that set forth ideas which were at the time quite unpopular. He said, "Must one then dismiss viruses as possible reasons for the generality of tumors? In doing so one would disregard the only activating cases for the neoplastic state thus far discovered and would consign the cancer problem to the imagination which so long had it in sole charge. And there are stronger reasons against such a course." He set forth these reasons with clarity and vigor, yet, as with his 1911 discovery, the scientific climate was not yet ready for such ideas and there was little immediate effect on the trend of research in this area. However, within about ten years, with the discovery of still more tumor viruses there began an upswing of interest in virus-cancer relationships which has continued with increasing vigor, and at long last Dr. Rous's early ideas and research contributions began to have a profound influence on the trend of tumor virus research. Today this is perhaps the most active and most promising area in all of cancer research. The RNA tumor viruses are providing their share of significant information, as we will learn from the speakers this morning. It is therefore a pleasure to dedicate this symposium to Peyton Rous.

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*A VIRUS RELEASED BY "NONPRODUCING" ROUS SARCOMA CELLS**

BY PETER K. VOGT

DEPARTMENT OF PATHOLOGY, UNIVERSITY OF COLORADO MEDICAL SCHOOL, DENVER

Infection of a chick embryo fibroblast with a single particle of the Bryan high-titer strain of Rous sarcoma virus (RSV) leads to transformation of the cell but apparently does not result in the production of progeny virus.[1] Transformed fibroblasts derived from such solitary RSV infection are therefore called nonproducing (NP) cells. NP cells contain the RSV genome and can be activated to release infectious RSV by superinfection with an avian leukosis helper virus. RSV produced in this fashion carries the envelope properties of the helper virus.[2-5] NP cells also synthesize the group-specific antigen of the avian sarcoma and leukosis complex.[6] This antigen appears to be an internal component of the virus particle.[7-9]

Investigations with physical techniques have revealed the presence of avian tumor viruslike particles in NP cells. Dougherty and Di Stefano studied NP cells with the electron microscope and discovered particles which were morphologically indistinguishable from avain tumor viruses and were associated with the cell surface.[10] A more recent survey of many independently isolated NP cell lines indicates that more than 90 per cent of these lines carry electron microscopically detectable virus particles.[11] These particles may be isolated from the nutrient medium of NP cultures and contain the high-molecular-weight RNA characteristic of the avian tumor virus group.[12] The particles demonstrable with physical techniques also seem to be active biologically. A previous report from this laboratory describes the finding of a subcellular, transforming agent in NP cultures.[13] The present paper deals with some biological properties of the new virus.

Materials and Methods.—Cells and virus: Chick embryo fibroblast cultures were prepared from single, ten-day-old embryos according to the methods described by Rubin.[14] Before use the cells were tested for congenital infection with avian leukosis virus by published techniques,[14] and cultures carrying such an infection were discarded. Four genetic types of chicken cells were used: type C/O cells were sensitive to subgroups A and B of the avian tumor viruses, type C/A cells were selectively resistant to subgroup A viruses, and type C/B cells could not be infected by avian tumor viruses of subgroup B. Type C/AB cells were insusceptible to subgroups A and B.[15] Type C/O and C/B chick embryos were obtained from a local branch of Hyline farms, and type C/A and C/AB embryos were the generous gift of Drs. B. R. Burmester and L. B. Crittenden of the Regional Poultry Research Laboratory, East Lansing, Michigan.

Quail fibroblast cultures were prepared from seven- to nine-day-old embryos in the same way as chick cell cultures. Fertile eggs came from a breeding flock of *Coturnyx coturnyx japonica* kept in this laboratory.

Two pseudotypes of the Bryan high-titer strain of Rous sarcoma virus (RSV) were used. The envelope of one was controlled by Rous-associated virus type 1 (RAV-1), a member of avian tumor virus subgroup A. The other RSV pseudotype had as a helper virus Rous-associated virus type 2 (RAV-2) which belongs to subgroup B of the avian tumor viruses.[15] RAV-50, an avian leukosis virus isolated from the Schmidt-Ruppin strain of Rous sarcoma virus[16] was used as prototype of subgroup C.[18]

Initiation and cultivation of NP lines: NP cultures were derived from individual Rous sarcoma foci according to published techniques.[1, 3] All NP cells were grown together with normal chick fibroblast feeder cells. Most of the NP lines used in this study were of the C/A type with either C/A or C/B feeder cells. Few were C/O NP cells cultivated with normal C/O cells, or C/B NP cells grown with C/B fibroblasts. Supernatant medium from NP cultures was assayed for free virus after passage through microfiber-glass disk filters (Millipore type AP 25). This procedure was shown to remove intact cells from the fluid.

Results.—A subcellular, infectious agent in the medium of NP cultures: In the course of several studies employing NP cells,[3, 6, 11, 13] many samples of fluid media from such cultures have been studied for infectious RSV. The great majority of these tests has given no evidence for the presence of free, infectious virus in NP cultures. However, occasional chick embryos yielded assay cultures which developed a few Rous sarcoma foci after inoculation with medium from NP cells. The appearance of these foci was first believed to be due to the inadvertent inclusion of whole, transformed cells in the supernatant samples. This trivial explanation was ruled out when focus-forming activity persisted in cell-free filtrates obtained by passing NP culture medium through a membrane filter of 0.45 μ average pore size. Since this subcellular, transforming agent was synthesized by NP cells, it appeared likely that it was free of helper virus. The abbreviation RSV(0) was therefore adopted at this point to designate the probable helper-independence of this agent and to differentiate the new RSV from common, helper-dependent pseudotypes such as RSV(RAV-1) or RSV(RAV-2). Chicken fibroblasts transformed by RSV(0) met the criteria of NP cells: infectious virus could not be detected in the supernatant medium by standard techniques but was de-

monstrable by assay on cells of rare, susceptible chick embryos. Superinfection with an avian leukosis helper virus activated the release of infectious RSV from these cells. RSV(0) was found in 25 out of 27 independently derived NP cultures of the C/A type. It was also present in 10 out of 12 C/B NP lines and in the 5 C/O NP lines tested so far.

Host range of RSV(0): Since infectivity tests with RSV(0) on standard assay cultures were only sporadically successful, further progress depended on the finding of a cell culture system which could be relied upon to support focus formation by RSV(0). Table 1 summarizes the search for a source of susceptible cells. RSV(0) produced foci of transformed cells in chick fibroblast cultures prepared from most C/A embryos and in all cultures derived from embryos of the Japanese quail. Only a small proportion of the C/O and none of the C/B and C/AB type chick embryos yielded cultures susceptible to RSV(0). Quail or pretested C/A chicken fibroblasts were therefore used in all further assays of RSV(0). As a rule, the plating efficiency of RSV(0) on quail cells was about two to four times higher than on C/A chicken cells. The release of progeny virus by quail and type C/A chicken cells infected with RSV(0) is now under study.

TABLE 1

HOST RANGE OF RSV(0)

Type of embryo cell culture	Fraction of embryos yielding cultures susceptible to RSV(0)
Chicken C/O	3/25
Chicken C/A	21/27
Chicken C/B	0/17
Chicken C/AB	0/5
Japanese quail	30/30

The findings compiled in Table 1 suggest that a certain genetic constitution is the basic prerequisite for cellular susceptibility to RSV(0). However, physiological factors which are as yet undefined appear to have important effects on RSV(0) infection. In the course of prolonged culture some susceptible quail and some C/A type chicken cells have become resistant and, less frequently, initially resistant quail or C/A type chicken cultures have turned susceptible to RSV(0).

The host range of RSV(0) also affected the persistence of transformation in various normal cell cultures inoculated with small numbers (20–50) of NP cells. NP cells seeded on feeder layers of the C/O, C/B, or C/AB types often disappeared in the course of a few transfer generations (20–30 days), but transformation persisted, and even spread through the whole culture, when the feeder layers were of the C/A type or consisted of quail fibroblasts. Regressions of transformation in NP lines have been observed before.[11, 13] The prevailing culture conditions appear to favor the growth of normal over that of NP cells, and the transformed state disappears from the cultures unless feeder cells can be infected by RSV(0) as is the case with C/A or quail cells.

The host range of RSV(0) is inconsistent with that of any established avian tumor virus subgroup.[17, 18] RSV(0) transforms C/A cells and is therefore not a member of subgroup A. Neither does it belong to subgroup B, because it infects quail fibroblasts which have been shown to be resistant to this subgroup.[19] Unlike either subgroup A, B, or C, RSV(0) often fails to transform C/O type cultures. This unusual host range, excluding RSV(0) from the great majority of chicken

TABLE 2

ABSENCE OF VIRAL INTERFERENCE FROM CULTURES INOCULATED WITH END-POINT DILUTIONS OF RSV(0)

Dilutions of RSV(0)*	Number of RSV Foci per Culture: Not superinfected	Number of RSV Foci per Culture: Superinfected with RSV(0)
1/10	78	425
1/20	70	432
1/40	0	393
1/80	0	356
1/160	0	397
1/320	0	392
1/640	0	384
Uninfected control	0	381

* Type C/A chick fibroblast cultures were inoculated with 0.1 ml of the dilutions of RSV(0) listed in the left column of the table. After three transfers (8 days) the cultures were divided. One set of subcultures was superinfected with about 400 focus-forming units of RSV(0), the other was left uninfected. Foci were counted 7 days after the third transfer.

cell types used for infectivity assays, explains why this agent has not been found earlier, and accounts for its erratic and elusive behavior which has hampered the earlier part of this investigation.

Tests for the presence of a helper virus associated with RSV(0): Since the release of infectious RSV from NP cells is usually controlled by a helper virus,[1] it was important to test for the presence of an avian leukosis virus in stocks of RSV(0). Type C/A chick embryo fibroblast cultures were inoculated with twofold serial dilutions of RSV(0) beyond the end point of focus formation. The cells were then transferred three times, and at the third transfer they were divided into two sets of subcultures. One set was superinfected with RSV(0) from the same stock which had been used for the primary infection. The other set was left without challenge infection. Foci were counted seven days after the third transfer (Table 2). Cultures inoculated with end-point dilutions of RSV(0) did not become resistant to a challenge infection with the same virus. This experiment was carried out with six stocks of RSV(0) derived from different NP lines. No evidence for the presence of an interfering virus was obtained.

These observations suggest that the production of RSV(0) by NP cells is not caused by a new helper virus, but they do not definitely rule out this possibility. Obviously, more data are needed before this important point can be considered as settled. Until then the helper-independence of RSV(0) should be regarded as hypothetical.

Levels of RSV(0) associated with different NP clones: The amounts of free RSV(0) present in the medium of NP cultures ranged from a barely detectable 5 to about 5×10^3 focus-forming units (FFU) per ml. However, the titers of RSV(0) associated with a given NP line remained remarkably constant throughout the culture history of the line. From the NP cultures listed in Table 3, supernatant samples were collected 12 times during a 3-months period and were assayed for RSV(0). The titers in Table 3 (column 2) were characteristic of the respective NP cultures. For instance, the medium of culture no. 70-22 contained upon repeated assays from 20 to 50 FFU per milliliter whereas culture no. 70-14 regularly produced from 1600 to 5000 FFU per milliliter. Microscopic inspection showed that all cultures listed in the table consisted only of transformed cells. The titer differences found could therefore not be due to the presence of varying numbers of

TABLE 3

CORRELATION BETWEEN RELEASE OF RSV(0) AND EFFICACY OF HELPER VIRUS ACTION

Culture no.	No Helper Virus Added		RAV-2 Added Three Days Before Harvest	
	RSV(0)*	RSV(RAV-2)†	RSV(0)*	RSV(RAV-2)†
70-13	1310	0	1190	390
70-14	1650	0	1270	810
70-22	48	0	21	6
70-24	1038	0	980	600
70-31	390	0	210	76

* FFU/ml of culture medium. Assayed on quail fibroblasts, which were insusceptible to RSV(RAV-2).
† FFU/ml of culture medium. Assayed on type C/O chick fibroblasts which were insusceptible to RSV(0).

NP cells. After superinfection with helper virus, the NP cultures produced helper-dependent RSV in amounts proportional to their release of RSV(0) (Table 3). NP cultures which made much RSV(0) before activation readily produced helper-dependent RSV after superinfection with RAV. NP cultures which released only little RSV(0) were also poorly activable.

These findings suggest that the level of free RSV(0) present in an NP culture may be used as a rough indicator of the intracellular amounts of Rous sarcoma viral genomes which are available for coating by helper virus.

Persistence of RSV(0) after activation of NP cells: Table 3 shows that RSV(0) continues to be made after superinfection of NP cells with helper virus. The same result was obtained in the experiment represented by Figure 1. There the titers of RSV(0) and of RSV(RAV-2) were determined separately in daily intervals after superinfection of a C/A NP culture with RAV-2. RSV(RAV-2) was plated on C/O-type chick fibroblasts which were insusceptible to RSV(0), whereas quail fibroblasts, used for the assay of RSV (0), could not be infected by RSV(RAV-2). Although RSV(0) remained present in the viral harvests during the short course of this experiment, it represented only a small fraction of the total free RSV during the last two days of observation.

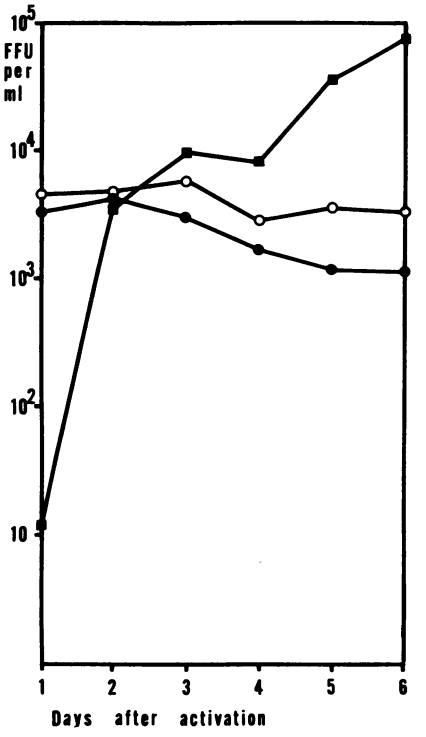

FIG. 1.—Titers of free RSV(0) and of RSV(RAV-2) in an NP culture activated with RAV-2 and in an nonactivated NP control. O——O RSV(0) in the medium of the nonactivated NP control. ●——● RSV(0) in the NP culture superinfected with RAV-2. ■——■ RSV(RAV-2) in the activated culture. No RSV(RAV-2) was demonstrable in the control NP culture.

In order to test whether RSV(0) was also present in previously prepared stocks of RSV pseudotypes obtained by activating NP cells with an avian leukosis virus, four preparations of RSV(RAV-2) which had been stored for three years were plated on types C/O and C/B chicken cells and on

TABLE 4

PRESENCE OF RSV(0) IN STOCKS OF RSV PSEUDOTYPES

RSV(RAV-2) (stock no.)	Titer (FFU/ml)		
	Chicken C/O	Chicken C/B	Japanese quail
1	8.8×10^4	<1	14
2	10.6×10^4	<1	25
3	4.9×10^4	<1	8
4	1.1×10^4	<1	<1

TABLE 5

INTERFERENCE BETWEEN AVIAN LEUKOSIS VIRUSES AND RSV(0)

Interfering virus	Plating efficiency of RSV(0)*
RAV-1†	1.2–1.8
RAV-2	<0.001
RAV-50	0.4–0.9
None	1.0

* Different C/A type chicken fibroblast cultures were infected with RAV-1, RAV-2, and RAV-50. After 2 transfers (6 days), the preinfected cells as well as an uninfected control culture were superinfected with RSV(0). Rous sarcoma foci were counted 7 days after challenge infection and the plating efficiency of RSV(0) on preinfected relative to normal C/A fibroblasts was determined.

† Carried out on quail fibroblasts.

quail fibroblasts. Again, C/O and quail cells were selectively susceptible to RSV (RAV-2) and to RSV(0), respectively. C/B cells could not be infected by either one of these viruses but would reveal any subgroup A contaminants of the stocks. Table 4 shows that none of the virus preparations produced foci on C/B cells, indicating absence of subgroup A particles. Titers on C/O cells were in the order of 10^4 to 10^5 FFU/ml. Three of the four preparations also produced small numbers of foci on quail cells. These foci were probably due to RSV(0). They indicate that some particles with the host range of RSV(0) may be present in stocks of RSV pseudotypes but the vast majority of virus particles appears to have envelopes controlled by the helper. The contribution of RSV(0) to progeny envelopes in this extreme case of phenotypic mixing seems therefore minute.

Viral interference with RSV(0): Cells infected with an avian leukosis virus become resistant to challenge infection with viruses of the same avian tumor virus subgroup, but remain sensitive to other avian tumor viruses. Interference by known avian leukosis viruses can therefore be used to classify an unknown avian sarcoma virus.[20] As judged by its host range, RSV(0) does not belong to either subgroup A, B, or C. Accordingly, it was expected that avian leukosis viruses of these subgroups would fail to interfere with RSV(0). However, the results of interference experiments compiled in Table 5 indicate that preparations of RAV-2, a member of subgroup B, induced strong cellular resistance to RSV(0). RAV-50 of subgroup C did not interfere with RSV(0), and RAV-1 (subgroup A) occasionally induced significant enhancement of the RSV(0) challenge. The interference between preparations of RAV-2 and RSV(0) is at present unexplained. It is restricted to type C/A chicken cells, which are susceptible to subgroup B, and does not occur in quail cells from which subgroup B viruses are excluded. Apparently, RSV(0) uses the same cellular receptors as do subgroup B viruses on C/A cells, but is also able to use quail cell receptors which seem not to interact with subgroup B viruses.

Discussion.—RSV(0) is only partially characterized by the data presented in this paper. The focus assay for RSV(0) should help in solving many of the re-

maining problems. These include a complete characterization of the envelope properties of RSV(0): host range, antigenicity, interference patterns, and particle stability. If indeed RSV(0) is not associated with an avian leukosis helper virus, it should have an envelope antigen of its own. NP cells have been shown to be free of viral envelope antigen, but the antigen which has been searched for is that of the helper virus.[2, 3] In order to detect helper-independent envelope antigen antibodies prepared specifically against RSV(0) must be employed. This work is now in progress. The interference between RAV-2 and RSV(0) does not appear to be reciprocal, because NP cells releasing RSV(0) can be superinfected with RAV-2 to yield RSV(RAV-2). This suggests that if RSV(0) produces an envelope antigen capable of competing for cellular receptors with RAV-2, the amounts of this agent are not sufficient to effectively exclude superinfecting RAV-2 from NP cells.

The most important question which remains to be answered concerns the possibility that an avian leukosis virus is associated with RSV(0) and is in control of envelope properties. The absence of an interfering agent from end-point dilutions of RSV(0) indicates that if a helper virus is present either its concentration is not greater than that of RSV(0) itself, or its growth rate is so slow that it cannot establish interference. It will now be necessary to isolate single foci of cells transformed by RSV(0) and to test such Rous sarcoma lines for the production of RSV(0) as well as for activability with avian leukosis helper virus. This study should uncover any helper virus in stocks of RSV(0) and should yield additional information on the correlation between the level of free RSV(0) in NP lines and their activability with helper virus.

The discovery of RSV(0) sheds new light on the problem of viral defectiveness in the avian tumor virus group. If future work confirms the suggested absence of helper virus from RSV(0), then the Bryan high-titer strain of Rous sarcoma virus is to be regarded as being defective only in a quantitative sense. There seems to be no absolute lack of the ability to direct the synthesis of functional viral progeny. Most NP lines release RSV(0) which can infect certain types of avian cells, but which is not detectable in most assay systems. However, even the highest titers of RSV(0) found in NP cultures are still 1000-fold lower than the titers of helper-dependent RSV produced by the same cells after superinfection with an avian leukosis virus. RSV(0) is thus quantitatively lacking in the ability to produce functional viral progeny. This may explain the common association with helper viruses which are responsible for increased virus titers and provide most of the envelope material for RSV. The basic observations supporting the concept of defective avian sarcoma viruses, namely activation of NP cells and envelope control by helper viruses, remain valid. But the emphasis has shifted from an absolute to a relative defect.

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[1] Hanafusa, H., T. Hanafusa, and H. Rubin, these PROCEEDINGS, **49**, 572–580 (1963).

[2] *Ibid.*, **51**, 41–48 (1964).

[3] Vogt, P. K., *Natl. Cancer Inst. Monograph*, **17**, 527–541 (1964).

[4] Hanafusa, H., *Virology*, **25**, 248–255 (1965).

[5] Vogt, P. K., *Virology*, **25**, 237–247 (1965).

[6] Vogt, P. K., P. S. Sarma, and R. J. Huebner, *Virology*, **27**, 233–235 (1965).

[7] Payne, F. E., J. J. Solomon, and H. G. Purchase, these PROCEEDINGS, **55**, 341–348 (1966).
[8] Bauer, H., and W. Schäfer, *Virology*, **29**, 494–497 (1966).
[9] Kelloff, G., and P. K. Vogt, *Virology*, **29**, 377–384 (1966).
[10] Dougherty, R. M., and H. S. Di Stefano, *Virology*, **27**, 351–359 (1965).
[11] Courington, D., and P. K. Vogt, *J. Virol.*, **1**, 400–414 (1967).
[12] Robinson, H. L., personal communication.
[13] Vogt, P. K., *J. Virol.*, in press.
[14] Rubin, H., these PROCEEDINGS, **46**, 1105–1119 (1960).
[15] Vogt, P. K., and R. Ishizaki, *Virology*, **26**, 664–672 (1965).
[16] Hanafusa, H., and T. Hanafusa, these PROCEEDINGS, **55**, 532–538 (1966).
[17] Vogt, P. K., and R. Ishizaki, *Viruses Inducing Cancer*, ed. W. J. Burdette (Salt Lake City, Utah: University of Utah Press, 1966), pp. 71–90.
[18] Vogt, P. K., R. Ishizaki, and R. Duff, in *Subviral Carcinogenesis*, ed. Y. Ito (International Symposium on Tumor Viruses, Nagoya, Japan, 1966), in press.
[19] Vogt, P. K., in *Virus-Directed Host Response*, ed. M. Pollard (New York: Academic Press, 1967), pp. 199–228.
[20] Vogt, P. K., and R. Ishizaki, *Virology*, **30**, 368–374 (1966).

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*VIRUS PARTICLES AND VIRAL ANTIGENS IN CHICKEN TISSUES FREE OF INFECTIOUS AVIAN LEUKOSIS VIRUS**

BY ROBERT M. DOUGHERTY, HENRY S. DI STEFANO, AND FRIEDA K. ROTH

DEPARTMENTS OF MICROBIOLOGY AND ANATOMY, STATE UNIVERSITY OF NEW YORK, UPSTATE MEDICAL CENTER, SYRACUSE

Two situations in which avian leukosis viral antigens and viral particles were found in chicken tissues that do not contain detectable infectious virus have been reported from this laboratory.[1–3] The first report described noninfectious virus particles associated with "nonproducer" Rous sarcoma cells.[1, 2] This finding was recently confirmed.[4] Work reported at this symposium showed that these particles had the same buoyant density as infectious avian leukosis/sarcoma viruses and that they contained an RNA component with the same molecular weight and base ratios as their infectious counterpart.[5] Further, Vogt[6] reported that the particles, which he termed "helper-independent virus," were infectious for certain specific avian tissues. It appears from this that nonproducer Rous sarcoma cells release Rous virus particles whose defectiveness is reflected in a restricted host range.

Recently, we observed viral particles in chick embryos that were free of infectious virus.[3] This report deals with an extension of this work to determine the nature and mode of transmission of these apparently noninfectious particles.

Materials and Methods.—Fertile hens' eggs were obtained from six different flocks, four of which are maintained in isolation specifically to exclude infection with avian leukosis virus (ALV). These include experimental flocks maintained by Dr. Roy Luginbuhl at the University of Connecticut; Dr. F. B. Bang at The Johns Hopkins University; Dr. B. R. Burmester at the Regional Poultry Research Laboratory in East Lansing, Michigan, and a commercial flock maintained by