CELLULAR IMMUNITY INDUCED BY ROUS SARCOMA VIRUS IN JAPANESE QUAIL

II. EFFECT OF THYMECTOMY AND BURSECTOMY ON ONCOGENESIS OF ROUS SARCOMA VIRUS

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SUMMARY: Suppression of the cellular and humoral immune capacities of Japanese quails was attempted by surgical thymectomy on the 7th day of hatch and surgical bursectomy on the day of hatch. Degree of immunosuppression on cellular immune response was evaluated by graft-versus-host reaction and skin graft rejection, and that on humoral immune response was evaluated by antibody production to sheep red blood cells and virus antigen. In thymectomized quails significant suppression of cellular immunity was demonstrated, whereas humoral immune capacity remained almost normal. In bursectomized quails, significant suppression of humoral antibody response was observed without any detectable suppression of cellular immune response. When the quails immunosuppressed either by thymectomy or bursectomy were inoculated with the Schmidt-Ruppin strain of Rous sarcoma virus, regression of primary tumors and induction of resistance to challenge with the same virus were significantly inhibited in thymectomized quails, but not in bursectomized ones. From the results, cell-mediated immune response was suggested to play a major role in the course of autochthonous virus-induced tumors in quails.

INTRODUCTION

High susceptibility of Japanese quails to the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV), and a high rate of regression of the virus-induced tumors led us to investigate the host's immune mechanism in the autochthonous virus-induced tumor system in quails. In the preceding report (Yamanouchi and Hayami, 1970), anti-lymphocyte serum (ALS), a potent immunosuppressant on cellular immunity, was shown to alter the course of primary tumors and to inhibit the induction of resistance to challenge. Since an involvement of virus-neutralizing antibody or interferon was unlikely as proved in other experiments (Yamanouchi et al., 1968), cell-mediated transplantation immunity was proposed as the mechanism responsible for tumor regr-

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ession and resistance induction.

Two major central lymphoid organs have been demonstrated in chickens; thymus controlling cellular immunity and bursa of Fabricius controlling humoral immunity. By thymectomy, cell-mediated immune responses such as delayed hypersensitivity, graft-versus-host (GVH) reaction and allograft immunity are markedly suppressed or abrogated, but humoral antibody responses remain almost normal. On the other hand, bursectomy inhibits humoral antibody responses, although cellular immune responses remain normal (Cooper *et al.*, 1966).

In the present study, thymectomy and bursectomy were applied to quails, and their effects on the course of SR-RSV-induced tumors in quails were examined.

MATERIALS AND METHODS

Virus: The SR-RSV of subgroup A was employed. Details for stock virus preparation were previously described (Yamanouchi *et al.*, 1968).

Japanese quail: Fertile eggs were obtained from a closed colony of Japanese quails (*Coturnix coturnix japonica*) in Nippon Institute for Biological Science, Tokyo. After hatch they were raised in brooder for 2 to 3 weeks, then in regular metal cages for quails.

Thymectomy and bursectomy: The small body size of the quail unlike the chicken made it difficult to conduct thymectomy within 24 hr after hatch. Therefore, thymectomy was performed at the age of 1 week. A total of 14 thymus lobes lying along jugular veins were surgically removed with ophthalmologic forceps. Surgical bursectomy was conducted on the day of hatch. Bursa of Fabricius located above cloaca was removed with forceps. At the termination of the experiment, all quails were autopsied and examined grossly for complete removal of thymus lobes or bursa. The quails having remnants of thymus lobes or bursa were excluded from the evaluation of the data. As controls, sham operations were conducted. Excision of the skin at the neck and the hind skin above cloaca were done for the thymectomy and for the bursectomy controls respectively.

Production of hemagglutinating (HA) antibody to sheep red blood cell (SRBC): To evaluate the capacity of humoral immune response, quails were inoculated intravenously with 0.2 ml of 10 % SRBC. After 5 days, they were bled. HA titers of their sera were expressed as reciprocals of the highest serum dilutions agglutinating 0.25 % SRBC on microplates.

Virus neutralization test: Details of the procedure were previously described (Yamanouchi *et al.*, 1968). Serum was tested at a dilution of 1:5.

Skin graft test: To estimate the capacity of cellular immune response, skin of about 1 cm² was cut from the back of each donor quail and placed on the peeled back of a recipient quail. It was protected by bandages for 6 days. After removal of the bandages, survival time of the donor skin on the recipient's back was measured. Since the quails employed were not inbred, the grafted skin was usually rejected within 9 days.

Graft-versus-host (GVH) reaction: As another measure to estimate capacity of cellular immune response, GVH reaction on chorioallantoic membranes (CAM) of chick embryos was employed by the technique described by Lafferty and Jones (1969) with slight modification. Since only a few pocks were produced on CAM of chick embryos

by spleen cells of normal quails, donor quails were immunized intravenously with spleen cells of normal chickens prior to the test. After 3 weeks, quail spleen cells thus sensitized with chicken tissue antigens were inoculated onto CAM of 11 day old chick embryos. Pocks were counted 5 days later.

Inoculation of virus to quails: Primary inoculation was made into a right wingweb with 100 ID 50 of the virus. Tumor sizes were measured as a rule every other day, and were expressed as a geometrical means of diameters. Regression rates of primary tumors were recorded in 4 weeks after virus inoculation. Quails were challenged with 1,000 ID 50 of the virus into a left wingweb in 4 weeks, and observed for further 3 weeks. Protection rates were calculated from the number of birds developing no tumors after challenge.

RESULTS

Tumor Growth in Thymectomized Quails

Two groups of quails, thymectomized and sham-operated ones, were inoculated with the virus at the age of 3 to 4 weeks. They were observed for tumor regression and protection to challenge.

Results of the tests repeated four times are summarized in Table 1. Regression rates of the primary tumors in the control groups were 90 to 100 %, whereas those in the thymectomized groups were only 0 to 30 %. The difference between the two groups was statistically significant. Growth curves of the tumors are depicted in Fig. 1. In the control group, tumors grew progressively by the second week, and then started to regress. Within 4 weeks complete regression was observed except one or two quails in Exp. 136, 147, and 149. Tumors in the thymectomized group showed a similar pattern of growth as those in the control group for the first 2 weeks followed by a tendency of regression. However, they started to grow progressively again between 3 to 4 weeks, and did not regress for 7 weeks. Resistance induction to challenge was also significantly inhibited in the thymectomized groups. In contrast to the protection rates of 90 to 100 % in the control groups, those in the thymectomized ones were 0 to 30 %.

Humoral and Cellular Immune Capacities of Thymectomized Quails

After the final observation for protection in 7 weeks after virus inoculation, the quails were examined for both humoral and cellular immune capacities by production of virus-neutralizing and HA antibodies, GVH reaction, or skin graft rejection. In Exp. 136, production of HA antibody was tested 4 weeks before challenge instead of 7 weeks. The results are shown in Table 1.

In two experiments, humoral immune capacity of the quails were examined. Production of virus-neutralizing antibody was demonstrated only in the thymectomized quails in Exp. 122. However, the result was not consistent as shown in Exp. 136 in which none of the thymectomized quails developed virus-neutralizing antibody. The capacity of production of HA antibody to SRBC of the thymectomized group was almost the same as that of the control group. Thus, there was no indication of suppression of humoral immune response resulting from thymectomy.

Cellular immune capacity was examined by GVH reaction and skin graft rejection.

Table 1. Effect of Thymectomy on Tumor Growth and Immune Responses

Exp.	Treatment	Regression rate (1)	Protection rate (2)	Humoral immune response			Cellular immune response	
				VN antibody (3)	HA antibody to SRBC		GVH (5)	Skin graft survival (6)
					Positive ratio (4)	Titer		
122	thymectomy	0/3*	0/3*	3/3	3/3	23.3	NT(7)	NT
	sham operation	4/4	4/4	0/4	4/4	22.5	NT	NT
136	thymectomy	3/10**	1/8**	0/10	9/10	22.2	7.3**	13.2**
	sham operation	9/10	9/10	1/10	10/10	23.0	36.0	7.1
147	thymectomy	3/9*	2/6**	NT	NT		NT	11.0
	sham operation	9/10	9/9	NT	NT		NT	8.0
149	thymectomy	2/12**	NT	NT	NT		5.2**	NT
	sham operation	14/16	NT	NT	NT		21.4	NT

* $P < 0.05$ ** $P < 0.01$.

(1) No. of quails with regressive tumor/No. of quails tested.

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

(3) No. of quails having antibody/No. of quails tested.

(4) No. of quails produced HA antibody/No. of quails tested.

(5) No. of pocks produced on CAM by 10^6 spleen cells.

(6) Mean survival time in days.

(7) Not tested.

In Exp. 136 and 149, there was a significant decrease in numbers of sensitized immunocytes responsible for GVH reaction in the thymectomized groups. Skin graft tests were conducted in Exp. 136 and 147. In both experiments, prolongation of skin graft survival by thymectomy was observed, although a statistically significant difference was obtained only in Exp. 136.

Thus, we conclude that the thymectomy conducted at the age of 1 week markedly suppressed the cellular immune capacity of the host, but not the humoral immune capacity.

Tumor Growth in Bursectomized Quails

Quails neonatally bursectomized or sham-operated were primarily inoculated with the virus at the ages of 3 to 4 weeks. Results of the duplicate experiments are shown in Table 2. Growth curves of the tumors are depicted in Fig. 2. In Exp. 119 complete regressions of the tumors were observed within 4 weeks both in control and bursectomized quails, showing the similar patterns of tumor growth. Although such a clear-cut regression curve as seen in Exp. 119 was not obtained in Exp. 136 because of two progressive tumors in nine bursectomized quails, there was no significant difference in the general pattern of tumor growth between the control and bursectomized groups.

Resistant states were also induced in both control and bursectomized groups; no difference in protection rate was observed between the two groups.

Fig. 1. Tumor development in thymectomized quails.
Ordinate: Average of mean diameters of tumors in each group (mm).
Abscissa: Weeks after virus inoculation.

Humoral and Cellular Immune Capacities of Bursectomized Quails

Degree of immunosuppression by bursectomy was examined in a similar way to that in the thymectomy experiment. Production of virus-neutralizing antibody was examined in Exp. 119 in 7 weeks at the end of the observation period of tumor growth. Production of HA antibody was tested in 7 weeks in Exp. 119 and in 4

Table 2. Effect of Bursectomy on Tumor Growth and Immune Responses

Exp.	Treatment	Regression rate (1)	Protection rate (2)	Humoral immune response			Cellular immune response
				VN anti-body (3)	HA antibody to SRBC		GVH (5)
					Positive ratio (4)	Titer	
119	bursectomy	4/4	4/4	0/4	0/4**	<2	NT(6)
	sham operation	8/8	7/8	2/8	8/8	2 ^{4.5}	NT
136	bursectomy	7/9	5/6	NT	0/9**	<2	8.0
	sham operation	9/10	9/10	NT	10/10	2 ^{3.0}	7.1

** P<0.01.
(1), (2), (3), (4), (5) Refer to Table 1.
(6) Not tested.

Fig. 2. Tumor development in bursectomized quails.

Ordinate: Average of diameters of mean tumors in each group (mm).

Abscissa: Weeks after virus inoculation.

weeks in Exp. 136. As shown in Table 2, none of the four quails in the bursectomized group developed virus-neutralizing antibody, but two of the eight quails in the control group developed it. However, this difference is not statistically significant. On the contrary, there was a marked difference in production of HA antibody. In contrast to the control group in which all eighteen quails developed HA antibody, none of the thirteen quails in the bursectomized group produced HA antibody.

Cellular immune capacity was estimated by skin graft rejection in Exp. 136 in 7 weeks. In both groups, skin grafts were rejected in 7 to 8 days. From the results, neonatal bursectomy was considered to be effective on suppression of the humoral but not of the cellular immune capacity.

DISCUSSION

In chickens the thymus was proved to control the ontogenic development of the cellular immunity system, and the bursa to control that of the humoral immunity system (Cooper *et al.*, 1966). The central immunity systems of a similar type is assumed to function in quails. As demonstrated in the present study, humoral and cellular immune responses in quails dissociated either by thymectomy or bursectomy. Bursectomy effectively suppressed the humoral immune responses. There was no apparent suppression of the cellular immune response in the bursectomized quails as examined by skin allograft rejection. Because of a technical difficulty, neonatal thymectomy was unsuccessful. Nevertheless thymectomy operated at the age of 1 week had a marked suppressive effect on cellular immunity as demonstrated by prolongation of skin allograft survival and decreased numbers of the sensitized lymphocytes capable of inducing GVH reaction. There was no suppressed production of humoral antibody to SRBC in quails in contrast to rodents in which thymectomy suppressed production of antibody to certain antigens such as SRBC through its suppressive effect on thymus-dependent antigen reactive cells (Good and Cain, 1970). Production of virus-neutralizing antibody in the three thymectomized quails regardless of the failure in the control quails was unexpected, although this tendency was not consistent. Generally, production of virus-neutralizing antibody to SR-RSV is not very efficient even in normal chickens or quails. Therefore, the persistence of large tumors for longer periods in thymectomized quails than in the control quails may have resulted in more antigenic

stimulation.

The present study indicated that regression of the primary tumors induced by SR-RSV was rarely observed in the thymectomized quails. On the other hand, bursectomy had no demonstrable effect on regression of the primary tumors. Radizichovskaja (1967 a, b) examined the effects of thymectomy of chicks and of bursectomy within 3 and 2 days after hatch, respectively, on the development of tumors induced by the Carr-Zilber strain of RSV (CZ-RSV). As a result, there was a slightly increased tumor formation rate in the thymectomized group compared to that in the control group. Mortality and metastasis rates also increased by thymectomy. Bursectomy had no such effect. Thus, a thymus-dependent immune mechanism was suggested to act in the tumor growth. However, effect of these operations on the course of primary tumors such as regression was not clearly demonstrated since CZ-RSV is generally virulent in chickens and spontaneous regression was rarely observed in this animal system. In the SR-RSV-induced quail tumor system, nearly 100 % regression of primary tumors can be expected by application of an appropriate virus dose. This is assumed to be the reason for the clear demonstration of the thymus-dependency of tumor regression in the present study.

Effect of thymectomy or bursectomy on the viral oncogenesis can be evaluated in different aspects. In general, increased tumor formation and tumor growth in thymectomized animals are considered to be caused by suppression of cellular immunity as demonstrated in the cases of Moloney sarcoma virus, adenovirus type 12, and simian virus 40 (Allison and Taylor, 1967). Yet there is a possibility of thymus dependency of target cells of the virus as in the case of leukemia induction by murine leukemia viruses (Siegel and Morton, 1970; Allison and Taylor, 1967). The target cells of lymphoid leukosis virus, another member of the avian tumor virus group, was shown to be bursa-dependent (Peterson et al., 1966). Relationship of the target cells of RSV to these central lymphoid organs, however, is still unknown. In the present experiments, we found no significant increase in susceptibility of quails to RSV from either thymectomy or bursectomy. Therefore, the increased incidence of progressive tumors in the thymectomized quails can be explained by suppression of cell-mediated immunity.

In an autochthonous virus-induced tumor system like as ours, Fefer et al. (1967, 1968) concluded that regression of primary tumors induced by Moloney sarcoma virus was mainly mediated by a cellular immune mechanism from the experiments in which the mice pre-irradiated by X-ray or pretreated with cortisone showed tumor regression less frequently than the untreated ones. Besides the cellular immune reaction, involvement of humoral immune reaction possibly mediated by protective antibody was also demonstrated by the colony inhibition technique in Moloney sarcoma virus-induced mouse tumors and Shope virus-induced rabbit tumors (Hellström and Hellström, 1969; Hellström et al., 1969). Jonsson (1969) showed development of cellular and humoral immune reactions in rats transplanted with SR-RSV-induced tumors. In these systems cell-mediated immune reactions were considered to play a major role in rejection of autochthonous or transplanted tumors, although humoral antibody was suggested to provide protection of tumor cells against attack of immune lymphocytes. From the present result of bursectomy, a possibility of serum-mediated protection of primary tumor growth cannot be excluded. Possibility of participation of such protective antibody in progressive tumors is now under investigation.

In a separate experiment, we succeeded in suppressing the growth of primary tumors by passive transfer of immune lymphocytes (unpublished). All these data including the previous ones with ALS (Yamanouchi and Hayami, 1970) strongly indicate a major role of cell-mediated immune response in the course of autochthonous virus-induced tumor in quails.

REFERENCES

- ALLISON, A. C. AND TAYLOR, R. B. (1967): Observations on thymectomy and carcinogenesis. *Cancer Res.*, **27**, 703-707.
- COOPER, M. D., PETERSON, R. D. A., SOUTH, M. A. AND GOOD, R. A. (1966): The function of the thymus system and bursa system in the chicken. *J. Exptl. Med.*, **123**, 75-102.
- FEFER, A., MCCOY, J. L. AND GLYNN, J. P. (1967): Induction and regression of primary Moloney sarcoma virus-induced tumors in mice. *Cancer Res.*, **27**, 1626-1631.
- FEFER, A., MCCOY, J. L., PERK, K. AND GLYNN, J. P. (1968): Immunologic, virologic and pathologic studies of regression of autochthonous Moloney sarcoma virus-induced tumors in mice. *Cancer Res.*, **28**, 1577-1585.
- GOOD, R. A. AND CAIN, W. A. (1970): Relationship between thymus-dependent cells and humoral immunity. *Nature*, **226**, 1256-1257.
- HELLSTRÖM, I., AND HELLSTRÖM, K. E. (1969): Studies on cellular immunity and its serum-mediated inhibition in Moloney-virus-induced mouse sarcomas. *Int. J. Cancer*, **4**, 587-600.
- HELLSTRÖM, I., EVANS, C. A. AND HELLSTRÖM, K. E. (1969): Cellular immunity and its serum-mediated inhibition in Shope-virus-induced rabbit papillomas. *Int. J. Cancer*, **4**, 601-607.
- JONSSON, N. (1969): Cell-mediated and humoral immune reactions against tumor-specific and viral antigens in relation to Rous sarcoma virus in rats. *Acta Path. Microbiol. Scand.*, **77**, 753-755.
- LAFFERTY, K. J. AND JONES, M. A. S. (1969): Reactions of the graft versus host (GVH) type. *Aust. J. Exptl. Biol. Med.*, **47**, 17-54.
- PETERSON, R. D. A., PURCHASE, H. G., BURMESTER, B. R. AND COOPER, M. D. (1966): Relationships among visceral lymphomatosis, bursa of Fabricius, and bursa-dependent lymphoid tissue of the chicken. *J. Natl. Cancer Inst.*, **36**, 585-598.
- RADIZICHOVSKAJA, R. (1967 a): Effect of bursectomy on the Rous virus tumor induction in chickens. *Nature*, **213**, 1259-1260.
- RADIZICHOVSKAJA, R. (1967 b): Effect of thymectomy on Rous virus tumour growth induced in chickens. *Proc. Soc. Exptl. Biol. Med.*, **126**, 13-15.
- SIEGEL, B. V. AND MORTON, J. I. (1970): Rauscher viral leukemogenesis in BALB/c mice treated with rabbits antimouse thymocyte serum. *J. Natl. Cancer Inst.*, **44**, 573-579.
- YAMANOUCHI, K., HAYAMI, M., FUKUDA, A. AND KOBUNE, F. (1968): Tumor development and induction of resistance by Rous sarcoma virus in Japanese quail. *Japan. J. Med. Sci. Biol.*, **21**, 393-404.
- YAMANOUCHI, K. AND HAYAMI, M. (1970): Cellular immunity induced by Rous sarcoma virus in Japanese quails. I. Effect of anti-lymphocyte serum on oncogenesis of Rous sarcoma virus. *Japan. J. Med. Sci. Biol.*, **23**, 395-402.

Note added in proof: After the submission of the present manuscript, presence of cellular immunity against tumor specific transplantation antigen was demonstrated by colony inhibition technique in chickens carrying primary SR-RSV induced tumors. (Sjögren, H. O. and Jonsson, M.: Cellular immunity to Rous sarcoma in tumor-bearing chickens. *Cancer Res.*, **30**, 2434-2437, 1970.)