Spontaneous Regression of Leukemia in Chickens Infected with Avian Myeloblastosis Virus[1] (37376)

R. F. SILVA AND C. MOSCOVICI

Tumor Virology Laboratory, Veterans Administration Hospital, and Department of Immunology and Medical Microbiology; Department of Pathology, University of Florida College of Medicine, Gainesville, Florida 32601

Avian myeloblastosis virus (AMV) strain BAI-A is capable of inducing osteopetrosis, nephroblastoma, lymphoid leukosis and myeloblastic leukemia in the chicken (1). The myeloblastic leukemia is characterized by an extensive proliferation of myeloblasts in the peripheral blood, spleen, liver and bone marrow (2).

Viral induced fowl leukosis is nearly always of a progressive nature. However, some early investigators have reported spontaneous recovery of leukosis in experimental birds. Olson (3) had reported four cases of spontaneous regression of leukosis in 98 experimental birds. One of the four birds was found to be viremic. In another instance, Oberling, Guerin and Guerin (4) reported a 10% incidence of spontaneous recovery following inoculation with a viral strain developed by them. More recently, a report described the spontaneous regression of a Friend virus induced leukemia of mice (5).

Studies of spontaneous regression have almost exclusively been confined to solid tumors. Rous sarcoma virus induced tumors have been extensively studied (6–8).

Tumors induced by Rous sarcoma virus (RSV) grow and regress as a function of virus inoculum and the age of the host. The younger the infected animal, the less the incidence of regression. Regression also correlates with virus dose (7). Regressing Rous tumors in the chicken have been shown to be infiltrated with lymphoid cells (6), indicating that cell mediated immunity might play an important role in regression. Chickens bearing a primary RSV sarcoma have been shown to have thymus cells which specifically inhibit the growth of the tumor cells *in vitro* (9). Spontaneous regression of murine sarcoma virus (10), feline sarcoma virus (11), and Shope virus (12) induced tumors have been reported. Reversal of the neoplastic state in other organisms such as plants and amphibia has been well documented in an excellent review by Braun (13).

This paper describes the occurrence of spontaneous regression of myelogenous leukemia induced in chickens by avian myeloblastosis virus (AMV).

Materials and Methods. Chickens. C/O and C/B eggs derived from the white leghorn breed K-137 of Kimber Farms were used for this study. C/AB eggs were originally obtained from USDA, East Lansing, MI, and subsequently bred in this laboratory.

Virus. A single stock of standard avian myeloblastosis virus (AMV) strain BAI-A was used for all studies. The stock was a pool of plasma derived from several leukemic Kimber chicks. A detailed description for collection and assay has been reported (14).

Cell and virus assays. Nomenclature for chicken cell types and methods for cell culture were the same as previously described (15). The focus assay for Rous sarcoma virus (RSV) followed the technique of Rubin (16).

Transforming activity of AMV was assayed *in vitro,* as previously described (14). The growth medium contained a polycation (polybrene at 2 μg/ml) which greatly enhances virus adsorption (17).

Leukemia production and regression. Kim-

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ber and C/AB were either injected with virus intravenously as 12 or 17 day old embryos or intraperitoneally as 1 day old chicks. Leukemic birds were considered to have regressed when myeloblasts disappeared from the peripheral blood.

Buffy coat cultures. Five milliliters of heparinized blood (Liquaemin sodium 10 from Organon Inc., Orange, NJ) were obtained from the wing vein. The buffy coat from the centrifuged blood was gently aspirated and seeded into Falcon plastic petri dishes, at about 2–3 $\times$ 10^6 leukocytes/ml. Buffy coat cultures were fed twice a week with a modified Eagle's medium (5% calf serum, 5% heat-inactivated chick serum, 10% tryptose phosphate, and 80% basal medium). The basal medium consisted of Eagle's minimum essential medium with quadruple the concentration of amino acids and vitamins and double the concentration of glucose (18).

Virus Neutralization test. Serial dilutions of heat-inactivated sera from regressed birds were mixed with an equal volume of AMV containing 2 $\times$ 10^3 FFU/ml. The mixture was shaken and incubated at 37° for 40 min. Serum from untreated chickens was concurrently incubated with AMV and served as the negative control. A known anti-AMV serum was used for the positive control. The serum–virus mixtures were added to C/O yolk sac cultures, overlayed, and foci were counted on Day 10 (19).

Myeloblast suspension culture. Cultures of myeloblasts from leukemic birds were routinely propagated in 50 ml Erlenmeyer flasks on an oscillating shaker at 37°. Cultures were maintained at about 10–30 $\times$ 10^6 myeloblasts/ml in a medium consisting of 25% double strength medium 199 and 75% chicken serum to which glucose (8 mg/ml) and folic acid (1.6 mg/liter) has been added (2).

Colony inhibition tests. A lymphocyte-rich suspension was prepared by forcing spleens through a fine mesh wire screen. A 1 ml suspension of washed spleen cells was gently pushed into a nylon-packed pasteur pipette (1.5 in., 3.0 denier, semidull, type 200 nylon from E. I. DuPont de Nemours Co., Wilmington, DE). The cells were incubated at 37° for 20 min and eluted slowly with 5 ml of warm barbital buffer. With this technique, greater than 95% of the eluted leukocytes are lymphocytes (20). The resulting lymphocyte suspension was diluted in the modified Eagle's medium to give 40–50 $\times$ 10^6 lymphocytes/ml. One-half milliliter of the lymphocyte suspension was mixed with an equal volume of myeloblasts (2 $\times$ 10^6 myeloblasts/ml) and incubated for 15–20 hr at 37° in a CO_2 incubator. After incubation, the myeloblasts were tested for their ability to form colonies in semisolid medium.

The semisolid medium consisted of the following ingredients: 20% 2X F-12 (GIBCO, Grand Island, NY); 6% calf serum; 2% heat-inactivated chick serum; 10% tryptose phosphate; 40% conditioned medium; 20% of 1.8% Bacto agar to which additional vitamins and folic acid (final concn, 0.8 mg folic acid/100 ml) has been added. The hard agar medium was prepared in the same manner except 3.6% Bacto agar replaced the 1.8% Bacto agar. Conditioned media was obtained by pooling the media from primary cultures of leukosis-free chicken fibroblasts on the third day of culture. A hard base layer was prepared by adding 1 ml of hard agar medium to a 35 mm Falcon tissue culture dish. After solidifying, 2 ml of soft agar medium containing the myeloblast–lymphocyte suspension was added to each dish. Each test was run in duplicate with 2 $\times$ 10^4 myeloblasts seeded/35 mm dish. The myeloblast colonies were counted after being incubated in a CO_2 incubator at 37° for 10 days. The number of colonies which developed from mixtures of myeloblasts and "regressor" lymphocytes from regressed birds was compared to the number which arose from mixtures of myeloblasts and control lymphoblasts and control lymphocytes from normal birds.

To test for the presence of blocking factors in the leukemic serum, 0.1 ml of inactivated serum (30 min at 57°) was incubated with 10^6 myeloblasts for 15 min at 37° in a total volume of 0.5 ml. Following this, the myeloblast suspension was mixed with the appropriate lymphocytes and seeded in semisolid agar medium as described above.

TABLE I. Spontaneous Regression of Leukemia in Kimber Chicks (C/O and C/B).

Virus	Route of injection	Dose of virus (TFU/embryo)[a]	Age of embryo (days)	No. injected	No. with leukemia	No. regressed	Percentage of leukemic birds that regressed
Standard AMV	Yolk sac	5×10^3	12	89	13	1	7
	Allantoic	5×10^3	12	14	1	0	—
	iv	50	12	218	76	0	—
	iv	5×10^2	17	196	64	10	15

[a] Transforming units determined by end point dilution in C/O yolk sac cultures.

Control lymphocyte preparations were obtained from normal Kimber which were approximately the same age as the regressed birds.

Histology. Autopsies were performed on all sacrificed birds. Tissue specimens were fixed in 10% formalin and stained with hematoxylin and eosin.

Results. In vivo *characteristics of regression.* The intravenous route of inoculation was found to be the most efficient for the induction of leukemia, followed by the yolk sac and the allantoic route. The rate of regression was found to be a function of the age of the embryo at the time of AMV inoculation. When Kimber chickens were injected intravenously as 12 day old embryos, about 35% (76/218) of the hatched chicks developed leukemia within 6 wk of hatching without any regressions being observed. When 17 day old Kimber embryos were injected intravenously with a 10-fold higher virus dose, approximately 33% (64/196) of the hatched chicks developed leukemia of which 15% (10/64) regressed. In spite of attempts to reproduce leukemia and regression in the group injected via the yolk sac, only one bird showed a definite disappearance of circulating myeloblasts in the peripheral blood (Table I).

The C/AB chickens were completely resistant to AMV if inoculated with the same virus dose used to infect the Kimber embryos. Thus, all viral inoculations of C/AB embryos were performed with a 10-fold higher concentration of virus than was used for the 17 day old Kimber. The allantoic route of inoculation produced no leukemia. The intravenous injection of the virus into 12 day old embryos was found to be the most efficient means of inducing leukemia. Even under these optimum conditions, one out of four leukemic C/AB chicks spontaneously regressed. Only 20% of the hatched C/AB chicks developed myeloblastic leukemia if they had been inoculated as 17 day old embryos. Of the 13 leukemic birds observed, 8 spontaneously regressed (Table II).

Table III follows the sequence of leukemia and regression for each Kimber chicken. The duration of the overt phase of the leukemia does not last over 2 wk. During this acute phase, the birds evidenced malaise as shown by stupor and ruffled feathers. In these in-

TABLE II. Spontaneous Regression of Leukemia in C/AB Chicks.

Virus	Route of inoculation	Dose of virus (TFU/embryo)[a] ($\times 10^3$)	Age of embryo (days)	No. injected	No. with leukemia	No. regressed	Percentage of leukemic birds that regressed
Standard AMV	Allantoic	5	12	12	0	0	—
	iv	5	12	8	4	1	25
	iv	5	17	66	13	8	62

[a] Transforming units determined by end point dilution in C/O yolk sac cultures.

TABLE III. Leukemia and Regression—Sequence of Events *in Vivo* (Kimber).

Bird no.	Onset of leukemia (wk post-hatching)	Duration of leukemia (wk)	Duration of regression (wk)	Autopsy
5653	4	2	7	Kidney lymphoma
7544	3	1	10	Kidney, liver, and lung lymphoma
7601	4	1	3	Relapse; 2nd leukemia fatal within 1 wk
7616	5	1	2	Relapse; 2nd leukemia fatal within 1 wk
7927	4	1	11	Normal
7932	3	1	3	Normal
7617	6	2	1	Relapse; 2nd leukemia fatal within 1 wk
8001	4	1	3	Liver lymphoma
8022	3	2	1	Normal
8194	3	1	6	Normal

stances, a second phase of leukemia appeared often less than 3 wk after regression. These birds all died within 1 wk of the appearance of the second attack of leukemia. Autopsies performed on the 10 regressed birds showed 3 birds with lymphomas.

Table IV shows the sequence of regression for each C/AB chicken. The onset of leukemia for C/AB chicks was about the same as that seen in the Kimber chicks. The duration of the leukemia was generally longer, and in the case of bird numbered 5968, leukemia lasted for 8 wk before regression occurred. Fatal relapses, similar to what occurred in the Kimber, were seen in two instances. Upon autopsy, four regressed C/AB were found to have lymphomas.

In vitro *characteristics of regression.* The plasma and buffy coat cultures were analyzed for the presence of virus during the leukemic and regressed states. The Kimber chickens were viremic during the entire period of study, even after the leukemia has regressed. Myeloblast proliferation occurred in buffy coat cultures that were established when peripheral blood smears were negative. The plasma of leukemic or regressed C/AB chickens was always negative for AMV when tested in C/O yolk sac cultures. Buffy coat cultures taken from regressed C/AB always resulted in the reappearance and proliferation of myeloblasts and were nonproducers of AMV. Thus, leukocyte cultures of regressed Kimber and C/AB became transformed when cultured *in vitro* (Table V).

Viral neutralizing antibodies. The role of viral neutralizing antibodies in nonviremic regressed C/AB chickens was analyzed. No AMV neutralizing antibodies were present in the regressed C/AB chickens.

Histological examinations. Kimber and C/AB birds were sacrificed at various inter-

TABLE IV. Leukemia and Regression—Sequence of Events *in Vivo* (C/AB).

Bird no.	Onset of leukemia (wk post-hatching)	Duration of leukemia (wk)	Duration of regression (wk)	Autopsy
5968	3	8	5	Kidney and liver lymphoma
6279	3	2	8	Runt: normal
6711	5	2	1	Normal
7563	5	4	1	Spleen lymphoma
8064	3	2	3	Normal
8226	5	2	1	Relapse; 2nd leukemia fatal in 3 wk
8230	5	1	6	Spleen lymphoma
8266	4	1	3	Relapse; 2nd leukemia fatal in 3 wk; liver and lung lymphoma

TABLE V. Leukemia and Regression—Sequence of Events *in Vitro*.

Chick phenotype	Presence of myeloblasts in peripheral blood	Viremia	Presence of myeloblasts in buffy coat cultures	Amount of virus in buffy coat cultures (TFU/ml)
Kimber (C/O or C/B)	+	+[a]	TFO[b]	2.0×10^3
Kimber (C/O or C/B)	0	+	TFO	2.0×10^3
C/AB	+	0	TFO	0
C/AB	0	0	TFO	0

[a] As tested for transformation activity in C/O yolk sac cultures—virus not titered.
[b] Transformation with myeloblast proliferation.

vals and examined grossly and microscopically. Gross examinations occasionally revealed lymphoma-like tumors. Histological examinations, however, revealed normal tissue had been replaced by blast-like cells. Some of these tumors were found to be of lymphoid origin, but several remaining tumors could not be conclusively identified. In some cases bone marrows of regressed birds also revealed that 30% of the marrow had been replaced by these blast-like cells. A more systematic study of tumors encountered in the regressed birds will be made in a separate report.

Effect of lymphocytes from regressed birds on colony formation by myeloblasts. Numerous *in vitro* tests have been used to demonstrate that regressed animals have sensitized lymphocytes which are active against their autochthonous tumors or closely related tumors (21–23). To determine if a similar mechanism was active in our system, we used a modified colony inhibition technique to determine if lymphocytes from regressed chickens could depress colony formation by leukemic myeloblasts.

Myeloblasts from leukemic Kimber chickens were routinely propagated in a shaker culture. The efficiency of colony formation in semisolid media by these myeloblasts varied from 1 to 10% depending upon the source of cells. Myeloblasts which had been growing in the shaker culture for 10 days or more produced colonies with a much higher efficiency than myeloblasts taken directly from the leukemic bird.

Greater than 50% inhibition of myeloblast colony formation occurred if lymphocytes from regressed birds were used within 3 wk of regression (Table VI). Splenic lymphocytes from Kimber No. 7932 inhibited nearly 93% of the colonies seen with the normal lymphocyte–myeloblast mixture. Experiments with bird Nos. 8022 and 8001 produced 52 and 76% inhibition, respectively. Colony formation by control lymphocyte–myeloblast preparations was always less than the number of colonies produced by myeloblasts alone. Splenic lymphocytes from normal or regressed birds did not produce myeloblast colonies. On the other hand, the splenic lymphocyte preparation from a leukemic animal produced 71 myeloblast colonies, which is not surprising since histological examination of leukemic spleens always revealed large numbers of myeloblasts.

Serum from leukemic birds inhibited the action of "regressor" lymphocytes to about the same extent as serum from the regressed bird. Colony formation under these conditions was 43 and 59% of the control, whereas lymphocytes from regressed birds, without serum treatment, resulted in colony formation of only 24% of the control (Table VI).

Discussion. We have described the spontaneous regression of leuemia, induced by a conventional strain of avian myeloblastosis virus (AMV) as evidenced by the disappearance of leukemic myeloblasts from the peripheral blood.

The regression of Rous sarcoma tumors in birds has been extensively studied (25). Regression of Rous tumors is accompanied by a decrease and eventual disappearance of RSV in the tumors. The regressing tumors are always found to be infiltrated with lymphoid

TABLE VI. Effect of Sensitized Lymphocytes on Colony Formation of Leukemic Myeloblasts.[a]

Regressed bird no.	Type of mixture[b]	No. colonies/dish ± SD	Percentage of control {[colonies/dish (test plate)]/[colonies/dish (control plate)]} × 100
7932	Myeloblasts + control lymphocytes	185 ± 43	100
	Myeloblasts + regressor lymphocytes	14 ± 10	7.5
	Myeloblasts	960 ± 180	—
8022	Myeloblasts + control lymphocytes	300 ± 7	100
	Myeloblasts + regressor lymphocytes	145 ± 18	48
	Control lymphocytes	0	—
	Regressor lymphocytes	0	—
	Myeloblasts	820 ± 103	—
8001	Myeloblasts + control lymphocytes	1670 ± 29	100
	Myeloblasts + regressor lymphocytes	400 ± 57	24
	Myeloblasts + regressor serum + regressor lymphocytes	990 ± 60	59
	Myeloblasts + leukemic serum + regressor lymphocytes	715 ± 18	43
	Leukemic lymphocytes	71 ± 31	—
	Regressor lymphocytes	0	—
	Myeloblasts	2950 ± 34	—

[a] Results based on: Incubation period of 17 hr at 37°.
[b] Ratio of lymphocyte per target cell approximately 25:1.

cells (6, 26). In addition, neonatal thymectomy enhances oncogenesis and significantly inhibits regression of Rous sarcoma tumors in quail, whereas neonatal bursectomy has no effect (27). These observations suggest that the regression of autochthonous Rous sarcoma tumors is mediated by a cellular immunologic mechanism (6). The results presented here indicate that the regression of viral induced leukemia differs in some respects from the regression of RSV induced tumors. The differences are suggested by: (a) Kimber chickens maintain a high level of circulating infectious virus during all phases of regression; (b) peripheral blood patterns return to normal in regressed birds, although their bone marrow harbors islands of leukemic myeloblasts.

Regression could reflect a characteristic of either the virus or the host. Evidence presented in this paper indicates that a host function is responsible for the spontaneous regression. We have used a conventional strain of AMV known to produce a rapidly fatal leukemia when a large amount of virus is used as inoculum, and the incidence of spontaneous regression is different depending upon the strain of chicken used. Only about 15% of the Kimber chickens spontaneously regress whereas 62% of C/AB chickens regress (Tables I and II). The sequence of events in the Kimber and C/AB chickens followed a similar pattern except that the duration of the leukemia was more prolonged in the C/AB and that relapsing C/AB birds lived for 3 wk as opposed to 1 wk survival for relapsing Kimber.

The incidence of regression in C/AB chickens may be related to their nonviremic state. It has long been suspected that virus transformed cells have a finite life-span and that progressive growth of a tumor is dependent upon the continuous conversion of normal cells by released transforming virus (28). Although this theory is not incompatible with our data, certain facts would seem to indicate that this mechanism is not of major importance in our system. Viremic Kimber chickens display a significant incidence of regression (15%) if injected at an appropriate age. Secondly, viral neutralizing antibodies could not be detected in regressed C/AB chickens. Thirdly, it has been shown that not all viremic chickens develop myeloblastosis.

Vogt and Rubin (29) have found that myeloblastic leukemia is not a necessary consequence of a high titer of circulating AMV in susceptible birds. Thus the presence or absence of infectious AMV in the serum would not seem to be related to myeloblastosis or to regression.

We do not have, at the moment, a good explanation for the reappearance of myeloblasts in buffy coat cultures obtained from regressed Kimber and C/AB chickens. One may speculate that regression *in vivo* is controlled by certain cells or factors present in the circulating blood which are absent in an *in vitro* condition. In the absence of these substances, myeloblast proliferation can occur. This theory is substantiated by the fact that the bone marrow of regressed birds contains islands of proliferating myeloblasts. Thus, myeloblasts arising from regressed buffy coat cultures may represent the progeny of the one or two leukemic myeloblasts out of 5×10^6 buffy coat cells seeded *in vitro* which would have been easily missed in a routine smear.

There appears to be a tumor-specific surface antigen (TSSA) common to all avian virus-transformed chicken cells, regardless of the virus subgroup. Kurth and Bauer (30, 31) have proposed that cytotoxic lymphocytes from an animal bearing an avian virus induced neoplasm recognize the TSSA. In our system, we have found that some of the regressed birds have died with lymphomas. Marek's disease is occasionally seen in a few of our chickens. Thus, we can not rule out the possibility that the lymphomas observed in regressed chickens resulted from the Marek's disease virus. Interference tests (14) have repeatedly been negative for the presence of avian oncorna viruses in the lymphomatosis tissue. If the lymphomas were induced by the associated viruses of AMV (1), the cells should carry the same TSSA of leukemic myeloblasts. An immunological response which has been successfully mounted against the leukemic myeloblasts should be effective against the virally induced lymphomas. Thus, regressed chickens should be refractory to challenge by another avian oncorna virus. We are currently testing this hypothesis.

We have shown that regressed Kimber chickens have splenic lymphocytes which are capable of inhibiting the growth of myeloblasts *in vitro* to a greater extent than lymphocytes from uninoculated chickens. Numerous reports have appeared which indicate that animals with progressive tumors have a factor in their serum which blocks the activity of their lymphocytes against autochthonous tumors (24). Because spleens of leukemic birds contain large numbers of myeloblasts, spleen preparations from these birds were not tested for their ability to inhibit colony formation of shaker grown myeloblasts. Instead, we tested the ability of leukemic serum to block the action of "regressor" lymphocytes against myeloblast colony formation. The data presented on the effect of progressor serum is only preliminary and further experiments are in progress. Nevertheless, the results suggest that blocking activity in some progressor sera may be absent. Based on these data it would be premature to consider the lymphocyte as the primary mediator of leukemia regression *in vivo*. Other possibilities such as interferon and macrophages are being tested as well as the role of circulating cytotoxic antibodies.

Our system may be comparable to leukemia remissions in man in some respects. Numerous cases have been reported where individuals have experienced complete peripheral remissions and still have had over 20% of the bone marrow replaced by leukemic cells. In fact, persistent tissue involvement and marrow infiltration is probably the most common type of remission observed (32). Our system may prove useful in determining the sequence of events which occur in the spontaneous regression of neoplasms.

Summary. The spontaneous regression of avian myeloblastosis virus (AMV) induced leukemia was observed in two chicken lines. When the virus was injected intravenously into 17 day old Kimber embryos, 15% of the resultant leukemias spontaneously regressed. If a relatively resistant chicken line (C/AB) was used, 62% of the leukemias spontaneously regressed. Nearly one-third of the regressed chickens experienced a fatal relapse. Over 30% of the bone marrow in both re-

gressed Kimber and C/AB chickens consisted of abnormal blast-like cells. Nearly 40% of the regressed birds evidenced lymphoma-like tumors upon autopsy. Kimber chickens were viremic during both the acute phase and during regression, while infectious AMV could not be recovered from the C/AB at any time. Buffy coat cultures from regressed Kimber and C/AB always resulted in the reappearance and proliferation of myeloblasts. Splenic lymphocytes from regressed Kimber chickens were found to inhibit colony formation of leukemic myeloblasts in semisolid medium.

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