CELLULAR IMMUNITY INDUCED BY ROUS SARCOMA VIRUS IN JAPANESE QUAIL

I. EFFECT OF ANTI-LYMPHOCYTE SERUM ON ONCOGENESIS OF ROUS SARCOMA VIRUS

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SUMMARY: Anti-lymphocyte serum (ALS) was prepared in rabbits by immunization with spleen cells of Japanese quails, and the effect of ALS on the course of tumor development by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) was examined. Treatment of quails with ALS significantly inhibited regression of primary tumors and induction of resistance to subsequent challenge with a large dose of the virus. ALS seemed to be effective when the treatment was started within 7 days after virus inoculation. In quails treated with ALS, incidence of tumor metastases was frequently detected, while no metastasis was observed in control quails. The possibility of involvement of cell-mediated transplantation immunity in tumor regression, resistance induction and tumor metastasis is discussed.

INTRODUCTION

Anti-lymphocyte serum (ALS) has been proved to act as an effective immuno-suppressant on various types of cell-mediated immune responses including allograft rejection (Levy and Medawar, 1966; Monaco et al., 1966), graft-versus-host reaction (Boak et al., 1967), experimental allergic encephalomyelitis (Leibowitz et al., 1968), and adjuvant arthritis (Currey et al., 1968). Although ALS was also demonstrated to suppress humoral antibody response (Barth and Southworth, 1968; James and Anderson, 1968), its immunosuppressive effect was considered to be mainly directed to cell-mediated immune response.

Participation of cell-mediated immune responses in virus infections and viral oncogenesis has been suggested by several workers (Allison, 1967). Recently, ALS was shown to suppress the cell-mediated transplantation immunity induced by adenovirus type 12 (Allison et al., 1967), polyoma virus (Allison and Law, 1968; Vandeputte, 1969), SV-40 (Tevethia et al., 1968), and mouse sarcoma viruses (Law et al., 1968; Varet et al., 1968), and also to modify the course of virus infections (Hirsch and Murphy, 1968).

Previously, we examined the response of Japanese quails to an inoculation of the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV). The quails were

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proved to be highly sensitive to SR-RSV belonging to subgroup A. The tumors induced by this virus showed a high rate of spontaneous regression, and the quails became resistant to subsequent challenge with a large dose of the same virus. Virus neutralizing antibody and interferon appeared not to be involved in the mechanism of autochthonous tumor regression and resistance induction (Yamanouchi *et al.*, 1968). Effect of ALS which was prepared in rabbits by quail spleen cells, on the course of tumor development by SR-RSV in quails is the subject of the present study.

MATERIALS AND METHODS

Japanese quail: Japanese quails (*Coturnix coturnix japonica*) obtained from a closed colony in the Nippon Institute for Biological Science, Tokyo, were employed in the experiments at the age of between 2 to 4 weeks.

Virus: The SR-RSV belonging to subgroup A was originally derived from the University Laboratories, Highland Park, New Jersey (lot SR-6). The chicken wingweb tumor extract served as a stock virus for inoculation to quails and also for virus neutralization test in chick embryo cells.

Preparation of ALS: Pooled spleens of normal quails were minced, passed through stainless steel wire screens of 200 mesh, and the cells were suspended in Eagle's minimum essential medium to make a suspension containing approximately 10^8 cells per ml. Rabbits were injected intramuscularly (IM) or intravenously (IV) 3 to 4 times at intervals of one week with 3 ml of the spleen cell suspension, and bled one week after the last injection. Sera were pooled and stored at -20°C .

In vitro properties of ALS: Leucoagglutinin (LA) titer of ALS was measured by the method of Gray *et al.* (1966). ALS employed in the present experiment had LA activity against quail spleen cells at titers between 1:512 to 1:1024. Heating at 56°C for 30 min did not decrease the LA activity of the ALS. ALS also had hemagglutinating (HA) activity against quail erythrocytes. However, the HA activity was removed successfully by repeated absorptions with packed quail erythrocytes. Only a slight decrease of LA activity was observed by the same absorption. For the administration to quails, heat-inactivated, unabsorbed ALS was routinely employed.

Treatment of quails with ALS: From a preliminary experiment in which the dose of ALS and route of injection were examined, IM injection of 0.2 ml of ALS was determined as a routine procedure for ALS treatment. Daily treatments with ALS for 4 weeks did not show any adverse effect on body weight or general conditions of quails.

Inoculation of virus to the quail: Primary inoculation was performed into the right wingweb of the quails with the virus having different infectivity titers. Challenge was made into the left wingweb with 1,000 ID₅₀ of the virus on the 4th week irrespective of the virus dose for the primary inoculation.

Measurement of tumor size: Tumor size was measured every other day, and expressed as a geometrical mean of the diameters. The tumor which showed a decrease in size to less than half the maximum size was recorded as regressive.

Titration of virus infectivity: Infectivity was titrated both in RIF-free chick

embryo cell cultures and in quails. The titers were expressed either as focus forming units (FFU) or as 50 % quail infecting dose (ID 50). There was a good correlation between FFU and ID 50, one FFU corresponding to one ID 50.

Virus neutralization test: The focus reduction test in chick embryo cells was used for virus neutralization. Details of the procedure were described previously (Yamanouchi et al., 1968).

RESULTS

Effect of ALS on Tumor Regression, Resistance Induction and Tumor Metastasis

Effect of ALS on the general course of tumor development was examined. The quails were inoculated with 100 ID 50 of the virus after daily injections of ALS or normal rabbit serum (NRS) for 5 days. The treatment with ALS or NRS was continued daily for the first week after the virus inoculation, and then every other day for further 3 weeks. As controls, an untreated group as well as an NRS treated group was set. Results of a typical experiment are summarized in Table 1. Details of the temporal changes in the tumor size are also depicted in Fig. 1. All the tumors of the control groups completely regressed within 5 weeks after virus inoculation. On the contrary, the ALS treated group did not show a sign of tumor regression during the observation period of 7 weeks.

The quails were challenged into the left wingweb with 1,000 ID 50 of the virus 4 weeks after the primary inoculation. All 5 quails in the NRS treated group and 6 out of 7 quails in the untreated group resisted to challenge. On the other hand, only 1 out of 7 quails in the ALS treated group resisted to challenge.

The quails were autopsied on the 8th week and examined grossly for metastasis of tumors. None of the quails in the NRS treated and untreated groups showed the presence of metastatic tumor. In the ALS treated group, 3 out of 7 quails had tumor metastases mostly in the liver, spleen and proventriculus. The metastatic tumors were diagnosed histopathologically as fibrosarcoma with the same characteristics as the primary wingweb tumors. The quails were bled

Table 1. Effect of ALS treatment on tumor growth

Treatment	Regression rate ¹⁾	Protection rate ²⁾	Metastasis rate ³⁾	Incidence of virus neutralizing antibody ⁴⁾	
				4 W	8 W
ALS	2/9 (P<0.01)	1/7 (P<0.05)	3/7	0/7	4/6
NRS	6/6	5/5	0/5	0/5	3/5
Untreated	9/9	6/7	0/6	0/7	3/6

1) No. of quails with regressive tumor/No. of quails observed.

2) No. of quails resisted to challenge/No. of quails challenged.

Difference in numbers of quails between primary inoculated ones and challenged ones was due to death of some quails during the experiment.

3) No. of quails with metastatic tumors/No. of quails examined.

4) No. of sera having antibody/No. of sera tested.

Fig. 1. Tumor development in quails treated with ALS or NRS. Tumor sizes are shown as averages of mean diameters with total quails in each group.

before challenge on the 4th week and also before autopsy on the 8th week. The sera were examined for the presence of virus neutralizing antibody at a dilution of 1:5. None of the sera collected before challenge contained antibodies. By the 8th week, 50 to 70 % of the quails developed antibodies. However, no significant difference was observed in the incidence of antibodies between the ALS treated and control groups.

Effect of ALS Treatment in Quails Inoculated with Different Doses of Virus

A group of quails received daily IM injections of 0.2 ml of ALS for 40 days. Untreated quails served as control. Stock virus having a titer of $10^{5.0}$ FFU/ml was diluted serially by 10-fold increments, and 0.1 ml of each dilution was inoculated into the right wingweb on the 5th day of the ALS treatment. Results are summarized in Table 2. No significant difference in the mean latent period before tumor appearance was observed between the ALS treated and untreated groups. The infectivity titers of the stock virus calculated from the tumor formation rate in quails were $10^{4.7}$ and $10^{5.1}$ ID 50/ml in the ALS treated and untreated group, respectively. Thus, the ALS treatment appeared not to influence the susceptibility of quails to the oncogenic effect of SR-RSV.

Regression rates of these primary tumors were recorded on the 5th week. The inhibitory effect of ALS on tumor regression was more clearly distinguished by tumors induced by higher doses of the virus. All surviving quails were challenged into the left wingweb on the 4th week. None of the quails which failed to form the primary tumors in both the ALS treated and untreated groups showed resistance to the challenge. Nine of 10 untreated quails which had primary tumors induced by various doses of the virus were resistant to the challenge. In contrast, all 10 ALS treated quails which had primary tumors (5 progressive and

Table 2. Effect of ALS in quails inoculated with different doses of virus

Treatment	Primary inoculation					Challenge Protection rate ³⁾
	Virus dilution	Tumor formation	No. of quails	Latent period ¹⁾	Regression rate ²⁾	
ALS	10 ⁻²	Positive	5	6.5	1/4	0/4
		Negative	0	—	—	—
	10 ⁻³	Positive	5	8.8	3/5	0/5(P<0.01)
		Negative	0	—	—	—
	10 ⁻⁴	Positive	0	—	—	—
		Negative	5	—	—	0/5
	10 ⁻⁵	Positive	1	10.0	1/1	0 1
		Negative	4	—	—	0/4
Untreated	10 ⁻²	Positive	5	6.6	3/3	2/3
		Negative	0	—	—	—
	10 ⁻³	Positive	5	8.4	5/5	5/5
		Negative	0	—	—	—
	10 ⁻⁴	Positive	3	9.6	3/3	2/2
		Negative	2	—	—	0/2
	10 ⁻⁵	Positive	0	—	—	—
		Negative	5	—	—	0/5

1) Mean latent period before tumor appearance in days.

2), 3) See Table 1.

5 regressive ones), failed to resist the challenge.

Comparison of Different Schedules of ALS Treatment

The effect of ALS was compared with different schedules of administration. The quails received injections of ALS starting on -7, 0, 7, 14, or 21 day of virus inoculation (100 ID₅₀), and the treatment continued every day until the end of the 4th week. The quails were challenged on the 4th week with 1,000 ID₅₀ of the virus. As shown in Table 3, a significant inhibition of tumor regression was observed in groups which received the ALS treatment starting before and at the same time of virus inoculation. The resistance induction was significantly inhibited when the ALS treatment was started after the 7th day.

DISCUSSION

In a previous report (Yamanouchi et al., 1968), we indicated the usefulness of Japanese quails as an experimental animal of avian species for the study of oncogenesis of SR-RSV. In the present study, effect of ALS on the oncogenesis of SR-RSV in quails was examined. Treatment of quails with ALS significantly inhibited tumor regression and resistance induction to challenge. Inhibition of tumor regression by ALS seemed to be more distinct in tumors induced by a high dose of the virus, while the ALS effect was not consistent in tumors induced by a low dose of the virus. A decrease in tumor size in parallel with a decrease

Table 2. Comparison of ALS treatments of different schedules

Schedule of ALS treatment	Regression rate ¹⁾	Protection rate ²⁾
-7 to 28 day	3/11 ($P<0.05$)	2/11 ($P<0.01$)
0 to 28 day	1/8 ($P<0.01$)	3/7 ($P<0.05$)
7 to 28 day	3/8 ($P<0.1$)	2/7 ($P<0.01$)
14 to 28 day	4/12 ($P<0.1$)	8/12
21 to 28 day	4/5	4/5
Untreated	7/8	7/7

1), 2) See Table 1.

in virus dose for primary inoculation was previously demonstrated. Regression was also observed more frequently with small tumors induced by a low dose of virus, probably because of the presence of a small amount of tumor cells transformed by the virus. Thus, balance between the strength of immune reaction and the amount of tumor cells as target for the immune reaction in large tumor-bearing quails might be more critical and might be more easily disturbed by the immunosuppressive treatment than in small tumor-bearing quails.

Thus far, metastasis has not been observed in tumors induced by SR-RSV in normal quails. In chicken tumors induced by the same virus, a high rate of metastasis was observed generally and this was considered as a main cause for its high mortality. The ALS treatment resulted in a high incidence of metastatic tumors in quails. The tumor metastasis may possibly be suppressed in normal quails by an immunological mechanism.

A significant inhibition of tumor regression was observed in quails which received ALS before or at the same time of virus inoculation. An inhibitory effect of ALS on resistance induction was observed in quails which received ALS starting from -7th to 7th days. The immune reaction involved in tumor regression and resistance induction seemed to develop by the 7th to 14th day of virus inoculation. The previous study (Yamanouchi *et al.*, 1968) also indicated that a resistant state was established approximately 10 days after virus inoculation.

Previously, virus neutralizing antibody and interferon were suggested not to be involved in the mechanism for tumor regression or resistance induction. In the present experiment, ALS did not exhibit a significant effect on the development of virus neutralizing antibody. Histopathologically, the quail tumors induced by SR-RSV are infiltrated with many lymphoid cells 2 to 3 weeks after virus inoculation. Subsequently, the tumors regress completely with concomitant appearance of lymphoid follicle-like structures (Uchida, N., and Yamanouchi, K., unpublished). The present results with ALS which is considered as a potent immunosuppressant on cellular immunity suggest the possible participation of cell-mediated transplantation immunity directed to the quail tumor cells in tumor regression, resistance induction, and tumor metastasis. In the following paper on the experiments with thymectomized and bursectomized quails, tumor regression and resistance induction were shown to be thymus-dependent, but not bursa-dependent. Since cellular and humoral immunities are thymus-dependent and

bursa-dependent, respectively, in avian species, the thymus-dependency of tumor regression and resistance induction also indicates an involvement of cellular immunity in the course of tumor development.

Thus far, an immunologic study on the regression of autochthonous virus-induced tumors has been limited to Moloney sarcoma virus-induced mouse tumors (Fefer et al., 1968) and Shope virus-induced rabbit tumors (Evans et al., 1962). In both systems, cellular immunity is postulated as a major mechanism for tumor regression (Hellström, 1969; Hellström et al., 1969).

Participation of specific transplantation immunity in the oncogenesis of RSV was first demonstrated in mice (Jonsson and Sjögren, 1966). However, immunological studies on the oncogenesis of RSV in chickens, a natural host of this virus, are few (Radichovskaja, 1967, 1968; Rubin, 1962), mainly because of a low incidence of tumor regression and also of their large body size as an experimental animal. Quails are as susceptible as chickens to subgroup A of SR-RSV. Furthermore, SR-RSV-induced quail tumors show a much higher rate of regression than the chicken tumors. Thus, the quail system may be useful for the immunologic study on the regression of autochthonous tumor.

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