Occurrence of Phenotypically Mixed Viruses in the Bryan High-Titer Strain of Rous Sarcoma Virus and RSV(RAV-1) Stocks[1] (34398)

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The occurrence of subgroups A and B of Rous sarcoma virus (RSV) and Rous-associated virus (RAV), and a range of host cells that are genetically susceptible or resistant to them, makes it possible for considerable phenotypic mixing in the avian tumor virus group. As previously reported by Vogt (1) phenotypic mixing of the avian tumor viruses was experimentally accomplished by infecting genetically susceptible chicken embryo fibroblasts with 2 dissimilar cloned viruses. The purpose and significance of this report, in addition to confirming the work of Vogt, is to demonstrate the occurrence of phenotypically mixed viruses in RSV stocks commonly used in laboratories today. By employing genetically different host cells and specific antisera it was possible to identify occult phenotypically mixed RAV in preparations of the Bryan high-titer strain of Rous sarcoma virus (BH-RSV) and its pseudotype designated as RSV(RAV-1).

Materials and Methods. Chicken fibroblast cultures. Chicken embryo fibroblasts (CEF) were obtained from 2 flocks[3] line 15-1 and 7 white leghorns (2). Avian tumor viruses have been classified into 2 major subgroups, A and B, on the basis of virus protein coat antigens (3-5). The CEF from line 15-1 are genetically susceptible to both subgroups A and B viruses, and designated as C/O cells. The CEF from line 7 are resistant to subgroup A or both A and B, and designated as C/A or C/AB cells, respectively (3–5). Primary cultures were prepared from 10-day-old chicken embryos according to the method described by Rubin (6). Secondary cultures used for the experiments were derived from 3- to 5-day-old primary cultures. Cultures were grown in an equal volume mixture of medium 199 and nutrient mixture F10[4] supplemented with 4–8% of heat-inactivated calf serum, and penicillin and streptomycin (100 units and 100 μg, respectively, per ml). For cell maintenance calf serum in the medium was reduced to 2%. All cultures were incubated at 37° in a humidified atmosphere of 3% CO_2. A portion of primary cultures from each cell preparation was tested by inoculation with RSV (RAV-1) and RSV(RAV-2) to determine genetic resistance.

Viruses. The seed virus (lot CT916) of Bryan high-titer strain of Rous sarcoma virus (BH-RSV) was supplied by Dr. W. R. Bryan, National Cancer Institute. The BH-RSV stock was prepared by the method of Moloney (7) from pectoral and wing-web tumors induced by the inoculation of young C/O chickens. Focus assays for RSV were done as described by Rubin (6). The titer of the BH-RSV stock was 3×10^7 focus-forming units (FFU)/ml in C/O and 2×10^2 FFU/ml in C/A cell cultures. Two pseudotypes of BH-RSV (8), RSV(RAV-1) and RSV (RAV-2), obtained from Dr. W. Okazaki, USDA Poultry Research Laboratory, East Lansing, Michigan, originated in the

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TABLE I. Presence of RAV in the Supernatant Culture Fluids of C/A Cells Inoculated with BH-RSV or RSV(RAV-1).

Virus used to preinfect C/O cells[a]	Relative plating efficiency of challenge virus[b]		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
C/A-BH-RSV	0.0057	0.0083	0.1635
C/A-RSV(RAV-1)	0.0069	0.0000	0.3478

[a] The C/A cells were inoculated with either BH-RSV or RSV(RAV-1). The supernatant culture fluids were harvested 5 days after infection and designated as C/A-BH-RSV or C/A-RSV(RAV-1), respectively.

[b] The C/O cells inoculated with C/A-BH-RSV or C/A-RSV(RAV-1) were challenged (RIF test) with the viruses indicated. Plating efficiency of RSV on C/O cells inoculated with control C/A cell-culture fluids was scored as unity.

laboratory of Dr. P. K. Vogt, University of Washington. Stock RSV(RAV-1) prepared from tumors in young C/O chickens had a titer of 10^7 FFU/ml in C/O cells. RSV(RAV-2) stocks were prepared from tumors in line 7 chickens. The titer of RSV(RAV-2) virus preparations in C/O cells was 2×10^5 FFU/ml.

Antisera. Anti-RSV(RAV-1) serum was produced by inoculating RSV(RAV-1) into crossbred chickens of line 7 and 15. Antiserum at a dilution of 10^{-4} neutralized more than 90% of RSV(RAV-1) activity when tested in C/O cells, and did not neutralize RSV(RAV-2) or the other subgroup B avian tumor viruses. Anti-RSV(RAV-2) serum was produced in line 7 chickens. A dilution of 10^{-3} neutralized 90% or more of RSV(RAV-2) and did not neutralize subgroup A viruses. Before use all antisera were inactivated at 56° for 30 min.

RIF Test. RAV-1 and RAV-2 were detected by virus induced cellular resistance (9) to superinfection by BH-RSV, RSV(RAV-1), or RSV(RAV-2). Secondary cultures of C/O cells were inoculated with 0.5 or 1.0 ml of virus preparation. After incubation at 37° for 3 days the culture fluid was discarded, the cell monolayer was rinsed with Hanks' balanced salt solution, fresh maintenance medium was added for 24 hr; after which time the cells were removed by trypsin, and transferred to 60-mm plastic plates.[5] The C/O cells received virus-uninoculated culture fluid served as controls. Cells were then challenged by the addition of graded amounts of BH-RSV, RSV(RAV-1), or RSV(RAV-2). Foci were counted on control and test plates 7 to 10 days following inoculation. The relative plating efficiency of each superinfected virus was scored.

Results. RAV in the supernatant fluids C/A cultures inoculated with BH-RSV or RSV(RAV-1). Plates containing C/A cells were inoculated with BH-RSV or RSV(RAV-1) stock. Five days later virus was harvested from supernatant culture fluids, centrifuged to remove cell debris, and designated as C/A-BH-RSV or C/A-RSV(RAV-1), respectively. Control fluids were obtained from uninoculated C/A cultures. The harvested viruses [C/A-BH-RSV or C/A-RSV(RAV-1)] were inoculated into C/O cells and RIF test was carried out. Table I shows the relative plating efficiencies of BH-RSV, RSV(RAV-1), and RSV(RAV-2) on C/O cells preinfected with C/A-BH-RSV or C/A-RSV(RAV-1). The plating efficiency of BH-RSV or RSV(RAV-1) on the test cells was reduced more than 100-fold. Resistance of C/O cells to superinfection with RSV(RAV-2) was approximately fivefold when cells were preinfected with C/A-BH-RSV, and threefold when preinfected with C/A-RSV(RAV-1). Not less than 5 replicate experiments were carried out by using C/A cells (Pooled line 7 embryos) for either BH-RSV or RSV(RAV-1) stock. Twelve additional experiments were performed by using C/A cells prepared from individual line 7 embryos and RSV

[5] Falcon Plastics Division of Becton, Dickinson Co., Los Angeles, California.

TABLE II. Serial Transmission of RAV in C/O but Not in C/A Cells.

Virus used to preinfect C/O cells[a]	Relative plating efficiency of challenge virus		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
C/O-[C/A-BH-RSV]	0.0000	0.0000	0.3592
C/A-[C/A-BH-RSV]	1.0787	0.9016	1.1000
C/O-[C/A-RSV(RAV-1)]	0.0000	0.0000	0.4980
C/A-[C/A-RSV(RAV-1)]	0.9290	0.9192	0.8398

[a] The C/O or C/A cells were inoculated with C/A-BH-RSV or C/A-RSV(RAV-1). The supernatant culture fluids harvested 5 days after inoculation were designated as C/O-[C/A-BH-RSV], C/A-[C/A-BH-RSV], C/O-[C/A-RSV(RAV-1)] or C/A-[C/A-RSV(RAV-1)], respectively. Tests were carried out as described in Table I.

(RAV-1) stock. These results indicate that C/A-BH-RSV or C/A-RSV(RAV-1) contained a virus that interfered with BH-RSV or RSV(RAV-1). The virus showing RIF activity appeared to be RAV-1 or one antigenically related.

Neutralization experiments using anti-RSV(RAV-1) and anti-RSV(RAV-2) sera confirmed the findings tabulated in Table I. When C/A-BH-RSV or C/A-RSV(RAV-1) was incubated at 37° for 40 min with antiserum to RSV(RAV-1), RIF activity against BH-RSV in C/O cells was eliminated. Since BH-RSV employed for RIF tests was used at a high dilution most of the focus-forming particles appeared to be RSV(RAV-1), and the interfering virus RAV-1 or a related virus.

Failure of RAV-1 to be serially transmitted in C/A cultures. The result of the preceding experiments indicated that RAV-1 or an antigenically related virus was a major component in C/A-BH-RSV or C/A-RSV(RAV-1). Presumably such virus would infect C/O but not C/A cells. To test this possibility C/O or C/A cells were inoculated with C/A-BH-RSV and virus was harvested from supernatant culture fluids. Such harvests were designated as C/O-[C/A-BH-RSV] or C/A-[C/A-BH-RSV], respectively. Similarly C/O or C/A cells were inoculated with C/A-RSV(RAV-1), thus producing C/O-[C/A-RSV (RAV-1)] or C/A-[C/A-RSV(RAV-1)]. The RIF tests were carried out in C/O cells using the 4 virus preparations. From the results tabulated in Table II it is evident that no foci occurred in C/O cells that were preinfected with C/O-[C/A-BH-RSV] or C/O-[C/A-RSV(RAV-1)] and challenged with BH-RSV or RSV (RAV-1). However, C/O cells exposed to C/A-[C/A-BH-RSV] or C/A-[C/A-RSV (RAV-1)] lacked RIF activity against the superinfecting RSV. These results indicate that the interfering virus in C/A-BH-RSV or C/A-RSV(RAV-1) preparations replicated in C/O but not in C/A cells and most likely was RAV-1 or a related virus. It should be noted that infection of C/A cells with BH-RSV or RSV(RAV-1) stock resulted in the replication of RAV-1 or a related virus (Table I) and this virus reinfected C/O cells but not C/A cells (Table II). The C/A cells supported only a single cycle of replication of the subgroup A RAV. The virus showing this effect is likely a subgroup A RAV genome enveloped in subgroup B RAV protein coat, *i.e.*, RAV-1 (RAV-2).

Absence of RAV activity of supernatant fluids from C/AB cultures inoculated with RSV(RAV-1). The results of the preceding experiments indicated that the BH-RSV and RSV(RAV-1) stocks contained a phenotypically mixed RAV virus, *i.e.*, subgroup A RAV genome enveloped in subgroup B RAV protein coat. Consequently, it was assumed that C/AB cells, which are resistant to infection by subgroups A and B avian tumor viruses studied to date, would not be infected with the phenotypically mixed virus. Three C/AB cultures prepared from individual line 7 chicken embryos received an inoculum of RSV(RAV-1). The supernatant fluids from these cultures were designated as C/AB-

TABLE III. Absence of RAV in the Supernatant Culture Fluids of C/AB Cells Inoculated with RSV(RAV-1).

Virus used to preinfect C/O cells[a]	Expt. no.	Relative plating efficiency of challenge virus		
		BH-RSV	RSV(RAV-1)	RSV(RAV-2)
C/AB-RSV(RAV-1)	1	0.77	0.80	0.91
	2	0.56	0.68	0.67
	3	1.02	1.01	1.04

[a] The C/AB cells were inoculated with RSV(RAV-1). The supernatant culture fluids were harvested 5 days after inoculation and designated as C/AB-RSV(RAV-1). Tests were carried out as described in Table I.

RSV(RAV-1). The RIF tests were carried out using the C/AB-RSV(RAV-1) in C/O cells. The results presented in Table III indicate that the C/AB-RSV (RAV-1) preparations were free of RAV.

Effect of antiserum to subgroup B viruses on the activity of RSV (RAV-1) in C/A cultures. The results of preceding experiments revealed that the RSV (RAV-1) stock contained RAV-1 (RAV-2). To eliminate viruses from the stock that had a RAV-2 protein coat equal amounts of RSV(RAV-1) virus (undiluted) and anti-RSV (RAV-2) serum at a dilution of 10^{-1} were mixed and incubated at 37° for 40 min. The C/A cultures received an inoculum of 0.4 ml of the virus–antiserum mixture. Control C/A culture received 0.4 ml of virus–normal serum mixture. The supernatant cell culture fluids were harvested and tested for their RIF activity in C/O cells. The results (Table IV) indicate that antiserum specific for subgroup B viruses neutralized viruses with a RAV-2 protein coat including RAV-1 (RAV-2) which was presumed to be present in RSV (RAV-1) stock.

Discussion. This report underlines the widespread occurrence of phenotypic mixing among the avian tumor viruses. Unawareness of this fact can lead to serious misinterpretation of experimental results. Experimental evidence demonstrated that when C/A cells were inoculated with either BH-RSV or RSV(RAV-1), the supernatant fluids from infected cultures contained a high concentration of a subgroup A virus, most likely RAV-1. The RAV-1 evidently had its origin as a phenotypically mixed virus that preexisted in BH-RSV and RSV (RAV-1) stocks and appeared to be a subgroup A RAV genome enveloped in the subgroup B RAV protein coat. This evaluation is based on the following evidence: (i) presence of RAV-1 in C/A-BH-RSV and C/A-RSV(RAV-1) preparations (Table I); (ii) serial transmission of the virus in C/O but not in C/A cells (Table II); (iii) absence of virus in the supernatant fluids from C/AB cultures inoculated with RSV (RAV-1) (Table III); and (iv) absence of virus activity in the supernatant fluid of C/A cultures which had been inoculated with RSV(RAV-1) stocks after neutralization with anti-RSV (RAV-2) serum (Table IV). Such results indicate that the phenotypically mixed virus was RAV-1 (RAV-2) and could infect C/A cells. The RAV-2 pro-

TABLE IV. Absence of RIF Activity in Culture Fluids of C/A Cells Inoculated with RSV(RAV-1) Neutralized by Anti-B Virus Serum.

Virus used to preinfect C/O cells	Relative plating efficiency of challenge virus[a]		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Culture fluid of C/A cells inoculated with RSV(RAV-1) neutralized by anti-B serum	0.87	0.96	1.03

[a] See Table I for experimental procedure.

tein coat facilitated the entry of RAV-1 genome into C/A cells. As a result RAV-1 progeny were produced. Such progeny could serially replicate in C/O but not in C/A cells. The possibility that C/A cells were not unequivocally resistant to infection by the RAV-1 present in BH-RSV and in RSV (RAV-1) stocks cannot be ruled out, however, such a problem exists whenever genetically resistant cells are used for experiments. Control studies carried out with each experiment proved that C/O, C/A, or C/AB cells had the anticipated spectrum of virus susceptibility.

The origin of RAV-1 (RAV-2) in BH-RSV stock can be accounted for as follows: when the BH-RSV stock was prepared in C/O chickens 2 viruses (RAV-1 and RAV-2) could have infected the same cell to yield progeny that were phenotypically mixed, RAV-1 (RAV-2). The origin of the phenotypically mixed viruses in the RSV (RAV-1) stock is less clear. A reasonable explanation is that the RSV(RAV-1) stock prepared in C/O chickens contained RAV-2 as a contaminant. Phenotypic mixing between RAV-1 (indigenous component of RSV (RAV-1) stock) and RAV-2 (contaminant) occurred to produce the phenotypically mixed virus. Selective force due to the early formation of anti-A virus antibody in the C/O chickens inoculated with RSV(RAV-1) stock may also contribute to the proliferation of subgroup B viruses. If one discounts the possibility of external contamination with a subgroup B virus, there is the alternative possibility of viral mutation during growth. The selective force of anti-A antibody makes it possible to manifest such a mutation.

A more detailed study of the genetic and antigenic properties of the viruses described in this report is beyond the scope of the present paper. However, the results emphasize the need to constantly recognize that complex mixtures of phenotypically mixed viruses can occur in avial tumor virus stocks and thus confuse the interpretation of experimental findings in work with such viruses.

Summary. RSV stocks in common laboratory use, BH-RSV and its pseudotype designated as RSV (RAV-1), were examined to demonstrate occult xenogenetic RAV by using genetically different host cells and antisera specific to subgroups A and B avian tumor viruses. The virus stocks contained a phenotypically mixed virus, *i.e.*, subgroup A RAV genome enveloped in subgroups B RAV protein coat. The virus replicated in C/A cells and yields the progeny of subgroup A RAV. The progeny replicated in C/O cells serially but not in C/A cells. The phenotypically mixed virus could not infect C/AB cells and was neutralized by an antiserum specific to subgroup B viruses. The possible origin and the significance of the virus were discussed.

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