. Hemagglutinin titer of control uninfected chickens and of chickens congenitally infected with RAV-1 and treated or not treated with niridazole[a]

Expt	RAV-1	Niridazole	No. of chickens	% Responders	Mean of titers (log_2)
1	−	−	10	100	5.80 ± 0.36
	+	−	27	100	5.66 ± 0.22[b]
2	−	−	10	100	9.6 ± 0.44
	+	−	22	100	9.4 ± 0.30[b]
3	+	+ (7 days)	10	100	8.9 ± 0.6[c]
	+	+ (21 days)	7	100	6.7 ± 1.06[c]
	+	−	8	100	8.0 ± 0.77

[a] Chickens were immunized with SE and *B. abortus* at 7 weeks of age; 7 days later, sera were evaluated for agglutinins.
[b] Not significant ($P > 0.05$) compared with uninfected chickens.
[c] Not significant ($P > 0.05$) compared with chickens not treated with niridazole.

for 7 days, the second group for 21 days, and the third group remained as the untreated control. Niridazole was suspended in distilled water, and 0.5 ml was given orally at a dose of 50 mg/kg of body weight daily. The response of representatives of the niridazole-treated and untreated groups to immunization with SE and *B. abortus* was evaluated at 7 weeks of age. At 14, 28, 35, and 42 days after the initiation of treatment, the mitogen (PHA-M) stimulation of peripheral blood lymphocytes was used to determine the cell-mediated immune function. The BF and thymus collected from three to five chickens of each group were grossly and microscopically examined for atrophic lesions at 1, 2, and 3 weeks after treatment stopped. The response of niridazole-treated and untreated groups to lymphoma development was determined by gross and microscopic examinations of affected tissues.

RESULTS

Virus assay. In all three experiments 85 to 100% of the embryo-inoculated chickens were positive for RAV-1 by testing meconia at hatching. The virus-negative chicks were excluded from the experiments.

Humoral immune function. The hemagglutinin titers of embryos infected with RAV-1 and niridazole treated or untreated and of control normal chickens are presented in Table 1. No significant difference was observed between the titers of infected and uninfected chickens. Furthermore, niridazole treatment for 7 or 21 days did not lower the hemagglutinin response to SE, and titers were comparable to untreated controls. The response of all of the groups to *B. abortus* showed a similarity to the response to SE.

Cellular immune function. Results of the mitogen stimulation of whole blood collected from infected and control chickens are presented in Table 2. No immunodepression of T-cell function, as determined by the mitogen stimulation assay, was detected. The skin response to PHA stimulation in infected and uninfected control chickens is given in Table 3. There was no significant difference in the thickness of the right wattles (injected with PHA) of infected and control chickens. A statistically significant ($P <$

TABLE 2. Whole-blood PHA-P response of chickens congenitally infected with RAV-1[a]

Age at testing (wk)	Mean [^{125}I]iododeoxyuridine uptake (cpm) for:			
	Expt 1		Expt 2	
	Control	Infected	Control	Infected
1	3,080 ± 465	4,157 ± 743[b]	3,797 ± 633	6,543 ± 344[b]
3	8,615 ± 837	12,028 ± 819	8,949 ± 295	9,566 ± 348
5	12,446 ± 715	13,339 ± 638	10,851 ± 554	12,053 ± 461
7	10,921 ± 926	10,704 ± 996	10,409 ± 715	9,274 ± 799
12	8,225 ± 824	8,720 ± 896	8,098 ± 386	8,376 ± 839
19	2,295 ± 306	6,308 ± 874	NT[c]	NT
21	NT	NT	3,574 ± 849	4,176 ± 971

[a] From 10 to 15 birds per group were tested at each time interval. The mean [^{125}I]iododeoxyuridine uptake in counts per minute ± the standard error of the mean is given. The background unstimulated culture for control groups is 136 ± 19; that for the infected groups is 120 ± 12.
[b] Not significant ($P > 0.05$) compared with uninfected chickens.
[c] NT, Not tested.

TABLE 3. Skin response to PHA-P stimulation of chickens congenitally infected with RAV-1 and of control uninfected chickens[a]

Expt	Age at testing (wk)	RAV-1	No. of chickens	Mean of thickness of right wattles (mm)
1	12	+	7	2.6 ± 0.16[b]
		−	10	2.58 ± 0.14
	19	+	6	2.8 ± 0.16[b]
		−	9	3.0 ± 0.14
2	12	+	8	2.43 ± 0.12[b]
		−	6	2.43 ± 0.1
	21	+	7	2.32 ± 0.1[b]
		−	12	2.4 ± 0.15

[a] Chickens were injected in the right wattles with 75 μg of PHA-P in 0.1 ml of PBS and in the left wattles with 0.1 ml of PBS. A significant difference ($P < 0.01$) was always seen between means of the thickness of right and left wattles. Infected chickens were proved to have LL tumors by cloacal palpation of the bursa at 19 weeks of age and by postmortem examination at termination.

[b] Not significant ($P > 0.05$) compared with uninfected chickens.

0.01) difference between the thickness of the right (injected with PHA) and left (injected with PBS) wattles of all chickens was detected at 24 h postinjection.

Histology of lymphoid organs. No atrophic lesions were seen in the BF, thymus, and spleen collected from five infected chickens at 1, 3, 5, and 7 weeks of age. The histological structure of these organs was compatible with that of the organs from control uninfected chickens.

Effect of niridazole treatment on the cellular immune function and lymphoma response. The mitogen stimulation of peripheral blood lymphocytes obtained from chickens congenitally infected with RAV-1 and treated with niridazole at 5 weeks of age is presented in Table 4. The counts per minute of PHA-M-stimulated cultures obtained from infected groups at 14, 28, 35, and 42 days after initiation of niridazole treatment were similar to those obtained from untreated groups of chickens. Atrophic changes in the BF and thymus were more frequently seen in chickens treated with niridazole for 21 days than in those treated for 7 days. The damage to these lymphoid organs persisted through at least 16 weeks as determined by examination of sections. Administration of niridazole for 7 days to 5-week-old chickens congenitally infected with RAV-1 had no effect on LL development. In contrast, administration of niridazole for 21 days in such chickens completely eliminated lymphoma as determined by gross and microscopic examinations of affected tissue (Table 5). However, about one-third of the chickens in this group died within 2 to 4 weeks after initiation of treatment, presumably from toxicity.

DISCUSSION

Evaluation of the immunocompetence of chickens congenitally infected with RAV-1 and undergoing lymphomagenesis revealed the following. (i) RAV-1 infection has no immunodepressive effects detectable by these methods in $15I_5 \times 7_2$ chickens which are highly susceptible to virus infection and readily develop LL. (ii) Lymphomagenesis can progress in hosts with an apparently intact immune system as determined by the criteria used in this study. Results reported herein indicate that susceptible chickens that were congenitally infected with a subgroup A ALV (RAV-1) had a normal blastogenic response to PHA, a normal skin response to PHA, and a normal response to immunization with SE and *B. abortus*. Our study also indicates that congenital infection with RAV-1 did not cause any atrophic changes in the BF, thymus, or spleen. Such atrophic lesions or immunodepression, or both, are usually seen in chickens infected with other avian oncogenic viruses such as those causing Marek's disease and reticuloendotheliosis (1, 18, 20, 21, 25). Atrophy of the BF and thymus was also seen in chickens bearing

TABLE 4. Peripheral blood lymphocyte PHA-M response of chickens congenitally infected with RAV-1 and treated or not treated with niridazole[a]

Days after initiation of treatment	Mean [^{3}H]thymidine uptake (cpm) for the following niridazole regimens:		
	None	7 days	21 days
14	163,837 ± 44,986	212,475 ± 42,555[b]	NT[c]
28	73,410 ± 21,515	88,314 ± 25,426	113,945 ± 19,559[b]
35	154,414 ± 36,506	99,464 ± 13,687	132,176 ± 12,868
42	138,893 ± 27,807	NT	103,087 ± 23,901

[a] Five birds per group were tested at each time interval. The mean [^{3}H]thymidine uptake in counts per minute ± the standard error of the mean is given. The background unstimulated culture for the untreated group is 1,313 ± 190; that for 7 days of treatment is 1,563 ± 390, and that for 21 days of treatment is 1,516 ± 275.

[b] Not significant ($P > 0.05$) compared with chickens not treated with niridazole.

[c] NT, Not tested.

TABLE 5. Effect of niridazole treatment on the incidence of lymphomas in chickens congenitally infected with RAV-1[a]

Duration of niridazole treatment (days)	No. of chickens with atrophy of BF and thymus/no. examined at the following age[b]		No. of chickens with bursal lymphoma/no. examined at the following age[b]		No. of chickens with neoplasms other than LL[c]		
	8 to 15 wk	16 wk	8 to 15 wk	16 wk	E	H	N
7	1/9	2/19	5/9	16/19	1	1	0
21	11/16	5/6	0/16	0/6	2	1	1
None	0/12	0/4	8/12	4/4	2	0	0

[a] Niridazole suspended in water was given orally to 5-week-old chickens congenitally infected with RAV-1 at a dose of 50 mg/kg of body weight daily.

[b] Determined by gross or microscopic examinations or both of tissues collected from chickens that died or were sacrificed and from those that survived the experimental period of 16 weeks; this does not include those that died during niridazole treatment or those that died from neoplasms other than LL.

[c] E, Erythroblastosis; H, hemangioma; N, nephroblastoma.

progressive Rous sarcomas, but not in those in which sarcoma regressed (6). Further, chickens infected as embryos with myeloblastosis-associated virus-2, a member of the subgroup B ALVs, had profound suppression of bursal and thymic lymphoid cell development within 2 to 3 weeks after hatching (22). The role of the immune system of chickens in lympho-magenesis induced by ALV is not clearly defined, and the presence of a tumor-specific antigen on tumor cells has not been reported. Our data agree with those of Dent et al. (4), Granlund and Loan (10), and Rup et al. (19). We have shown that chickens that are highly susceptible to virus infection and readily develop LL remained immunocompetent after congenital infection with a prototype of subgroup A ALVs (RAV-1).

Since viruses of ALV subgroup A are the most commonly isolated viruses from the field and because chickens in the field are not as susceptible as the $15I_5 \times 7_2$ chickens used in this study, it is unlikely that infection with subgroup A ALV in chicken flocks in the field will lead to immunodepression. Field studies indicated that performance was significantly lower and that nonspecific mortality was significantly higher in chickens that shed group-specific ALV antigens into their egg albumin than in those that did not (8). Whether such low performance and high mortality were due to an impairment of the immune function was not determined. Recent experiments have shown that RAV-1 infection can cause immunodepression only in chickens that lack endogenous virus gene expression (3). In addition to the immunodepression, nonneoplastic lesions which include hepatitis, anemia, and severe atrophy of lymphoid organs were also associated with RAV-1 infection of such chickens. Whether the presence of endogenous virus expression in the $15I_5 \times 7_2$ chickens used in this study is the principal reason for lack of immunodepression by RAV-1 is not known.

Results from the third experiment indicate that treatment with niridazole of 5-week-old chickens congenitally infected with RAV-1 for 7 to 21 days did not alter the immune function. Donahoe et al. (5) and Walser (26) observed significant depression of the cell-mediated immune response of niridazole-treated chickens when examined at 1 week after initiation of treatment. In our study, however, we examined the cellular immune response at 2, 4, 5, and 6 weeks after initiation of the treatment, and the chickens used were congenitally infected with ALV (Table 4). Because of the detrimental effect of niridazole on the structure of the BF, niridazole can be used to experimentally bursectomize chickens at 5 weeks of age without compromising the humoral immune function. However, lower doses of niridazole or other regimens of application may effectively bursectomize chickens with minimal toxic effects. Walser (26) also reported that niridazole in dosages of 100 and 200 mg/kg of body weight was severely toxic to 5-week-old chickens. Elimination of lymphoma development in the group which received niridazole for 3 weeks can be explained by the severe atrophy of the BF. Other chemicals, hormones, and surgical bursectomy have been shown to reduce or eliminate LL (17). However, use of such chemicals or hormones sometimes requires their application in the early stages of the animal's life and thus introduces the risk of compromising the immune function of the host.

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