Resistance to Rous Sarcoma Elicited by Immunization with Live Virus[1] (35531)

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Previous attempts at immunization against Rous sarcoma have met with little success. Injections of inactivated Rous sarcoma virus, RSV(RAV-1)[2], engendered in chickens high levels of neutralizing antibody but such chickens manifested little, if any, resistance to tumor induction (1). There have been instances, however, when certain preparations which were initially thought to be free of infectious virus elicited significant levels of protection. Based on preliminary data, this protective immunization could be attributed to the presence of low levels of live virus in these preparations. The present report describes experiments on immunization with subtumorigenic doses of live RSV(RAV-1) or large doses of RAV-1, leading to the induction of strong resistance to Rous tumor induction.

Materials and Methods. White Leghorn chickens, free from avian leukosis virus and antibodies, were purchased from SPAFAS, Inc. Immunizations were begun when the birds were 12–15 days of age. Birds were bled by cardiac puncture and the serum was stored at —20° until use.

RSV(RAV-1), originally obtained from Dr. Peter Vogt, was maintained in our laboratory by tissue culture passage in SPAFAS chick embryo fibroblasts (CEF) or as a semipurified preparation (2) of SPAFAS chicken wing web tumors. The potency of the virus was determined by assay on CEF (3) and by tumor induction in the chicken wing web. The 50% tumor-inducing dose in chicks (TID_{50}) was calculated by the method of Reed and Muench. RAV-1 obtained from Dr. Robert M. Dougherty, and maintained by tissue culture passage, was assayed by means of the COFAL test (4). Both RSV(RAV-1) and RAV-1 are members of subgroup A of the avian leukosis/avian sarcoma group of viruses and are antigenically identical (5).

Neutralization tests for chicken antiviral antibodies were performed on CEF cell cultures essentially as previously described (6). The neutralization index (NI), using a 1:10 dilution of serum was calculated as the ratio of the number of foci (usually 100) produced by a virus control to the number of foci produced by the serum–virus mixture. A NI greater than 5.0 was considered positive. In some cases, assay of antibody was performed *in vivo*. Serial dilutions of chicken serum were mixed with 1000 focus-forming units of RSV(RAV-1), incubated overnight at 4° and 0.1 ml of the serum–virus mixtures were inoculated into the wing web of chickens. Usually, six birds were inoculated for each serum dilution along with the appropriate virus controls, and tumors were scored until no new tumors appeared.

Results. An experiment was undertaken to determine the effects of immunization with subtumorigenic doses of live RSV(RAV-1) on subsequent tumor formation upon challenge with the same virus. SPAFAS chickens were immunized beginning at 15 days of age, with three biweekly injections (*i.e.*, one injection every second week for 6 weeks) of 0.1 ml of a 10^{-8} dilution of RSV(RAV-1). The

[1] This investigation was supported by U.S. Public Health Service Contract No. PH-43-67-1187 from the National Cancer Institute.

[2] The standard nomenclature is used to designate strains of RSV and leukosis virus. Thus the Rous-associated virus, RAV-1, a leukosis virus, may function as a "helper virus" with "defective" strains of RSV to give rise to a sarcoma virus with the envelope of the helper. RSV(RAV-1) is the designation for the Bryan strain RSV genome with the coat protein of RAV-1.

TABLE I. Protective Effect of Low Dose RSV(RAV-1).

Group	Tumor incidence after challenge		Antibody titer at time of challenge[b]			
	—[a]	(%)	No.	Dilution	No.	Dilution
Control	15/15	100	9	<1:10	1	1:300
Immunized	1/16	6	1	<1:10	9	1:500–>1:10,000[c]

[a] Number with tumor/number challenged.
[b] Ten individual sera were tested in each group.
[c] Range.

virus stock had been previously assayed in chickens and had a titer of 10^8 TID_{50}/ml. Although no tumors developed after the first immunizing injection, ultimately tumors appeared in about one-half of the birds upon repeated immunizing injections. Three weeks after the last injection, 16 of the immunized birds which showed no signs of tumors, and 15 unimmunized controls were challenged with 100 tumor-inducing doses of RSV(RAV-1). Sera were taken from representatives of both groups, for assay of neutralizing antibodies at the time of challenge. The results presented in Table I show that the immunized birds manifested solid resistance to tumor induction by RSV(RAV-1), and that such animals had generally high serum titers of antiviral neutralizing antibody in contrast to the control group.

Since a 10^{-8} dilution of RSV(RAV-1) induced tumors in a substantial number of birds during the course of immunization, the experiment was repeated with the inclusion of a 10-fold higher dilution of immunizing virus. Following three biweekly injections, 11/46 (25%) chickens immunized with the 10^{-8} dilution of virus, and 2/46 (4%) chickens immunized with the 10^{-9} dilution developed tumors. Two weeks after the last immunization injection, chickens which showed no evidence of tumors, as well as unimmunized controls, were challenged with RSV(RAV-1) at dilutions of 10^{-4}, 10^{-5}, and 10^{-6}. As Table II shows, all control chickens developed tumors while chickens immunized with the 10^{-8} dose of virus showed protection at the rate of 50–60% regardless of the challenge dose. Chickens immunized with 10^{-9} virus showed only slight protection (10–25%), again regardless of the challenge dose. Thus resistance, when manifested, was against both 1.5 and 3.5 logs of challenge virus, *i.e.*, was "all or none." Therefore, protection was dose-dependent and the immunizing dose was not far enough removed from the threshold of tumorigenicity to be consid-

TABLE II. Immunization with Subthreshold Doses of RSV(RAV-1).

Immunizing virus dilution[a]	Cumulative tumor incidence before challenge		Tumor incidence after challenge with RSV(RAV-1) diluted:[b]					
			10^{-6}		10^{-5}		10^{-4}	
	—[c]	(%)	—[c]	(%)	—[c]	(%)	—[c]	(%)
None	0/34		11/11	100	12/12	100	11/11	100
10^{-8}	11/46	25	4/9	44	5/10	50	4/10	40
10^{-9}	2/46	4	10/11	90	11/13	89	11/14	74

[a] Pretitration of virus gave a titer of 10^8 TID_{50}/ml; 0.1 ml of the appropriate dilution was injected.
[b] Only chickens free of tumors were challenged.
[c] Number with tumors/number challenged.

TABLE III. Immunization of Chickens with RAV-1.

Immunizing inoculum	Immunization schedule (injections biweekly)	Time of challenge (weeks) in relation to: First immunization	Time of challenge (weeks) in relation to: Last immunization	Presence of antibody at time of challenge —[a]	Presence of antibody at time of challenge (%)	Geometric mean antibody titer	Tumor incidence after RSV(RAV-1) challenge —[b]	Tumor incidence after RSV(RAV-1) challenge (%)
None (control)	—	—	—	0/21	0	<1:10	21/21	100
RAV-1[c]	2	7	3	15/22	68	>1:5000[d]	6/22	27
RAV-1[e]	3	7	5	21/21	100	>1:6000[e]	2/22	9

[a] Number with positive (>5.0) arithmetic neutralization index/number tested.
[b] Number with tumors/number challenged.
[c] 10^6 infectious units.
[d] 6; positive sera tested.
[e] 4; positive sera tested.

ered a safe procedure.

It was therefore decided to attempt immunization with a leukosis virus, RAV-1, which causes no overt tumor formation in chickens of the age used in our experiments. Additionally, it was desired to determine whether the RSV(RAV-1) used for immunization was itself responsible for protection or whether protection was caused by RAV-1. The latter possibility was a real one, since stocks of RSV(RAV-1) by nature of the "defectiveness" of this virus inevitably contain high concentrations of RAV-1 (7) and thus the very low doses administered during our immunization procedures must contain mostly RAV-1 alone. Accordingly, chicks were injected in the wing web with 10^6 infectious units of RAV-1, either two or three times at biweekly intervals. Seven weeks after initiation of the immunization program the two groups of chickens and a group of unimmunized controls were challenged with 100 tumor-inducing doses of RSV(RAV-1). The results in Table III show that three biweekly injections of RAV-1 afforded a high level of protection against challenge while two injections elicited a lesser, but still marked degree of protection. Table III also shows the results of neutralization tests with sera of these chickens collected at the time of challenge. None of the sera of unimmunized chickens had antibody, whereas 68 and 100% of chickens receiving two and three injections, respectively, had antiviral antibody (NI usually 69 or higher). Six sera of the second group (2 injections) and 4 sera of the third group (3 injections) were titrated in the *in vivo* neutralization test and found to yield titers higher than 5000.

Discussion. Successful immunization against virus-induced Rous tumors can be achieved by the inoculation of live virus. In the present work protection was achieved with either RSV(RAV-1) in subtumorigenic doses, or with RAV-1, a virus which causes no overt tumors in chickens of the age employed in the experiments. Since RAV-1 is present in high titer in the stocks of RSV(RAV-1), it is possible that the former virus may have contributed to the immunization achieved with low doses of RSV(RAV-

1). The sera of the chickens rendered resistant to tumor induction contained high levels of neutralizing antibody, whereas the sera of susceptible chickens had no antibodies or, at best, low levels of antibodies. The question of the role of humoral antibody in immunity to Rous sarcoma has interested investigators for a long time. Starting with the experiments of Rous and Murphy in 1914 (8), a number of investigators have failed to find a correlation between neutralizing antibodies and resistance (1, 9, 10). Maternal antibodies have not afforded solid protection to young chicks against Rous tumors (1, 9, 10). Immunizations with killed virus preparations which led to antibody formation (1, 11) as well as passive immunization (10) have also failed to bring about significant resistance. In view of these findings, one would be inclined to conclude that humoral immunity is of little relevance to tumor resistance in Rous sarcoma. In line with the immunologic facts pertaining to other cancers, one would have to invoke the dominant role of cell-mediated immunity. Evidence for the existence of tumor-specific antigens and cell-mediated immunity has been provided by the studies of Jonsson and Sjögren (12) and Bauer and his associates (13) in mammalian Rous systems in which the tumors are free of infectious virus. This form of immunity could doubtless curtail growth of tumors whose progress depends on cellular proliferation, but cell-mediated immunity may not suffice for rapid elimination of infectious virus in the natural host, the chicken, where the virus can infect, transform, and recruit new cells into the tumor mass or initiate new tumors. Elimination of virus may require the intervention of neutralizing antibody. The observations of Fink and Rauscher (11) that a correlation existed between antibody levels evoked by immunization and tumor regression and the findings of Vigier (14) that the growth rate of the tumor diminished at the time of appearance of antiviral antibodies lend some support to the idea that a neoplasm which contains the etiologic agent in infectious form may require a cooperative effect of cellular and humoral immune factors. Our current data on the presence of antibody in chickens which resisted challenge with RSV(RAV-1), could also be interpreted in the same vein. Following immunization with subtumorigenic doses of RSV(RAV-1), 90% of the resistant birds had antibodies in high titer, and following injection with RAV-1, 68 to 100% of the resistant population had antibodies (in sera subjected to titrations all titers were high, *i.e.*, >5000). Moreover, among the 8 chickens which proved to be susceptible to challenge virus, 6 had no antibody, 1 had a strong activity (NI $\geqq 138$) and 1 had a borderline reactivity (NI $= 5.3$).

Little is known about the operation of cell-mediated immunity in the Rous sarcoma in the chicken beyond Rubin's observations of the infiltration of lymphocytes in regressing Rous tumors (15). Although Sjögren and Jonsson (16) recently reported that thymocytes of chickens bearing Schmidt-Ruppin RSV tumors manifested "transplantation immunity" by the colony inhibition test against mammalian Rous sarcoma cells, this type of immunity may not be entirely sufficient, as discussed above, under conditions where infectious virus is being generated.

We attribute the failure of killed vaccine to provide protection to its failure to induce cell-mediated immunity. The same explanation could apply to lack of resistance of chickens possessing maternal antibodies or antibodies administered passively. Under such circumstances, antibody alone may be incapable of halting or reversing the progress of the neoplasm. A point requiring further investigation is the previously observed resistance in chickens whose sera had no detectable antibodies (1), and the finding of occasional antibody-free resistant chickens (2/44) in the present studies. Even though the present findings show a correlation between resistance and antibody, they do not prove a causal relationship, as the antibody may simply be a by-product of virus infection (whose more relevant function may be to impart to cells transplantation antigens and thereby evoke cell-mediated immunity). However, data obtained from preliminary experiments in our laboratory designed to measure the effects of immune chicken spleen lymphocytes and antiviral antibody on transplanta-

tion of RSV-transformed cells to chickens, suggest that a maximal effect required both lymphocytes and antibody. There is an obvious need therefore for an investigation of the several parameters of cell-mediated immunity in the chicken immunized with live virus and assessment of the comparative contributions of this type of immunity and humoral immunity to the initiation and progress of Rous tumors in the natural host. This work is currently under way.

Summary. Immunization of chickens with live avian sarcoma virus RSV(RAV-1) or with its helper leukosis virus, RAV-1, afforded high levels of protection against sarcoma induction by RSV(RAV-1). The sarcoma virus was used in subtumorigenic doses and the RAV-1, which is of low overt oncogenicity, was used in a concentration of 10^6 tissue culture infectious units. In most cases, birds resisting tumor formation possessed high levels of virus neutralizing antibody. The role of antibody and cell-mediated immunity in avian sarcoma in which the etiologic agent persists in infectious form is discussed.

The authors thank Mantley Dorsey, Jr., Eva K. Hultqvist, Anna Iosiphidis, and Samuel L. Watkins, Jr., for their excellent technical assistance.

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Received Nov. 9, 1970. P.S.E.B.M., 1971, Vol. 137.