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[33] Our recent experiments, with improved experimental methods, do not support our earlier conclusion[3] that TPN is also required for cyclic photophosphorylation.

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*THE DEFECTIVENESS OF ROUS SARCOMA VIRUS**

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The Bryan high-titer strain of Rous sarcoma virus (RSV) produces foci of transformed cells after infecting monolayers of chick embryo cells in tissue culture. The transformed cells take on the typical rounded appearance of cultivated Rous sarcoma cells and are no longer restricted to growth in a monolayer. When a stock of RSV is diluted beyond the end point for focus formation, another virus can readily be isolated which has been called Rous-associated virus (RAV).[1] RAV does not cause a noticeable morphological change in cells, but it induces resistance to RSV within a few days, and this resistance is the basis for the assay of RAV. RAV is indistinguishable from RSV in heat sensitivity, cellular site of maturation, growth rate, and immunological specificity.[1] It is known to differ from RSV only in its failure to produce either foci in tissue culture or sarcomas in the chicken. It does, however, produce leukosis in chickens, and can be considered a virus of the avian leukosis complex. In view of its close relationship to RSV and its presence in RSV stocks in higher titer than RSV itself, it seemed unlikely that RAV was a mere accidental contaminant of the RSV stock. A study was undertaken to clarify the relationship of the two viruses. Attempts were made to isolate a stock of RSV free of RAV by picking single foci of transformed cells. Although the transformed cells present in such foci multiplied indefinitely and maintained their distinctive morphology, they failed to produce either RSV or RAV. However, when RAV was added to such cells, they quickly produced large amounts of both RAV and RSV. We conclude from these observations that RSV is a defective virus which can only produce mature virus in the presence of a helper virus such as RAV.

Material and Methods.—RSV stock and assay: The high titer strain of RSV was used in the present studies. The stock was obtained from the medium of heavily infected monolayers of chick embryo cells. The medium was subjected to sonic vibration at 9 kc to disperse virus aggregates. The virus titer was assayed by focus formation on chick embryo cells.[2] The RSV stock contained 5×10^6 focus forming units (FFU) of RSV per ml and about 5×10^7 infectious units of RAV per ml (see below).

RAV stock and assay: RAV was isolated from a stock of RSV and was purified twice by limiting dilution *in vitro*. The stock used was obtained by disrupting infected chick embryo cells by sonic vibration. The virus was assayed by its interference with RSV infection as described

in a previous paper.[1] The titer was calculated from the terminal dilution which induced interference, and was expressed in terms of infectious units. The RAV stock contained about 10^8 infectious units of RAV per ml. No evidence for the presence of RSV could be found upon inoculation of the stock into chickens, chick embryos, and cultivated chick embryo cells.

Other viruses: Avian lymphomatosis virus (RIF)[2] was obtained from the plasmas of congenitally infected chickens. Avian myeloblastosis virus was obtained after one passage in tissue culture of virus from plasmas of infected chickens. The Bryan standard strain of RSV (H_2C_1 strain) which had been purified by isolation of a single focus was obtained from Dr. John Bader.

Antiserum to RAV: Four-week-old chickens were infected intravenously with 10^6 infectious units of RAV and were bled for antibody one month later. At a dilution of 1:1,000 these sera reduced the titer of both RSV and RAV by a factor of 1,000 in 40 min at 37°C.

Chick embryo cell culture: The techniques for preparing cultures of chick embryo cells have been described in detail elsewhere.[2] The only modification was in the medium used to grow cells which had been transformed by infection with RSV. These cells could be maintained more readily if overlaid with a medium containing 0.4% agar. All media contained 199 as the basic ingredient plus 10% tryptose phosphate broth and 5% calf serum.

Results.—The absence of virus production in foci of transformed cells: As the first step in studying the relationship between RSV and RAV, an attempt was made to obtain stock of RSV which was free of RAV. The RSV stock containing RAV was added to chick embryo cultures at a very high dilution so that only 2 or 3 foci appeared per plate in order to minimize likelihood of contamination of the foci by RAV. The cultures were overlaid with an agar medium containing anti-RAV serum as a further precaution against contamination of the RSV foci by RAV. After allowing 7 days for the development of foci, trypsin was added to loosen the attachment of the cells to the dish; the isolated foci were removed with a capillary pipette and added to 10^6 normal chick embryo cells. Serial transfers of the cultures were made at intervals of 3–4 days. At each of the early transfers an aliquot of the cells was diluted and plated on normal chick embryo cells to determine the number capable of growing into foci. Before each transfer the supernatant fluid was harvested and assayed for RSV and RAV.

In the course of many such experiments, it was found that more than 80 per cent

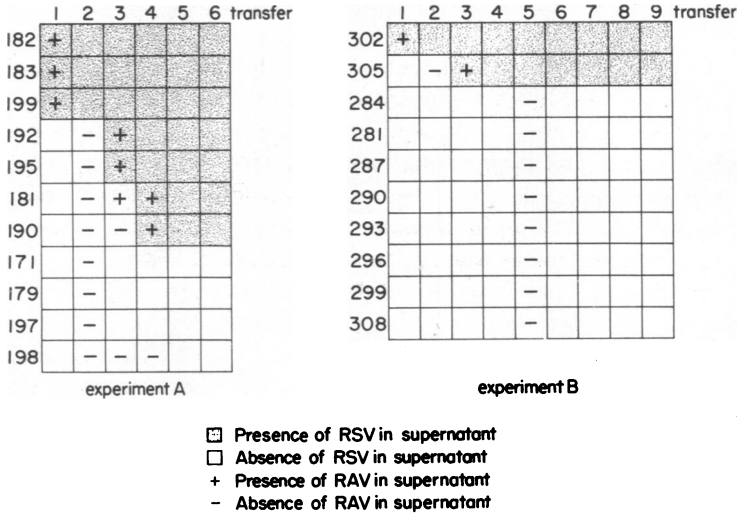

FIG. 1.—Pattern of RSV production from transferred foci.

TABLE 1

NUMBER OF TRANSFORMED CELLS CAPABLE OF GROWING INTO FOCI

Focus no.	Foci produced after plating original focus	Foci produced by plating at transfer (per 10^6 cells) 1st transfer	2nd transfer	3rd transfer	4th transfer
Virus producers					
302	32	1,100 (v)*	39,000 (v)	...	...
305	80	1,200	7,200	31,400 (v)	>100,000 (v)
Nonproducers					
281	160	2,300	55,000	45,000	>100,000
284	18	24	4,700	25,000	>100,000
287	80	380	12,000	54,000	25,000
290	90	460	12,000	...	40,000
293	12	28	140	555	1,100
296	10	72	1,900	4,700	40,000
299	4	70	224	1,500	5,100
308	50	78	810	1,600	5,800

* (v) = RSV demonstrated in the medium.

Individual foci were picked and added to cultures of uninfected chick embryo cells. The mixed cultures were transferred serially. At each transfer, an aliquot of cells was plated separately to determine the number able to initiate a focus. The cultures presented in this table are the same as those of experiment *B*, Fig. 1.

of the original foci failed to release either RSV or RAV at the first transfer despite the fact that some of the cultures contained over 1,000 transformed cells. Cells with the characteristic morphology of transformed Rous sarcoma cells but not producing virus will be referred to as nonproducing (NP) cells. The presence or absence of virus in two experiments is shown in Figure 1, and the number of transformed cells present during the first four transfers of one of these experiments is shown in Table 1. The transformed NP cells clearly had a growth advantage over the normal cells, since they formed an increasingly large fraction of the population with continuing transfer (Table 1). It can be seen that the transformed cells derived from many individual foci failed to produce virus at any time over as many as 9 transfers (Fig. 1), even though they already represented over 10 per cent of the population in some cultures by the 4th transfer and constituted a majority in some of the cultures of the 9th transfer. Cultures which failed to release virus into the medium also failed to yield virus when the cells were disrupted by sonic vibration. The medium of the NP cells had no inhibitory effect on the infectivity of RSV.

A further attempt was made to detect virus production from NP cells which had been transferred 3 times, by exposing them to 5,000 r of X irradiation and plating them on normal chick embryo cultures. The dose of X rays was large enough to block cell division, but not large enough to affect virus release.[3, 4] The plating of irradiated cells is a highly sensitive technique for detecting virus production, because the released virus has an immediate opportunity to contact susceptible cells. The results of this experiment are presented in Table 2. It can be seen that the cells of all NP cultures which failed to release detectable virus into the medium during the course of the experiment, also failed to register as infective centers after irradiation. The cells of virus-producing culture #302, which was included as a control for the sensitivity of the technique for detecting virus-releasing cells, induced focus formation as effectively after X irradiation as before. The results show, therefore, that even the most sensitive technique available for the detection of virus fails to reveal any virus production by the NP cells.

Some of the foci which did not produce RSV in the early transfers began to release RSV spontaneously after several transfers. Every one of the total of 53 such foci

TABLE 2

INABILITY OF NP CELLS TO INITIATE FOCI AFTER X IRRADIATION

Culture no.	Foci produced per 3.6×10^5 cells: Unirradiated	Foci produced per 3.6×10^5 cells: Irradiated	RSV in medium before transfer (FFU/0.1 ml)
281	16,600	0	0
284	9,200	0	0
287	19,400	0	0
293	200	0	0
296	1,900	0	0
299	550	0	0
305	11,200	0	0
308	560	0	0
302*	18,800	17,000	3,800

* A virus releasing control culture.
At the third transfer the media from mixed cultures of NP and normal cells were assayed for RSV and the cells were plated for focus formation before and after receiving 5000 r of X irradiation.

found to date began to release RAV at the same time or slightly before releasing RSV, and the titer of RAV was always somewhat higher than that of RSV. There was not a single case of RSV production in the absence of RAV production.

RSV production after infection of NP cells by RAV: The consistent failure to obtain RSV production without RAV production suggested that RAV might be essential to the production of mature RSV. To investigate this possibility further, RAV was added to NP cultures and the type of virus released into the medium determined. It was found that the addition of RAV to 42 NP cultures was followed by the release of infectious RSV and RAV in every case. (The simple mixing of RAV with the cell-free medium from such cultures did not unmask a putative RSV, showing that RAV actually initiated the production of RSV by the cells.) The daily yield of RSV following the addition of RAV was determined in three of the NP cultures, and the results are shown in Figure 2. RSV release became detectable in large amounts within a day after the addition of RAV, and continued with only a slight increase in rate thereafter.

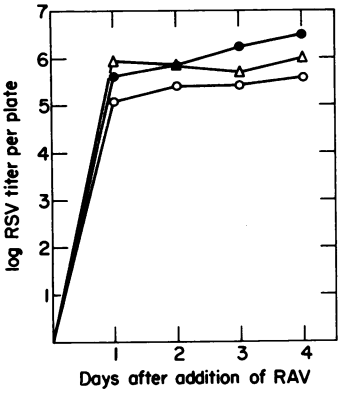

FIG. 2.—Release of RSV following the addition of RAV to NP cultures. 5×10^7 infectious units of RAV in 0.5 ml were added to monolayers of NP cultures. There were 4.9×10^4 NP cells in #287 (●) 1.8×10^4 NP cells in #290 (○), and 3.1×10^4 NP cells in #299 (△). Titer of RSV in the supernatant medium was assayed at daily intervals.

The unfailing initiation of infectious RSV production by RAV superinfection can be explained if we assume that RSV is incapable of producing infective progeny in solitary infection, and that RAV plays the role of a helper virus. This assumption is supported by the finding that the spontaneous onset of RSV production in some foci after several transfers was always preceded or accompanied by RAV production. The presence of RAV in such cases can plausibly be attributed to contamination of the original foci by the progeny of RAV present in the initial inoculum despite the precautions taken to prevent such an occurrence.

If RAV production by transformed cells of the foci were the result of accidental contamination, it would be expected that normal cells from some of the interfocal areas of the original plates would also have been contaminated. The proportion

of such interfocal areas which produces RAV should be about the same in a given experiment as the proportion of transformed foci which produces RAV and RSV, but the former should produce only RAV. As part of experiment *A* of Figure 1, 8 interfocal areas were picked, transferred, and tested for virus production. Cells from 5 of the 8 interfocal areas produced RAV, and only RAV, at the 3rd transfer. Since 7 out of the 11 foci in this experiment eventually produced both RAV and RSV, the findings support the suggestion that spontaneous RSV production was due to contamination with RAV. When rigorous precautions were taken to avoid contamination (exp. *B*, Fig. 1), most NP foci could be maintained as nonproducers indefinitely.

The effect of a variety of agents other than RAV on initiating RSV production by NP cells was tested. Successful initiation has been attained with all the viruses of the avian leukosis complex thus far tested, including a strain of visceral lymphomatosis virus (RIF), myeloblastosis virus, and an RAV-like agent isolated from Bryan's standard strain by RSV.

Absence of RSV production in the early stages of solitary infection: The foregoing experiments have shown that RSV is not produced by the cells on fully developed foci following solitary infection with RSV. Since these foci had been picked one week after infection, and had developed in the presence of neutralizing antibody, it could not be ascertained whether transient RSV production had occurred at an early stage of infection. The following experiment was therefore carried out to detect such early virus production by singly infected cells.

Approximately 100 FFU of RSV were adsorbed for one hr to chick embryo cell monolayers containing 10^6 cells. These will be referred to as RSV-infected cultures, since the low multiplicity of infection assured solitary RSV infections at the cellular level. Half of the cultures were then infected with 10^7 infectious units of RAV so that each RSV-infected cell was superinfected with RAV. All cultures were washed and overlaid with agar medium containing antibody to RAV. On each subsequent day the agar was removed from one RSV-infected culture and one RAV-superinfected culture and the cells were suspended with trypsin. An aliquot of the suspended cells from each culture was X-irradiated with 5,000 r. Irradiated and unirradiated cells were centrifuged, resuspended in medium and plated for focus formation on chick embryo cells.

It was anticipated that unirradiated RSV-infected cells would register as focus formers regardless of whether they produced virus since they could multiply as transformed cells. The irradiated RSV-infected cells, however, could only initiate foci if they released virus. Their failure to initiate foci could be interpreted as a failure to release virus only if X irradiation did not suppress focus formation by the cells superinfected with an excess of RAV, which were expected to release RSV. It can be seen in Table 3 that the unirradiated cells from cultures infected with 100 FFU of RSV produced about 100 foci when plated one day after infection. The number of foci produced by the RSV-infected cells increased by a factor of about 60 in the next 4 days, indicating an average generation time for the NP cells over this period of about 18 hr. Irradiation reduced the number of foci produced by these cells more than 100-fold during the first 3 days, and more than 30-fold on the 4th and 5th days.

The RSV-infected cells superinfected with an excess of RAV produced as many

TABLE 3

EFFECT OF X IRRADIATION ON FOCUS FORMATION BY CELLS DURING EARLY STAGES OF SOLITARY RSV INFECTION

Inoculum	Treatment	Days after infection 1	2	3	4	5
RSV (100 FFU)	Unirradiated	96*	460	1660	3300	6160
	Irradiated	0	4	14	100	238
RSV (100 FFU) plus RAV (10^7 infectious units)	Unirradiated	72	980	2280	7400	...
	Irradiated	60	616	2200	7300	...

* Calculated number of foci produced by plating all the cells from a single infected culture.
Cultures were infected with 100 FFU of RSV, or 100 FFU of RSV plus 10^7 infectious units of RAV. On each subsequent day the cells were suspended, an aliquot was X-irradiated with 5,000 r, and the unirradiated and irradiated samples were plated for focus formation on fresh chick embryo cell cultures.

foci after irradiation as before, indicating that irradiation did not suppress the initiation of foci by virus-producing cells. The results, therefore, show that the vast majority of cells infected with RSV alone fail to produce virus during the early stages of infection. The few foci produced after irradiation of the cells infected with RSV alone were probably due to unavoidable contamination by RAV during the various experimental manipulations.

Discussion.—The failure of the high titer strain of RSV to produce infectious virus in single infection may be compared to other systems in which deficiencies in the production of infectious virus have been found. Three such systems which have been subjected to critical analysis are the production of incomplete influenza virus in the Von Magnus phenomenon, the absence of virus-like materials in cells transformed by infection with polyoma virus, and the inability of defective bacteriophages to produce infectious virus in the absence of a helper virus.

The conditions for infecting cells so that they release incomplete influenza virus are the opposite of those required to produce NP Rous sarcoma cells. Cells which release incomplete influenza virus result only after multiple infection by influenza virus,[5] while NP cells can only be produced with low multiplicities of infection by RSV. This indicates a fundamental difference between influenza and RSV, since single influenza virus particles are fully capable of producing infective progeny, while single RSV particles are not.

In the case of polyoma virus, attempts to demonstrate the presence of virus-related material such as infectious DNA or virus protein in certain lines of transformed cells have failed.[6] When such cells are superinfected with another variant of polyoma virus, they produce only the superinfecting type, i.e., there is no indication of a helper virus action.[7] Therefore, the state of the polyoma virus, if it is present at all, in transformed cells is different from that of RSV in NP cells.

There are, however, striking similarities between the defective bacteriophages and RSV. Like RSV, the defective bacteriophages are unable to produce infectious progeny unless a helper virus is present.[8-11] Therefore, stocks of defective bacteriophage, like RSV, are mixtures which always contain helper virus. That the genome of the defective bacteriophage can multiply when it is established as prophage is shown by the fact that it is present in the progeny of a lysogenic bacterium. The genome of RSV also multiplies since a high proportion, if not all, of the NP cells produce infectious RSV when superinfected with RAV. Therefore, we conclude that the high titer strain of RSV is a defective virus analogous in some respects to the defective bacteriophages.

However, it is unwarranted to carry the analogy with defective bacteriophages

too far since fundamental differences are likely to be found. One difference already known is that RSV contains RNA as its genetic material and the defective bacteriophages contain DNA. Speculation about the precise nature of the defectiveness of RSV will be postponed until the results of current studies are available.

If it seems remarkable that the defectiveness of RSV has gone unrecognized for so long, it should be pointed out that the existence of the helper virus in the RSV stock was unsuspected until two years ago. The opportunity for its detection became available only with the advent of the interference test for avian leukosis viruses.[2] Even with full knowledge of the presence of RAV, rigorous precautions must be taken to prevent superinfection of transformed non virus-producing cells by RAV, since its concentration in the RSV stock is 10 times higher than RSV itself. Since production of RSV by transformed cells follows quickly upon RAV superinfection, previous growth curves obtained with this strain of RSV[12] must be reinterpreted in terms of the interaction with RAV.

Some confusion may arise from the fact that RAV, which plays an essential role in the production of RSV, is assayed by interference with RSV. RAV infection must precede RSV infection by several days for a highly effective interference to occur. There is no indication that the interference is reciprocal, since NP cells which have carried the RSV genome for many generations can readily be superinfected with RAV. The other avian leukosis viruses have a similar nonreciprocal interfering relationship to RSV.[2, 13] The fact that they do not appear to be defective suggests an association between their ability to produce infectious virus (i.e., to carry out a late stage of virus growth) and their ability to interfere (i.e., to suppress an early stage of RSV growth).

Thus far, clear evidence for the defectiveness of RSV has been provided only with the high titer strain. There are two findings which suggest that the standard strain of RSV is also defective. The first is the discovery by Temin that a large fraction of the chick embryo cells which are infected with this strain at a relatively low multiplicity and then are cloned on a feeder layer of mouse cells do not produce RSV unless another avian tumor virus is added.[14] When cloned on chicken cell feeder layers, however, the infected cells do produce RSV. The second is that a virus similar to RAV has been isolated from the standard strain (Shimizu, personal communication). A straightforward explanation for Temin's observations can be made, based on the fact that the avian tumor viruses grow poorly, if at all, on mouse cells while they flourish on chicken cells. A background of mouse feeder cells would minimize the spread of RAV to the RSV-infected chicken cells, while a background of chicken cells would facilitate such spread, and thereby elicit RSV production in the emerging clones.

It is of great importance to determine whether all strains of RSV are defective and are consequently always associated with helper viruses. If this is so, a rational explanation can be offered for such puzzling features of the biology of RSV as the failure to isolate infectious virus from tumors induced in chickens by low doses of RSV.[15] The absence of virus in such tumors cannot be explained on the same basis as the disappearance of virus from aging tumors induced with higher doses of RSV. The latter phenomenon occurs only in immunologically competent chickens and has been shown to be due to a cellular response by the host against the tumor cells.[16] The low dose noninfective tumors can be induced in baby chicks which are immu-

nologically incompetent, and the frequency of noninfective tumors does not depend on the age of the tumor. It seems likely such tumors are due to solitary infection and transformation of cells by defective RSV, which would be made possible by the low concentration of both RSV and RAV in the dilute inoculum.

Finally, attention should be drawn to the possible relationship between the defectiveness of RSV and its carcinogenic properties. The failure to perform the late virus functions could result in unrestrained production of the early virus products and thereby impair the regulatory functions of the cell.

Summary.—In an attempt to obtain the high titer strain of RSV free of RAV, isolated foci of transformed cells were produced by infecting chick embryo cultures with very high dilutions of the RSV stock. Agar, containing antibody to RAV, was added to the cultures to minimize the spread of RAV. The transformed cells were added to uninfected chick embryo cells and retained their altered morphology through repeated serial transfers. Most of the foci maintained in this way yielded no detectable virus. In every such case, however, RSV production could be elicited by adding RAV or any one of several other avian tumor viruses. A high proportion, if not all, of the transformed cells produced RSV upon superinfection with RAV, even when they had undergone well over 20 divisions in the absence of virus production.

A minority of the foci produced RSV spontaneously upon transfer and in every such case RAV was also produced. The proportion of foci producing RSV and RAV spontaneously was matched by the proportion of interfocal areas of normal cells which produced RAV alone. This indicated that RAV from the inoculum had infected cells at random and that the "spontaneous" production of RSV by transformed cells was due to the delayed spread of RAV following the removal of antiserum.

It is concluded that the high titer strain of RSV is defective since it cannot generate the production of new infectious virus unless a helper virus such as RAV is multiplying in the same cell. By contrast, however, the ability to cause the malignant transformation is expressed continuously, and consequently does not require the production of mature virus. Both the potential for virus production and the ability to transform cells are perpetuated in a cell line by hereditary transmission.

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