*DETERMINING FACTOR IN THE CAPACITY OF ROUS SARCOMA VIRUS TO INDUCE TUMORS IN MAMMALS**

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Since Zilber and Kriukova[1] and Svet-Moldavsky[2] demonstrated that some strains of Rous sarcoma virus (RSV) are capable of inducing hemorrhagic cysts or sarcomas in newborn rats, numerous attempts have been made to induce tumors by RSV in various species of mammals.[3-9] One of the remarkable aspects revealed by these investigations is the strain differences in RSV for this capacity.[6-9] Certain strains, such as the Schmidt-Ruppin strain, are active, while others, such as the Bryan strain, are generally inactive in mammals. The cause of such strain differences has not been elucidated.

Our previous studies have shown that the Bryan high-titer strain of RSV is a defective virus which cannot reproduce without the help of any one of the avian leukosis viruses.[10] The defectiveness derives from the inability of this strain of RSV to induce the synthesis of its own viral envelope.[11] An essential role played by the helper virus is to provide the envelope to the RSV genome, whose replication occurs in the infected cells without the aid of helper virus. Thus, several properties of RSV which presumably depend in some way on the character of the viral envelope are controlled by the helper virus used to activate the RSV.[11-13] These properties include the antigenicity, the sensitivity to the interference induced by the helper virus, and the host range among genetically different chick embryos.

These findings raised the question whether certain strains of RSV are active in mammals because they have a special envelope or because they have a special genome. Developments in the ways of changing the RSV envelope without changing the genome and of discriminating among viruses with different envelopes by their phenotypic properties have made it possible to investigate this problem. The present communication provides evidence that the viral envelope is the main determining factor in the tumor induction of RSV in mammals.

Materials and Methods.—Notation: It is now possible to obtain variants of Bryan high-titer or Schmidt-Ruppin RSV by the use of various helper viruses to provide different coats. However, these RSV variants differ only in the character of the envelope; their genome remains the same.[13] To distinguish these phenotypic variants of RSV, a notation has been introduced giving the respective helper virus in parentheses after RSV.[11] To distinguish the different strains of RSV where the viral genomes are different, the abbreviation RSV will be preceded by the initials of the strain name.[14] For example, *BH*-RSV(RAV-2) and *SR*-RSV(RAV-1) refer, respectively, to the Bryan high-titer strain of RSV whose envelope-dependent characters are controlled by Rous associated virus-2, or RAV-2, and to the Schmidt-Ruppin RSV whose envelope-dependent characters are controlled by Rous associated virus-1, or RAV-1.

Viruses: The preparations of RAV-1, RAV-2, *BH*-RSV(RAV-1), and *BH*-RSV(RAV-2) have already been described.[13, 15] The helper viruses were assayed by the capacity to induce interference to their cognate *BH*-RSV.[15]

The original stock of *SR*-RSV obtained from Dr. H. Rubin, who had received it from Dr. Ahlström, was purified by the single focus isolation.[16] Two properties, the nondefectiveness[14, 16, 17] and the morphology of foci of transformed cells, were used to distinguish the Schmidt-Ruppin strain from Bryan strain of RSV. These properties of RSV were proved to be under the control of RSV genome and were not affected by altering viral envelopes with those of various helper viruses.[14, 16]

Both *BH*-RSV and *SR*-RSV were assayed by focus formation in cultures of chick embryo cells.[18]

Chick embryo cells: These were prepared from individual chick embryos derived from the eggs of Kimber Farms White Leghorn chickens, strain 813, according to the method of Rubin.[18] Each embryo was classified as to two phenotypes, K and K/2, depending on whether they were, respectively, susceptible or resistant to *BH*-RSV(RAV-2).[13] The non-virus-producing, transformed cells (NP cells) were prepared by isolating single foci of cells transformed by either *BH*-RSV (RAV-1) or *BH*-RSV(RAV-2) as described previously.[11]

Infection of newborn hamsters by viruses: One-tenth ml of virus suspension in tissue culture medium was injected subcutaneously into the right hip of newborn Syrian hamsters within 1 day after birth. They were palpated daily, beginning 7 days after injection, and presence or absence of tumor was finally confirmed by autopsy at 2 months after infection.

Results.—Enclosure of SR-RSV in the envelope of RAV-1: Although *SR*-RSV can reproduce in the absence of helper virus, the yield of *SR*-RSV from infected cultures was significantly lower than that of *BH*-RSV produced from NP cells superinfected with RAV-1 or RAV-2.[16] The average titer of RSV from 1×10^6 transformed cells was about 1×10^4 FFU (focus forming units)/ml for *SR*-RSV and 4×10^7 FFU/ml for *BH*-RSV(RAV-1). However, if RAV-1 was added to the cells transformed by *SR*-RSV, the yield of RSV, which formed foci characteristic of *SR*-RSV, increased to 50- to 100-fold. Some properties of the RSV obtained from cultures to which RAV-1 was added (stock #1) are presented in Table 1. It can be seen that this RSV was strongly interfered with by RAV-1 and neutralized significantly by antiserum to RAV-1, while the original *SR*-RSV was insensitive to both treatments. This indicated that the majority of RSV in this stock was *SR*-RSV(RAV-1). However, the results also showed that the stock still contained the parental *SR*-RSV in about 1 per cent. This was deduced from the relatively poor sensitivity of this stock to these two treatments compared with *BH*-RSV-(RAV-1). Further purification of *SR*-RSV(RAV-1) was achieved by treating the above stock #1 with antiserum to *SR*-RSV which neutralized *SR*-RSV exclusively. This treatment reduced the amount of *SR*-RSV in that stock to the level of less than 0.002 per cent of *SR*-RSV(RAV-1), judging from the sensitivity of the treated virus to interference by RAV-1 (Table 1). This virus preparation was used for further studies as *SR*-RSV(RAV-1).

The following experiments were performed to prove the presence of intact *SR*-RSV genome in the *SR*-RSV(RAV-1). Foci were produced on K-type chick embryo cells with high dilutions of the *SR*-RSV(RAV-1) in the presence of antiserum to RAV-1, and ten of these foci were isolated. All ten foci produced RSV after several transfers. Examination of phenotypic properties of RSV produced from each focus showed that RSV obtained from six foci were pure *SR*-RSV and those from four foci were a mixture of *SR*-RSV and *SR*-RSV(RAV-1) (Table 1). This result can be well explained if one assumes that the cells infected with *SR*-RSV(RAV-1) alone produce only *SR*-RSV and that some foci coinfected with RAV-1 which exists in the stock of *SR*-RSV(RAV-1) produce both *SR*-RSV and *SR*-RSV(RAV-1).

From these results it was concluded that *SR*-RSV(RAV-1) consists of the genome of *SR*-RSV and the viral envelope which expresses the phenotypic characters of RAV-1.

Isolation of RAV-50 and preparation of BH-RSV(RAV-50): In the course of purification of *SR*-RSV, a helper virus stock was obtained from K-type chick

TABLE 1

SOME PROPERTIES OF *SR*-RSV AND *SR*-RSV(RAV-1)

RSV stocks	Type of foci	Ratio of the infectivity on K/2 cells to that on K cells	Relative sensitivity of RAV-1 infected K cells[a]	Surviving Fraction after Treatment with Antiserum to: RAV-1	*SR*-RSV
BH-RSV(RAV-1)	BH[f]	1	0.00015	<0.002	1
SR-RSV	SR[f]	0.1–1[g]	5–20[h]	1	<0.001
Stock #1[b]	SR	1	0.05	0.015	1
SR-RSV(RAV-1)[c]	SR	1	0.0001	<0.001	1
Stock #3[d]	SR	0.5–1	5–20	1	<0.002
Stock #4[e]	SR	1	0.01–0.05	0.01–0.1	1

[a] Infectivity of RSV on RAV-1 infected K cells was compared with that on uninfected K cells.
[b] RSV obtained from cultures doubly infected with *SR*-RSV and RAV-1.
[c] Prepared by treating stock #1 with antiserum to *SR*-RSV.
[d] Recovered from four foci induced by *SR*-RSV(RAV-1).
[e] Recovered from six foci induced by *SR*-RSV(RAV-1).
[f] Foci characteristic of *BH*-RSV and *SR*-RSV, respectively.
[g] Cf. ref. 19.
[h] Cf. ref. 31.

embryo cells infected with dilutions of a crude preparation of *SR*-RSV which had once been passed in hamsters. The presence of the helper virus was recognized from the fact that the dilution beyond the end point of *SR*-RSV conferred on the cells either a resistance to the crude stock of *SR*-RSV or the cytopathic changes resembling those seen in RAV-2-infected K cells. This helper virus stock was used to activate Bryan RSV from K-type NP cells. It was found that the activated RSV stock possessed the capacity to produce sarcomas in newborn hamsters. Since both *BH*-RSV(RAV-1) and *BH*-RSV(RAV-2) were known to be inactive in hamsters from other experiments, it was suspected that the above RSV stock might contain an unknown helper virus capable of endowing RSV with this capacity. In order to purify the putative new agent, the RSV stock was passaged twice in K/2 cells, since RAV-2 and *BH*-RSV(RAV-2) were found to exist in the stock as contaminants. The K/2-passaged RSV produced generally the same number of foci on K and K/2 cells[19] and was not subject to interference by RAV-1, indicating that the RSV obtained was different from all the variants of Bryan RSV hitherto isolated.[13, 20–22] A pure helper virus, designated as Rous associated virus-50 or RAV-50, was isolated from this RSV stock. Finally, a stock of *BH*-RSV(RAV-50) was made by superinfecting NP cells with the purified RAV-50 and used for the further studies. Some properties of *BH*-RSV(RAV-50) are compared with those of other variants of Bryan RSV in Table 2.

Tumor-inducing capacity of various stocks of RSV in hamsters: The capacity of

TABLE 2

SOME PROPERTIES OF *BH*-RSV(RAV-50)

Viruses	Ratio of the infectivity on K/2 cells to that on K cells	Relative Sensitivity of Cells Infected with:[a] RAV-1	RAV-50[d]
BH-RSV(RAV-50)	0.1–1[b]	5–20[c]	0.01
BH-RSV(RAV-1)	1	0.00012	0.1–1
BH-RSV(RAV-2)	<0.00004	5–20[c]	0.01
SR-RSV	0.1–1[b]	5–20[c]	Not done

[a] Infectivity of RSV on RAV-1 or RAV-50 infected K cells was compared with that on uninfected K cells.
[b] Cf. ref. 19.
[c] Cf. ref. 31.
[d] According to the cytopathic changes in RAV-50 infected chick embryo cells, the accurate sensitivity could not be determined.

TABLE 3

ONCOGENICITY OF VARIOUS RSV'S IN HAMSTERS

Viruses	Virus dose inoculated[a]	Day tumor first noted	Hamsters with Tumors on Postinoculation Day Indicated 15	25	60
SR-RSV	$0.5–2.3 \times 10^3$	18	0/12[c]	12/12	12/12
SR-RSV[b]	1.2×10^3	16	0/5	5/5	5/5
SR-RSV	0.5×10^2	22	0/14	7/14	7/14
SR-RSV(RAV-1)	$0.4–1.2 \times 10^5$	—	0/20	0/20	0/20
SR-RSV(RAV-1)	1.2×10^4	—	0/12	0/12	0/12
BH-RSV(RAV-1)	$2.0–8.3 \times 10^5$	—	0/31	0/31	0/31
BH-RSV(RAV-2)	3.2×10^4	—	0/16	0/16	0/16
BH-RSV(RAV-50)	$0.6–5.1 \times 10^3$	9	21/24	24/24	24/24
RAV-50	1.0×10^4	—	0/22	0/22	0/22
Supernatant of NP cultures	—	—	0/11	0/11	0/11
SR-RSV + RAV-1	1.2×10^3 / 2.0×10^6	16	0/10	10/10	10/10

[a] Infectivity of viruses (FFU or infectious units/0.1 ml) on K-type chick embryo cells.
[b] *SR*-RSV recovered from single foci induced by *SR*-RSV(RAV-1).
[c] Number with tumors/number on test.

various stocks of RSV to produce tumors in mammals was tested by inoculating them into newborn Syrian hamsters. Syrian hamsters were chosen as test animals because of their high susceptibility to *SR*-RSV.[4] Table 3 summarizes such experiments. As Ahlström has observed,[4] *SR*-RSV was quite active in hamsters. All of 17 hamsters infected with about 10^3 FFU and 7 of 14 hamsters infected with about 10^2 FFU of *SR*-RSV developed a subcutaneous sarcoma in the site of injection within 25 days. The tumors generally grew rapidly so that their presence could be recognized grossly as early as 18 days. In contrast, despite the inoculation of 10- to 100-fold higher doses, *SR*-RSV(RAV-1) induced no tumor in any one of 32 hamsters injected. The inability of *SR*-RSV(RAV-1) to produce tumors is not due to an inhibitory effect upon tumor development of RAV-1 present in the stock of *SR*-RSV(RAV-1), because simple mixing of high concentrations of RAV-1 with *SR*-RSV did not diminish the activity of the latter. Nor is it due to genetic change in *SR*-RSV genome. *SR*-RSV recovered from single foci formed by solitary infection with *SR*-RSV(RAV-1) was found to have regained the same activity in hamsters as the original *SR*-RSV.

Tumor induction was not observed in any hamster inoculated with high concentrations of either *BH*-RSV(RAV-1) or *BH*-RSV(RAV-2). However, prompt tumor production was accomplished by the use of *BH*-RSV(RAV-50). More than 80 per cent of hamsters receiving this agent developed palpable tumors, some over 1 cm in diameter, by 15 days and all 24 animals developed tumors by 20 days. Both the stock of RAV-50 and the supernatant fluid of NP cultures from which *BH*-RSV(RAV-50) was obtained were inactive in sarcoma formation either in chicks or in hamsters. Histological examination of two hamster tumors induced by *BH*-RSV(RAV-50) showed that they are typical fibrosarcomas. Trypsinized tumor cells were transplantable to all of 13 newborn hamsters. The supernatant of tumor homogenate produced no tumors in any of 7 newborn hamsters, and was not infectious to chick embryo cells, indicating that no infectious RSV was produced from the tumor cells.

In preliminary tests of the oncogenicity of *BH*-RSV(RAV-50) in other species of animals, it was found that 8 out of 9 newborn rats developed multiple hemorrhagic cysts and solid tumors by 15 days after injection.

Recovery of RSV from hamster tumors: In all of the above-mentioned properties, the *BH*-RSV(RAV-50)-induced hamster tumors were indistinguishable from those induced by *SR*-RSV. However, they were different in the type of RSV genome they carried, and the two strains of RSV could be recovered from their respective hamster tumors by the following procedures. About 2×10^5 cells taken from each of four individual hamster tumors induced by *BH*-RSV(RAV-50) or *SR*-RSV, respectively, were co-cultivated *in vitro* with 1×10^6 chick embryo cells in the presence or absence of RAV-1, and the cultures were serially transferred at 4-day intervals. In the mixed cultures derived from *SR*-RSV-induced tumors, transformed chick cells appeared in all four cultures after one to two transfers, regardless of the presence or absence of RAV-1. These transformed chick cells always produced *SR*-RSV in the absence of RAV-1. However, in the mixed cultures derived from *BH*-RSV(RAV-50)-induced tumors, *BH*-RSV(RAV-1) was produced in all four cultures after four to five transfers *only* in the presence of RAV-1. Without RAV-1, neither transformed chick cells nor infectious RSV appeared in the mixed cultures. *In vitro* cultivation of both types of hamster tumor cells without chick embryo cells yielded no infectious RSV whether in the presence or absence of RAV-1. These results could be explained in the following way. Both *SR*-RSV and *BH*-RSV(RAV-50) are incapable of producing infectious progeny in hamster cells, but their genomes replicate at least in some proportion of hamster tumor cells. When these hamster tumor cells come in contact with chick embryo cells, the RSV genome is transferred by some mechanism from the former to the latter, as postulated by Svoboda[23] for the similar finding on the RSV production following direct contact between chicken cells and rat tumor cells induced by Prague strain of RSV. As *SR*-RSV is nondefective, it can generate infectious RSV in the chick embryo cells without helpers, whereas the genome of defective Bryan RSV matures into infectious virus only in the presence of helper virus. In fact, the viruses recovered from the hamster tumors in this way produced foci characteristic of the respective strains used for tumor induction. These findings indicate that two types of hamster sarcoma were indeed produced by infection with *BH*-RSV(RAV-50) and *SR*-RSV, respectively.

Discussion.—The methods used here for changing the viral envelope largely depend on the inability of RSV to produce the viral envelope. The complete dependence of the Bryan RSV on helper virus for its envelope has repeatedly been demonstrated. Variants of Bryan RSV could be obtained simply by superinfecting NP cells with various helper viruses. Despite its nondefectiveness, the Schmidt-Ruppin RSV seems to produce a limited amount of envelope in the infected cells. Thus, the addition of RAV-1 resulted in a 50- to 100-fold increase in the yield of Schmidt-Ruppin RSV progeny, a majority of which were coated by the envelope of RAV-1 alone. Such a mixed virus could be separated from parental *SR*-RSV based on the difference in phenotypic characters. The results presented show that such alteration of viral envelope of RSV does not cause any stable hereditary change in its viral genome.

Contrary to the ease with which *SR*-RSV(RAV-1) was obtained in a relatively pure form, our original intention to enclose the Bryan RSV genome in the envelope of *SR*-RSV encountered a serious difficulty. It was not difficult to create such a virus by superinfecting Bryan NP cells with *SR*-RSV, but the product always con-

tained almost equal amounts of *SR*-RSV itself. There were no available means to separate these two viruses, because they had the same envelope. Attempts at clonal isolation have also failed; foci initiated by the single infection with *BH*-RSV coated by the envelope of *SR*-RSV were found to be NP cells. However, a new form of Bryan RSV with tumor-inducing properties associated with a different coat was found in *BH*-RSV(RAV-50) when we tested the oncogenicity of various stocks of Bryan RSV. In its phenotypic properties the *BH*-RSV(RAV-50) differs from any one of the variants of Bryan RSV so far isolated. It rather resembles the *SR*-RSV in the poor virus yield and in the faster growth in K cells than in K/2 cells despite the equal plating efficiency on two types of chick embryo cells.[16] Dougherty[17] isolated an agent designated ARC (Another Rous Contaminant) from crude stock of *SR*-RSV. Although preliminary experiments suggest that RAV-50 differs also from the ARC, which was obtained from Dr. Dougherty, in its plating efficiency on two types of chick embryo cells, further comparison with this agent as well as virological characterization of RAV-50 is now under way.

The loss of the capacity of *SR*-RSV to induce hamster tumors by enclosing its genome in the RAV-1 envelope, together with the acquisition of this capacity by Bryan RSV following its coating with RAV-50 envelope, strongly supports the idea that the viral coat determines this capacity of RSV. The determining influence of viral envelope on this capacity of RSV was not entirely unexpected since the intraspecies host range of Bryan RSV has been found to depend on its viral envelope,[13, 20, 21] where the susceptibility of certain chick embryo cells to a given variant of Bryan RSV is strictly determined by the susceptibility to the helper virus used to activate RSV. Some evidence indicates that a particular RSV is unable to infect resistant chick cells because of its failure to carry out some early step of virus infection, such as adsorption, penetration, or uncoating.[13] Probably some similar barrier controls the ability or inability of RSV to infect hamster cells. Viruses like *SR*-RSV may have some specific macromolecules in their viral envelope which enable them to attach to or to penetrate the hamster cell membrane. Once the viral genome is incorporated into the hamster cells, RSV genome of either strain would generate malignant changes in these cells. Such specific interaction between virus envelope protein and cell membrane was first demonstrated by Holland with poliovirus.[24] In a more recent study, Cords and Holland[25] clearly showed that Type 1 poliovirus acquires the ability to infect ordinarily insusceptible mouse tissue by enclosing its RNA within the protein capsid of Coxsackie virus.

In contrast to failure in a number of attempts to induce mammalian tumors by the Bryan strain of RSV, which probably consists principally of *BH*-RSV(RAV-1), a few successes have been reported on the transformation of cultivated rat cells,[26, 27] on the production of characteristic lesions in cultures of human and monkey cells,[28] and on the production of tumors in hamster brains inoculated with Bryan RSV.[29] Stewart and Landon[30] have reported the acquisition of mammalian tumor-inducing capacity by Bryan RSV following *in vitro* incubation with homogenates of various actively growing tissues, including hamster sarcoma induced by *SR*-RSV. Some of these results may be due to selection of oncogenic Bryan RSV variants, with a viral envelope permitting mammalian cell infection. However, there may be other factors besides viral envelope which facilitate entry of Bryan RSV genome, though they have not as yet been determined.

Summary.—Enclosure of the genome of Schmidt-Ruppin strain of RSV within the viral envelope of RAV-1 rendered it inactive in tumor induction in newborn hamsters. Among the variants of Bryan strain of RSV coated by RAV-1, RAV-2, and a newly isolated helper virus (RAV-50), only the last variant produced sarcomas in hamsters. The results are consistent with the hypothesis that the ability of RSV to induce mammalian tumors is chiefly determined by the viral envelope.

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