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# *EVIDENCE FOR THE IN VITRO TRANSFER OF DEFECTIVE ROUS SARCOMA VIRUS GENOME FROM HAMSTER TUMOR CELLS TO CHICK CELLS**

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Rous sarcoma virus (RSV) induces tumors in a variety of mammalian and avian species.[1] In the homologous avian host, the virus usually reproduces to form infectious progeny virus. Tumors induced in heterologous mammalian hosts usually contain no directly demonstrable infectious virus,[1–6] although they contain virus-specific complement-fixing (CF) antigens[5, 6] and transplantation antigens.[7, 8] Injection of viable mammalian tumor cells into young chickens generally results in the production of infectious RSV and the induction of typical Rous sarcoma.[5, 9–12] Infectious RSV has also been retrieved by cocultivation *in vitro* of transformed mammalian cells with chicken cells;[13, 14] however, the mechanism of the synthesis and release of the infectious RSV resulting from contact between mammalian cells and chicken cells is not well understood.

The Bryan strain of RSV was recently shown to be defective and to require the help of an associated avian leukosis virus to form infectious progeny virus in chick embryo fibroblasts (CEF).[15, 16] When CEF cells are infected with a low multiplicity of Bryan strain RSV and subsequently grown in the presence of antiserum to Rous associated virus (RAV), a proportion of cells singly infected with the defective RSV undergo morphological transformation but do not release infectious RSV. Such transformed cells, called nonproducer cells (NP cells), synthesize RSV upon reinfection with one of several strains of RAV or any one of a number of noncytopathogenic avian leukosis viruses.[15, 16] The NP cells contain virus-specific internal capsid antigens demonstrable in the CF test,[17, 18] and can be transplanted into chicks where they grow into virus-free tumors.[15, 16]

When it was shown recently that the Bryan high-titer strain of RSV (BH-RSV) caused tumors in hamsters[19] and that these tumors contained the leukosis group-specific CF antigen,[6] it became of interest to determine the fate of defective RSV

and RAV in such tumors. This report contains preliminary results of an investigation which suggests that the defective RSV genome in the hamster cells was transferred to chicken cells when the chicken and hamster cells were grown in the same cultures. Chicken cells in the mixed cultures acquired the transformed and antigenic characteristics of chicken NP cells and synthesized RSV when superinfected with RAV. The mixed cells also induced virus-free tumors in chicks which yielded chicken NP cells in tissue cultures.

*Materials and Methods.—Virus:* BH-RSV, lot TV 39,[20] used to induce tumors in hamsters, was serologically identical with RAV-1.[21] RAV-1 used in these experiments also contained a leukosis virus of the B subgroup as a minor component of the virus stock.[22-24] RAV-2 was used in one activation experiment.[22] The infectious titer of RAV and the amounts used for RSV recovery experiments were determined in the COFAL test.[25]

*Hamster tumors:* Brain tumors (gliomas) were induced by inoculating golden Syrian hamsters on the day of birth with BH-RSV.[19, 20, 26] A transplant line of hamster tumor was established by serial subcutaneous transplantation of minced brain tumors into newborn hamsters.[26] Two first transplant passage tumors and a tumor in second passage were used in these studies. The tumors were removed 3–4 months after transplantation when they were about 50 mm in diameter.

The tumor cells were processed and established in tissue cultures as follows: necrotic tissue was carefully removed and the viable tumor tissue washed several times with tris buffered saline containing antibiotics. Each tumor was divided into four portions and used for the following purposes: (*a*) tissue cultures were prepared as described below; (*b*) extracts (20% clarified) were prepared and tested for avian leukosis group complement-fixing antigen titer and for infectious virus in the COFAL test;[5,25] (*c*) minced pieces were inoculated into breast and pectoral muscles of 4–5 leukosis-free chicks as a test for tumor induction; and (*d*) pieces were placed in 10% formalin for histological examination.

*Hamster tumor tissue cultures:* Primary monolayer cultures of the hamster tumor were prepared by a trypsinization procedure described previously.[21] Cell suspensions were planted in disposable plastic Petri dishes (100-mm Falcon) at a concentration of $5 \times 10^5$ cells per ml in Eagle's minimum essential medium,[27] supplemented with 5% agamma calf serum, 5% bacto tryptose phosphate broth, glutamine, penicillin, and streptomycin.

*Chicken embryo fibroblasts:* Secondary culture suspensions of leukosis virus-free CEF were obtained by trypsinizing primary monolayer cultures grown in 32-oz screw-capped bottles.[21] In each experiment, cells derived from 3–6 embryos of the same hen were used.

*Mixed cultures of hamster tumor cells and CEF cells:* Mixed cultures of hamster cells and CEF cells were established by planting each Petri dish with $2 \times 10^6$ CEF cells mixed with varying concentrations of hamster cells ($10^2$–$10^6$). Since the hamster tumor cells tended to outgrow and eventually eliminate the CEF cells, long-term studies of mixed cultures (experiment 1) were carried out with mixed cultures which initially contained no more than 80–120 colonies of hamster tumor cells on a confluent background of CEF cells. Cells derived from the same hen were added twice to each mixed culture, using $2 \times 10^6$ cells per dish at approximately 6 and 9 weeks after initial preparation of the culture when it appeared that the CEF cells were being excluded from the mixed cultures.

In each experiment, several CEF and hamster tumor Petri dish cultures were included as controls. The media of all cultures were replaced at intervals of 3–5 days with a maintenance medium containing a reduced concentration of serum (1%) to prevent cell overgrowth. The cultures were serially transferred only at intervals of 7–20 days. After 20 days of cultivation *in vitro*, each lot of CEF used was checked for leukosis virus contamination by testing cell pack preparations for leukosis antigens in the CF test using high-titered specific antiserum from tumor-bearing hamsters.[25]

*Tests for induction of tumors in chicks:* Mixed tissue culture cells and control hamster tumor cells and CEF cells were tested for their ability to cause tumors in chicks. Avian leukosis virus-free chicks, derived from the same source which furnished the embryos for tissue culture, were used. The chicks were usually inoculated at 6–10 days of age. The cells were inoculated into the breast and pectoral muscles of 3–8 chicks using 5–10 million cells per site.

*Complement-fixation tests:* CF tests were conducted by the micro procedure described pre-

viously, using 4–8 units of avian leukosis group CF hamster antibodies.[5, 6, 25, 28] The hamster antibody pool was derived by pooling individual high-titer sera (titer > 1:40) of hamsters bearing transplanted Schmidt-Ruppin hamster tumors. The antibody pool was specifically reactive with tumor and tissue culture antigens of various avian leukosis and sarcoma viruses, and failed to react with antigens prepared from normal hamster or chicken tissues, Fortner sarcoma 3 tumors,[29] or with antigens prepared from hamster tumors induced by adenoviruses,[30] SV40,[31] and polyoma.[32]

*Test for infectious virus:* Hamster tumor cell cultures and mixed cultures of hamster tumor cells and CEF cells were tested for infectious virus by inoculating supernatant media and sonicated, clarified 20% cell pack suspensions into CEF cultures. The CEF cultures were observed for Rous foci and for leukosis-specific antigens. The latter were determined by testing CEF cell pack preparations made up 20 days after infection (the COFAL test).[25]

*Test for the synthesis of RSV following superinfection with RAV:* Monolayer cultures of hamster tumor cells grown with and without chicken cells, and the control cultures, all of which failed to yield infectious RSV or RAV, were infected at various intervals after preparation (11–90 days) with $10^4$-infectious doses of RAV.[22, 25] Daily samples of supernatant fluids were collected for 2 days prior to and also immediately before medium replacement and the addition of RAV. During the following 14 days, 1–2 ml of supernatant fluids of each Petri dish (3 per group) of the inoculated and uninoculated control cultures were collected at 1–2-day intervals, and the amount removed was replaced with fresh medium. Each day's collections of like samples were pooled and clarified by centrifugation at 1600 *g* for 15 min in the International refrigerated centrifuge, model PR-2. Samples were stored in 2 or more aliquots at −70°C.

The various fluid specimens were tested by inoculating each into two freshly planted secondary culture suspensions of CEF and observing these cultures for Rous foci for a period of 14 days. Supernatants of selected RSV positive cultures were serially passed in CEF cultures. Prior to inoculation, in order to eliminate cells, the culture fluids were filtered through a Swinney filter fitted with a 0.45-$\mu$ Millipore filter.

*Results.*—Tables 1 through 3 summarize results obtained in three experiments. In experiment 1, mixed cultures which contained 80–100 colonies of hamster tumor cells on a confluent background of CEF cells were studied in detail. Infectious RSV or RAV could not be demonstrated in culture fluids and pellets derived by ultracentrifugation of culture fluids or from sonicated cell suspensions at any time during 4 months of mixed cultivation. Virus was also not demonstrable in cultures of hamster cells and CEF cells grown separately.

*Synthesis of RSV by mixed cultures upon superinfection with RAV:* In experiment 1, addition of RAV to the cultures on the 43rd day of cultivation was followed by the release of infectious RSV into the culture medium of mixed culture cells but not in the media of cultures of hamster tumor cells or CEF cells (Table 1).

TABLE 1

SYNTHESIS OF RSV BY MIXED CULTURES OF BRYAN STRAIN RSV HAMSTER TUMOR CELLS AND CHICKEN EMBRYO FIBROBLASTS AFTER SUPERINFECTION WITH RAV

| Expt. no. | Type of culture | Test for infectious virus (RSV, RAV) | CF antigen titer | Synthesis of RSV after superinfection with RAV |
|---|---|---|---|---|
| 1 | HTC | 0 | 1:16 | 43 (−), 90 (−)* |
| | CEF | 0 | <1:2 | 43 (−), 90 (−) |
| | HTC + CEF | 0 | 1:4 | 43 (+), 90 (+) |
| 2 | HTC | 0 | 1:16 | 21 (−), 62 (−) |
| | CEF | 0 | <1:2 | 21 (−), 62 (−) |
| | HTC + CEF | 0 | 1:4 | 21 (+), 62 (+) |
| 3 | HTC | 0 | 1:16 | 11 (−), 62 (−) |
| | CEF | 0 | <1:2 | 11 (−), 62 (−) |
| | HTC + CEF | 0 | 1:4 | 11 (+), 62 (+) |

HTC = hamster tumor cells; CEF = chicken embryo fibroblasts; and 0 = negative for infectious RSV and RAV.

* Number denotes days after preparation of culture when RAV was added to culture. Symbol within parentheses indicates result: + = positive for RSV, − = negative for RSV.

TABLE 2

INDUCTION OF TUMORS IN CHICKS BY MIXED CULTURES OF BRYAN STRAIN RSV HAMSTER TUMOR CELLS AND CHICKEN EMBRYO FIBROBLASTS

| Expt. no. | Inoculum | Induction of Tumors: Days after culture preparation | Induction of Tumors: No. chicks with tumors |
|---|---|---|---|
| 1 | Tumor mince | 0 | 0/5* |
| | HTC | 36 | 0/7 |
| | | 49 | 0/4 |
| | CEF | 49 | 0/4 |
| | HTC + CEF | 49 | 4/8 |
| 2 | Tumor mince | 0 | 0/5 |
| | HTC | 63 | 0/6 |
| | HTC | 87 | 0/5 |
| | CEF | 63 | 0/5 |
| | HTC + CEF | 15 | 2/5 |
| | | 21 | 3/8 |
| | | 63 | 2/5 |
| 3 | Tumor mince | 0 | 0/4 |
| | HTC | 21 | 0/5 |
| | CEF | 50 | 0/3 |
| | HTC + CEF | 50 | 1/3 |

HTC = hamster tumor cells; CEF = chicken embryo fibroblasts.
* Numerator indicates number with tumor; denominator indicates number inoculated.

Karyotype analysis of mixed cultures at the time of inoculation indicated that 97 per cent of the cells were hamster cells and 3 per cent were chick cells.[23] Since the hamster tumor cells tended to outgrow and eliminate CEF cells, further additions of CEF were made on the 45th and 66th day of cultivation. The mixed cultures demonstrated the ability to synthesize RSV and RAV upon infection with RAV as late as the 90th day after initial planting (Table 3).

A virus preparation produced in CEF culture with a RSV-containing fluid of activated mixed culture had a focus-forming titer of $10^{3.7}$/ml and a titer of $10^{4.7}$/ml of RAV by COFAL test in CEF cultures.[25]

The phenotype of the activated RSV was immunologically similar to the RAV added to mixed cultures in that it consisted of virus particles with envelope antigens of RAV-1 and also of a B subgroup leukosis virus.[21, 23, 24]

Experiments 2 and 3, using tumor cells derived from different hamsters, furnished results similar to those obtained in experiment 1 (Tables 1 and 3). In both these experiments, mixed cultures containing an initial concentration of $10^6$ hamster cells and $2 \times 10^6$ CEF cells per Petri dish were observed for various periods.

In experiment 3, cultures of hamster and CEF cells yielded RSV when they were infected with RAV 11 days after initiation of mixed cultivation (Tables 1 and 3). Other serially propagated mixed cultures were fortified by additional normal CEF cells on the 48th day and were infected with RAV on the 62nd day; RSV and RAV were released into culture medium 10–11 days later (Table 3).

*Induction of tumors in hamsters and chicks by mixed cultures of hamster tumor cells and CEF:* Hamster tumor cells grown with or without CEF readily induced tumors in hamsters. These tumors contained CF antigens in titers of 1:16 to >1:64 but contained no demonstrable infectious RSV or RAV. Tissue cultures of a hamster tumor developing from mixed culture cells failed to yield RSV upon infection with RAV.

Leukosis-free chicks inoculated with minced hamster tumors, hamster tumor cells, or chick cells failed to develop tumors, whereas those inoculated with mixed cultures

of hamster tumor cells and chick cells developed tumors measuring 8–12 mm within 7–15 days (Table 2). A transplantable line of chicken tumor was established from one of the tumors induced by mixed cultures. On serial passage of explants, large tumors (30–50 mm) developed within 7–12 days. Some of these tumors eventually killed the avian hosts while others regressed completely. Tumors examined at the 2nd and 3rd transplant passages had the histological structure of fibrosarcoma.[33] Clarified 20 per cent extracts of the primary- and transplant-passage chick tumors had CF antigen titers of 1:32 to >1:64, but contained no demonstrable infectious RSV or RAV. Tissue culture cells derived from a 3rd-passage chick tumor also had the karyotype of chicken cells.[33]

*Synthesis of RSV by tissue cultures of chicken tumors upon superinfection with RAV:* Cells derived by trypsinization of a 2nd-passage chicken tumor were established in tissue cultures by seeding the cells on monolayers of normal CEF in Petri dishes. These cultures were free of demonstrable infectious RSV and RAV, but after addition of RAV to the culture, RSV was recovered in the culture medium (Table 3).

Cultures were also prepared from a 30-mm tumor taken at the 3rd passage of the serially transplanted line (*vide supra*). This tumor contained CF antigen, but no demonstrable infectious virus. The tumor was removed from chicks 20 days after transplantation and established into monolayer cultures without using feeder layers of chicken cells. These cells had the characteristic morphology of transformed Rous sarcoma cells described for chicken NP cells[15] and chicken karyotype (*vide supra*). Addition of RAV-2 to these virus-free CF-antigen-containing cultures was followed by the release into the culture medium of RSV with the antigenic character of RAV-2 (Table 3).

TABLE 3

RELEASE OF RSV INTO CULTURE MEDIUM OF SPECIFIED TISSUE CULTURES AFTER SUPERINFECTION WITH RAV

| Culture | No. days in culture prior to addition of RAV | Tests for RSV on Days before and after Addition of RAV | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | −2 | −1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Mixed culture, expt. 1 | 43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | +* | + | + | + | + | + | +* | +* | :* | : |
| | 90 | 0 | 0 | 0 | 0 | : | 0 | 0 | : | 0 | : | 0 | : | : | + | + | + | : |
| Mixed culture, expt. 2 | 21 | 0 | : | 0 | : | : | 0 | : | : | + | : | + | : | + | : | +* | :* | : |
| | 62 | 0 | 0 | 0 | 0 | : | 0 | 0 | : | 0 | : | 0 | : | : | + | +* | + | : |
| Mixed culture, expt. 3 | 11 | 0 | 0 | 0 | 0 | 0 | 0 | : | : | : | 0 | 0 | 0 | : | : | + | + | ++ |
| | 62 | 0 | 0 | : | 0 | : | 0 | : | 0 | : | 0 | : | 0 | : | + | + | : | : |
| Culture of chick tumor† | 7 | 0 | 0 | 0 | 0 | : | 0 | : | : | + | : | : | + | : | + | + | + | : |
| Culture of transplant line chick tumor‡ | 4 | 0 | : | 0 | : | : | : | : | : | + | : | + | : | + | : | + | : | + |

+ = Positive for Rous foci in 2 transfers of supernatant medium in chicken embryo fibroblast cultures (CEF); 0 = negative for Rous foci as tested above; : = not done.
* Selected samples were filtered through Millipore 0.45 filter prior to inoculation into CEF culture.
† Derived from chick tumors induced by mixed cultures, expt. 2.
‡ RAV-2 was used in this experiment.

*Discussion.*—It appeared from these experiments that the hamster cells transformed *in vivo* by BH-RSV had some of the characteristics described for the chicken NP cells induced by the same virus; they contained no demonstrable RAV or RSV, but did, however, contain virus-specific CF antigens and thus, presumably, the genetic information necessary for the synthesis of the RSV. However, unlike the chicken NP cells, the BH-RSV-induced hamster tumor cells were unable to synthesize RSV upon superinfection with RAV, and unlike mammalian cells transformed by Schmidt-Ruppin or Prague strains of RSV,[5, 9–12] they did not produce tumors when transplanted into chicks. The fact that mixed cultures of hamster tumor cells and chicken cells did not yield infectious virus but did so after superinfection with RAV, suggested that CEF cells in the cultures acquired the characteristics of RSV NP cells through direct contact with the BH-RSV hamster tumor cells. The development of noninfectious Rous sarcoma tumors in chickens given transplants of cells from the mixed cultures, but not by hamster tumor cells alone, also supports this hypothesis. Cells grown from tumors produced in hamsters by transplant of cells from the mixed cultures did not release infectious virus after superinfection with RAV, thus behaving in the same fashion as the original hamster tumors.

Mixed cultures of hamster tumor cells and chicken cells generally did not release RSV into culture medium until 5–11 days after addition of RAV, which are unexpectedly long periods. This suggested that relatively few chicken cells were available in the mixed cultures to acquire the RSV genome and to replicate new RSV. On the other hand, transfer of the noninfectious genome from mammalian to chicken cells may be accomplished only in rare instances. However, this transfer occurred regularly in the three experiments reported herein.

The nature of the defective RSV that was transferred from hamster tumor cells to chicken cells remains to be determined. Gerber[34, 35] obtained evidence to indicate that SV40 virus-induced, virus-free hamster tumor cells released infectious nucleic acid which infected adjoining African green monkey kidney cells. Temin[36] reported similar evidence which suggested that virus-free chicken cells transformed by Fuginami virus transferred viral nucleic acid to adjoining uninfected chicken cells.

The Schmidt-Ruppin (S-R) and Prague strains of RSV, which have not been shown to be defective in chicken cells, readily induced tumors in hamsters and other mammals,[5, 9–12] and infectious RSV was retrieved by transplantation of viable mammalian tumor cells into chicks.[5, 9–12] Hamster and rat tumor cells induced *in vivo* by S-R RSV[14] and the Prague strain,[13] respectively, induced RSV in normal chicken cells when grown in mixed tissue cultures, as described above. In neither instance was it necessary to superinfect with RAV. One must conclude that the genomes of the Schmidt-Ruppin and Prague strains contain information for the production of their outer envelopes and that this information is present in the transformed mammalian cells as well as in chicken sarcoma. On the other hand, the Bryan strain genome apparently does not contain this information, whether it is present in a chicken or a hamster tumor.

*Summary.*—Monolayer culture cells of hamster tumor cells induced by the defective Bryan strain of Rous sarcoma virus (RSV) contained no demonstrable RSV or Rous associated virus (RAV), despite the presence of avian sarcoma and leukosis

group-specific complement-fixing antigens. The cultured hamster tumor cells failed to induce tumors in chicks and to yield RSV or RAV upon superinfection of tissue cultures with RAV. However, when hamster tumor cells and chick cells were grown together for variable periods in the same tissue cultures, the mixed cultures acquired the ability to induce virus-free but CF antigen-containing sarcomas in leukosis-free chicks.

The mixed cultures of hamster tumor cells and chick cells and cultures derived from chicken sarcomas were free of demonstrable infectious RSV or RAV, but on superinfection with RAV, both RSV and RAV were released into the culture medium. We therefore concluded that the cultured hamster cells contained defective RSV but no RAV and that upon mixed cultivation of hamster cells with chick cells, the defective RSV was transferred from hamster cells to the chick cells, the latter remaining as nonproducers until superinfected with RAV. Thus, it appears that when the Bryan RSV genome induces hamster tumors, it is present in the hamster tumor cells in a more defective state than is true of the Schmidt-Ruppin and Prague RSV genomes under similar circumstances.

*Addendum:* While this paper was in preparation, Hanafusa and Hanafusa[37] reported that the defective Bryan strain RSV genome present in hamster tumor cells was able to generate the synthesis of infectious RSV only in the presence of chicken cells infected with RAV.

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## *DNA-STRAND SCISSION AND LOSS OF VIABILITY AFTER X IRRADIATION OF NORMAL AND SENSITIZED BACTERIAL CELLS**

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Although radiochemical lesions in DNA appear to be responsible for the loss of viability in X-irradiated cells,[1, 2] the nature of such lesions has not been established. Recently, Freifelder[3] correlated the inactivation of X-irradiated coliphage T7 with the yield of double-strand scissions in DNA. In alkaline sucrose gradients, McGrath and Williams[4] observed a decreased sedimentation rate, attributed to single-strand scission, in alkali-denatured DNA of X-irradiated *E. coli;* reincubation of irradiated cells of a radioresistant strain (B/r) restored the sedimentation rate essentially to the preirradiated level, whereas reincubation of a radiosensitive strain ($B_{s-1}$) had no effect. In the studies reported here, alkaline and neutral pH sucrose gradients were used to study the effect of X rays on the sedimentation behavior of DNA from normal and sensitized cultures of *E. coli.*

*Materials and Methods.*—The bacterium employed was a mutant of *E. coli* K12, substrain JE-850, kindly provided by Dr. Y. Hirota, Department of Biology, University of Osaka, Osaka, Japan, who has characterized it as $F^-$, $Thy^-$, $Try^-$, $Pur^-$, $Lac_{85}^-$, $Xyl_2^-$, $Ara_2^-$, $Mtl^-$, $Gal_2^-$, $Sm^r$, $Phos^-$, $Tb^r$, $\lambda_{ind}^-$, $Rhamnose_2^-$. The culture media, incubation conditions, irradiation procedures, and viability determinations have been previously described.[2–4] Cultures were supplemented during log phase growth with 5 $\mu$c/ml of tritiated thymidine ($H^3$-TdR) to label the bacterial DNA. The pyrimidine analogue 5-bromodeoxyuridine (BUdR) was purchased from California Corporation for Biochemical Research, Los Angeles.