Correction

PERSPECTIVE

The author notes that on page 14390, column 3, the reference to work by Groupé and Manaker should instead appear as “Manaker and Groupé.” The corresponding reference, ref. 21, should also appear as “Manaker RA, Groupé V.”

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The early history of tumor virology: Rous, RIF, and RAV

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One hundred years ago Peyton Rous recovered a virus, now known as the Rous sarcoma virus (RSV), from a chicken sarcoma, which reproduced all aspects of the tumor on injection into closely related chickens. There followed recovery of causal viruses of tumors of different morphology from 4 more of 60 chicken tumors. Subsequent studies in chickens of the biology of the first RSV isolated moved slowly for 45 y until an assay of ectodermal pocks of the chorioallantoic membrane of chicken embryos was introduced. The inadequacies of that assay were resolved with the production of transformed foci in cultures of chicken fibroblasts. There followed a productive period on the dynamics of RSV infection. An avian leukemia virus (ALV) was found in some chicken embryos and named resistance-inducing factor (RIF) because it interferes with RSV. Its epidemiology in chickens is described. Another ALV was found in stocks of RSV and called Rous-associated virus (RAV). Cells preinfected with RAV interfere with RSV infection, but RSV does not produce infectious virus unless RAV is added during or after RSV infection. Intracellular RAV provides the infectious coat for the otherwise defective RSV. The coat determines the antigenicity, host range, and maturation rate of RSV. RSV particles carry reverse transcriptase, an enzyme that converts their RNA into DNA and allows integration into the cell’s DNA, where it functions as a cellular gene. This was the bridge that joined the biological era to the molecular era. Its relation to oncogenes and human cancer is discussed.

defective virus | helper virus | leukemia virus | DNA provirus

One hundred years ago Peyton Rous published an article that was to prove the foundation stone of tumor virology (1). It reported the isolation of a “filterable agent” from the sarcoma in the breast muscle of a Plymouth Rock hen. This “agent” was later called the Rous sarcoma virus (RSV). Although RSV was the first virus isolated from a solid cancer that could transmit the cancer serially when inoculated into other chickens, it was not the first virus to be isolated from a neoplastic condition. That honor goes to two Danes, Ellermann and Bang, who isolated a filterable agent from the blood cells of chickens with erythro-myzeloblastic leukemia (2). At the time such leukemias were not considered cancers, and little attention was paid to the report of its transmission by a filterable agent. In some of the inoculated chickens the blood picture remained essentially nonleukemic, but they developed infiltration of the internal organs with leukemic cells, without increase in the tissue cells themselves. However, the sarcomas induced by RSV were typical of the connective tissue tumors of mammals, although some questioned the relevance for human cancer because they yielded a transmissible agent (or agents) that were considered viruses of the avian leukemia complex (ALV) and were only relevant to chickens. Later work showed that RSV and ALV were closely related and each other. For these reasons and others to be considered later, continuing study of ALV was largely neglected, but Rous and his colleagues continued intense study of RSV and two other viral-induced tumors in the years following its discovery. Much later other kinds of oncogenic viruses were discovered, and the field of tumor virology has grown enormously. However, this article will be restricted to Rous’s early work and the biology of RSV and ALV, with emphasis on quantitative studies in chicken embryos and cell culture from the mid-1950s to the mid-1970s that led into the molecular era. More emphasis will be placed on the dynamics of ALV infection than is shown in most of the recent reviews. Consideration will be given to the relevance of these studies for cell growth regulation and human cancer.

Cellular Transmission of Chicken Sarcomas and Their Causation by Filterable Agents

Peyton Rous (Fig. 1) joined the Rockefeller Institute 2 y after graduation from medical school (3). He was put in charge of the laboratory for cancer research and soon received a hen approximately 15 mo old with a tumor in its right breast that had been noticed 2 mo earlier (4). Widespread necrosis was seen at autopsy in the center of the tumor, with a translucent, finely striated tissue at the rim. Small bits of tissue were implanted by trocar in the left breast of the same fowl and in its peritoneal cavity. Similar implantations were made into two young hens of the same inbred flock. The original tumor was identified as a spindle cell sarcoma with frequent mitoses in the rim and cells with two to five nuclei where necrosis began. The necrosis was due to insufficient blood supply and hemorrhage from thin-walled vessels. The hen died 25 d later from growths of the transplant in the peritoneal cavity.

One of the two inoculated hens developed tumors that were similar to the original tumor. It was the first successful transplantation of a tumor in fowls (chicken tumor #1). Four generations of transplantation were carried out in the same intimately related flock, with only one-fourth of them successfully transplanted (Fig. 2). None of the market-bought Plymouth Rock chickens accepted transplants, nor did mixed breeds. Among birds younger than 3 mo there was transient growth and regression, but there was no sign of growth in adults. No attempt was made to transmit the tumors by cell-free fragments or their derivatives because of the difficulty of propagating the intact tissue.

By the sixth serial transplantation the tumors grew quickly and were highly malignant, producing widespread metastases (1). Pieces of tumors were ground with sand in Ringer’s solution and centrifuged twice at low speed. The supernatant fluid was collected, passed through a bacteria-retaining Berkefeld filter, and injected into one breast of the closely related barred Plymouth Rock chickens, and a tumor fragment was transplanted in the other. All of the latter developed sarcoma at the site of injection; the same growth appeared more slowly at the point where the presumably cell-free fluid had been injected.

When the filtrate of the tumor was injected alone, only a small proportion of the chickens developed a minute growth and did so in the track of the needle (5). When it was injected in the form of dried and powdered tumor tissue, the sarcoma appeared as a diffuse mass in many of the fowl (later found to be composed of many nodules) (6). It seemed that the filterable causative agent required for its action cell derangement or proliferation, such as the needle prick or the presence of dried

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Second-generation transplant of chicken

The probable primary tumor was in the gizzard, and it metastasized to muscle and liver. It was a spindle-shaped sarcoma, fissured and subdivided by flattened sinuses emptied of blood. A virus was isolated from a late transplant generation that produced tumors of the same histological features as the primary tumor, although they tended to resemble chicken tumor #1 after serial viral injections. It is difficult to reconstruct a century later how transplantation promoted the discovery of active tumor viruses; one can speculate that virus arose during the course of transplantation or that preexisting viruses were selected during serial transplantation. Differential filtration indicated that the viruses were approximately the same size. Two other tumors out of a total of 60 yielded sarcoma viruses but were not studied to the same extent as those above (9).

Quantitation of RSV by Pocks on the Chorioallantoic Membrane of Chicken Embryos

Until the 1950s the titration of RSV was by injection into chickens to determine its latent period and its incidence in serial dilutions (10). This required maintenance and observation of many animals for long periods of time, which was expensive and required large amounts of space. I came as a postdoctoral fellow to the laboratory of Renato Dulbecco at the California Institute of Technology in 1953 with the intention of developing a cell culture method for assaying RSV. The idea was to modify Dulbecco’s plaque assay in cell culture for cytocidal viruses to allow transformed focus formation by RSV. I arrived with samples of RSV from Rous and the high-titer strain of RSV from Ray Bryan of the National Cancer Institute. My initial attempts at the focus assay were unsuccessful, so I turned to the in vivo chorioallantoic membrane (CAM) of the chicken embryo, where RSV had been shown by Keogh to produce small, countable ectodermal tumors (11). The method had its irregularities, but it was adequate for estimating the rate of virus production by cells from chicken sarcomas as well as the number of virus-producing cells (12). The experiments showed that the sarcoma cells released RSV into the medium at a very slow rate and that single infectious units of the virus produced individual pocks on the CAM. Virus from the ectodermal tumors led to sarcomas in the underlying mesoderm, which countered the strict connective tissue specificity of the virus in posthatching chickens (13). The cells continued to release virus over many hours without being visibly damaged, which allowed the cells to multiply. That suggested a relationship to lysogenic bacteria carrying temperate bacteriophage, but there were enough differences to raise questions about the analogy. Most prominently, the genome of RSV was RNA (14, 15) rather than DNA of the bacteriophage, and it was produced continuously, whereas the lysogenic bacterium lysed and releases a large number of bacteriophage particles. The persistence of virus production in the sarcoma cells suggested that the virus plays a direct and continuing role in perpetuating the malignant state of the cell and its descendants.

The CAM was later improved (16) and used by Fred Prince in a series of experiments on infection by RSV. He found that the resistance of the CAM of occasional embryos is an inheritable property controlled by a single pair of alleles (17). Heavy infection of the CAM gave rise to new infectious virus at approximately 15 h, followed by an exponential increase that leveled off several days later (18). Ectodermal cells began to proliferate at approximately 1 d after infection, and underlying mesodermal cell proliferation was seen at approximately 2 d. Ectodermal proliferation reached its peak at a little more than 3 d, after which massive necrosis set in. At the same time rapidly increasing numbers of mesodermal cells could be seen, resulting in a large sarcomatous mass at 7 d, with no necrosis.

Prince followed up on the observation of Bryan that some of the sarcomas produced in chickens by low concentrations of RSV contained no infectious virus (19). Such tumors were serially transplanted by Prince and periodically tested for infectious virus on the CAM (20). Some of the noninfectious tumors regressed when observed over a long period, whereas most of the virus producers continued to grow, indicating that spread of the virus to newly infected cells is required to perpetuate tumor growth.

Quantitation of RSV in Chick Fibroblast Culture

In 1956 I reviewed for Virolology a note by Groupé and Manaker reporting an assay of RSV in cell culture by production of transformed foci (21). A significant difference from my efforts to assay RSV in culture was their addition of tryptose phosphate broth to the medium. I enlisted Howard Temin, a first-year graduate student in embryology, to help in adaptation of the RSV focus assay to Dulbecco’s monolayer assay of cytocidal viruses (22). An important difference from the method of Groupé and Manaker was to use an agar overlay to prevent secondary infection of cells at a distance by RSV released from the originally infected cells. Part of the inducement to Temin was that after achievement of the modified assay he could work on the virus Rous had isolated from chicken tumor #7, which


A virus was isolated from a late transplant generation that produced tumors of the same histological features as the primary tumor, although they tended to resemble chicken tumor #1 after serial viral injections. It is difficult to reconstruct a century later how transplantation promoted the discovery of active tumor viruses; one can speculate that virus arose during the course of transplantation or that preexisting viruses were selected during serial transplantation. Differential filtration indicated that the viruses were approximately the same size. Two other tumors out of a total of 60 yielded sarcoma viruses but were not studied to the same extent as those above (9).

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transformed connective tissue cells in chickens into bone. By reducing the density of seeded cells and adding tryptose phosphate broth to the medium, countable numbers of transformed foci were produced in the monolayer (23). The number of foci was proportional to a wide range of concentration of virus, indicating that each focus was initiated by one infectious unit of virus entering a cell that then multiplied for 5–7 d to form a multicellular, transformed focus.

There were a number of variables in the assay that had to be taken into consideration to increase the reproducibility of the assay. Foci were not reliably produced if the assay was delayed until 2 d after seeding the cells. Maximal efficiency was attained by adding RSV when the cells were seeded (24). FBS at conventional concentrations (5–10%) prevented the formation of transformed foci but did not affect the production and release of virus (25). Higher concentrations of calf serum (15%) also suppressed focus formation, so an optimal concentration of calf serum (5%) was regularly used.

When high enough concentrations of the Bryan high-titer strain of RSV were used to transform all of the cells in a population, they stopped multiplying if they were passaged when transformation began (26). Medium taken from such a culture was damaging to normal cells, suggesting that it reflects leakage from the sarcoma cells and may play a role in transformation. It may be related to the observation that the ectodermal CAM cells heavily infected with RSV became necrotic after the cells reached maximal proliferation (18). It has also been noted that transformation of RSV form nodal plaques in cell culture after they have initially made foci and were kept long enough at temperatures higher than 37°C (27–29). Rous himself called attention to certain strains of vaccinia virus that cause cells to multiply and die soon thereafter (30). These results raise the possibility that the transforming effects of RSV mimic the early stages of some cytocidal viruses.

**Radiation Studies of Infection with RSV**

The cell culture assay for RSV was used to explore the radiosensitivity of the virus and of the cell’s capacity to establish and perpetuate infection. Parallel studies were made with Newcastle disease virus (NDV), similar in size and RNA content to RSV. Both viruses were similar in their sensitivity to X-rays, but RSV was far more resistant than NDV to inactivation by UV (31, 32). The relative resistance of RSV to UV was similar to that of temperate compared with virulent bacteriophage (33). In contrast, the ability of cells to support the initiation of RSV infection was far more sensitive than NDV to both UV and to X-rays. Once RSV production began, however, the radioresistance of the cells to continue virus production became comparable to that of NDV-producing cells. The radiation sensitivity of cells to initiate RSV infection was similar to that of their ability to divide. The resemblance of the patterns of radiosensitivity of RSV to those of temperate bacteriophages suggested that RSV can repair UV damage by recombination with related host DNA sequences and that it required replication of cellular DNA for integration. However, the RNA genome of RSV indicated that the mechanism of integration is different. Separate findings that both conflicted with and supported this concept will be considered later.

**Resistance-Inducing Factor, a Virus in Chicken Embryos That Induces Resistance to Infection with RSV in Culture**

Focus formation by RSV under standardized conditions was usually reproducible, but occasionally it was reduced 100-fold in cells from a combined batch of embryos. I found that this resistance to RSV infection could be transferred to susceptible cells by adding medium from the resistant cells, indicating the resistance was induced by an infectious agent that produced no histopathological changes (34). The infectious agent was named resistance-inducing factor (RIF). It proved to be a strain of ALV that was introduced into cultures by one embryo among the several that were combined in making a working set of cultures. Apparently that embryo had been congenitally infected with RIF; and the virus had multiplied for a few days to cells from the other embryos, thereby inducing resistance in the entire culture to RSV. Henceforth cultures from individual embryos were tested for resistance to RSV, and the positives were discarded. RIF was morphologically and physically indistinguishable from RSV (35). RIF and RSV were antigenically related but not identical when tested by inactivation of infectivity by antiserum to RSV. However, RIF was antigenically indistinguishable from RPL12, a classic strain of ALV. The above characteristics warrant classifying RAV as a member of the ALV family.

**Epidemiology of RIF-ALV Infection in a Flock of Chickens**

RIF was indistinguishable from RPL12, a classic strain of ALV. Studies were initiated with RIF as a model for the congenital transmission and spread of ALV and the antibody response to infection, using interference with RSV as a quantitative assay (37, 38). Approximately 20% of the hens had a persistent high-titer viremia of RIF, and all of them transmitted the virus through the egg. The resultant embryos became viremic and congenital transmitters. There was no congenital transmission of RIF by roosters that had become viremic through congenital transmission of RIF by roosters. A minority of nonviremic hens were congenital transmitters but more erratically than viremic hens. There were no antibodies in viremic hens or roosters. They seemed to be immunologically tolerant to the virus. The absence of congenital transmission by viremic roosters raised questions about the integration of the viral genome into host DNA, which will be considered later.

Congenital infection of RIF spread horizontally from the progeny of viremic hens to the progeny of nonviremic hens. The latter exhibited low to moderate viremia, which rose to approximately 40% of the population at 3 mo and declined to zero by 5 mo. Approximately one-third of the progeny of nonviremic hens displayed maternally derived antibody to RIF 2 wk after hatching but lost that antibody by 4 wk and began producing their own.
antibody at 8 wk. The proportion of progeny with antibody increased to 80–90% by 18 wk and remained at that level many months. The presence of antibody would account for the decline of virus in the blood of these birds beginning at 8 wk and sharply decreasing after 14 wk. When progeny from nontransmitting hens were raised in isolation, none of them developed RIF viremia or antibodies to RIF, showing that infection in this class resulted from exposure to viremic birds.

Vascular lymphomatosis occurred approximately six times more frequently in congenitally infected birds than in those infected by contact. It was diagnosed at autopsy 4–14 mo after hatching, with an average of approximately 8 mo in both congenitally and horizontally infected birds.

**Murine Leukemia–Sarcoma Virus Complex**

Chickens have no peripheral lymph nodes, whereas mice and humans do (39). Therefore, there can be no enlargement of lymph nodes in advanced cases of visceral lymphomatosis in chickens, whereas it is characteristic of mouse and human lymphatic leukemia. The thymus of mice and humans is important in certain forms of leukemia, whereas it does not play an important role in the common forms of avian leukemia. For these and other reasons, efforts were made to detect causal viruses in leukemias in mice that might apply to understanding human leukemias.

Initial attempts to isolate viruses from mouse leukemia failed (39). Strains of inbred mice were developed that had a high incidence of leukemia, and serial transplants of leukemic cells were successful in those strains. Transmission of the disease by filtrates from the extracts of the leukemic cells was not successful until the extracts were inoculated into newborn mice of a low-leukemia strain (40). Virus was also found in a transplantable rat sarcoma that could be detected by the complement fixation test for antibodies to the virus (41). That test for murine leukemia virus was successfully applied to infected cultures of mouse embryo fibroblasts. It also detected virus activated from a sarcoma induced in hamsters by a mouse sarcoma virus. Subsequent studies brought out “certain remarkable similarities between these viruses and those of the avian leukemia–Rous sarcoma complex” (41). These similarities included the detection of murine leukemia viruses by interference with mouse sarcoma virus and the occurrence of defective forms of the latter that could be activated by superinfection with the mouse leukemia viruses. To my knowledge no systematic study of the epidemiology of mouse leukemia viruses equivalent to those done in chickens has been done.

**Defectiveness of RSV**

As mentioned earlier, both Bryan and Prince found that some tumors induced in chickens by very low doses of RSV yielded no infectious viruses (19, 20, 42). Related findings were made by Temin with individual foci of cells transformed by RSV in culture (43). In the latter case the addition of ALVs induced the production of RSV from the “non-producing” foci. Serial passages of noninfectious tumors sometimes resulted in production of the virus (20, 42), as did prolonged culture of nonproducing clones from transformed foci induced by very low doses of RSV (44).

As also noted earlier, an ALV RAV was found in our stock of the Bryan high-titer strain of RSV (36), and I asked a new postdoctoral fellow, Hidesaburo Hanafusa, to isolate transformed foci free of RAV from cultures infected with very low doses of RSV (45). He found that most of the foci yielded neither RSV nor RAV in serial passage. They were called nonproducer (NP) foci. Intact cells from the NP foci produced foci when seeded with an excess of normal chick fibroblasts. The addition of RAV to NP cultures yielded RSV. Other nontransforming viruses of the ALV, including RIF, induced infectious RSV production from NP cultures. The growth curve of RSV from RAV-activated NP cells, designated RSV (RAV), paralleled that of RAV, consistent with the rate-limiting step in the production of infectious RSV (RAV) being the helper-dependent maturation process.

The NP cells were incapable of either absorbing RSV-neutralizing antibodies from antisera or of stimulating the synthesis of neutralizing antibodies when inoculated with inoculated cells, indicating that the virus-specific coat protein is that of the helper virus used (46). The possible presence of viral coat antigen was also tested by inoculating NP cells into chickens to produce tumors and examining them for neutralizing antibodies. The inoculation of relatively small numbers of intact NP cells into chickens induced visible tumors within 5 d, which continued to grow for approximately 12 d, when they started to regress (46). The regression of the NP tumors is presumably the result of an immunological reaction to antigens in the tumor cells. Biopsy of the tumors yielded cells that grew in culture with characteristics of cells from Rous sarcomas. None of the NP tumors yielded virus spontaneously, but all of them retained the capacity for RSV activation by RAV.

Chickens that had borne NP tumors developed new tumors when inoculated with RSV, whereas those chickens inoculated with RAV were resistant to inoculation with RSV. The overall results showed that there were no viral-specific coat antigens in the NP cells, and defective RSV depends entirely on the helper virus to mature as infectious particles (46).

A new helper virus, designated RAV-2, was isolated from the stock of the Bryan high-titer strain of RSV (47). RAV-2 is antigenically unrelated to RAV-1, which occurs in the same RSV stock. RAV-2 fails to grow in the cells of some chicken embryos that support the growth of RAV-1, but other embryos support the growth of both helper viruses. Each of the helper viruses confers its host range properties on RSV activated by that helper. RSV activated by RAV-2 (i.e., RSV(RAV2)) is not susceptible to interference induced by RAV-1. The host range of RSV, the antigenicity of the virus, and its susceptibility to interference are all determined by the helper virus that provides the virus coat. Growth curves of infectious RSV in NP cells activated by helper viruses of different growth rates revealed that the helper virus was the regulating factor (48). Experiments were also done in which RSV was added to cells previously infected with helper viruses and therefore were in full production of viral coats. The latter case included superinfection with RSV(RAV-2). The rate of RSV production was independent of helper virus because a large supply of viral coat was already available. Hence all of the properties measured of RSV except transformation are controlled by the coat of the helper virus (49).

Different viral coats with the same transforming genome are known as pseudotypes. They might be likened to a wolf in sheep’s clothing.

The acute leukemia viruses of myeloblastosis and erythroblastosis are also defective (50, 51). These lethal viruses productively infect many cell types but transform only their cognate hematopoietic cells (52, 53).

**Virus Particles in NP Cells**

Some results from other laboratories were not fully consistent with those described above. Examination of chicken NP cells in the electron microscope by Dougherty and Di Stefano (54) revealed the presence of extracellular particles with the morphology of ALVs. The number of virus particles in the cells was estimated at 1/10 to 1/100 the number found in culture of Rous sarcoma cells releasing infectious virus. The morphology of the NP cells in the electron microscope was similar to that of cells releasing infectious particles of RSV (55). The failure of NP cells to produce infectious virus indicated that transformation was dependent only on infection by RSV. Labeling of NP cells with 3H-uridine and purifying the labeled virus particles revealed that they had the same sedimentation characteristics as RSV from
NP cells activated by RAV-1, but they were present in much smaller numbers. The RNA of the particles from the NP cells labeled with \(^3\)P had the same base composition as that of RSV(RAV-1) and of RAV-1.

Vogt found that NP particles were noninfectious on commonly used C/First cells that are susceptible to viruses like RAV-1 and its pseudotype RSV(RAV-1) (56). In contrast, cells of the C/A type that were resistant to infection with RAV-1 and its pseudotypes but susceptible to infection by RAV-2 and its pseudotypes were also susceptible to infection by the particles of NP cells (56). The NP particles could also infect and transform quail cells. Hence, those particles were called RSV(0), viruses that can infect certain types of avian cells but were not detectable in most assay systems. However, even the highest infectivity titer of RSV(0) found in NP cultures was 1,000-fold lower than the titers of helper-dependent RSV produced by the same cells after superinfection with an ALV. Therefore, RSV(0) is quantitatively lacking in the ability to produce functional viral progeny. The basic observations supporting the concept of defective avian sarcoma viruses, namely the activation of NP cells and envelope control of helper viruses, remain valid, but the emphasis has shifted from an absolute to a relative defect (56). The envelope of the infectious RSV(0) was subsequently shown to be provided by endogenous (genetically transmitted) RAV(0) or related defective endogenous viruses (57–59).

The great majority of the NP lines produced virus of relatively low titer that was infectious on C/A (cells resistant to the A class of RSV) and quail cells, as described for RSV(0) above (60). However, a minority of the NP lines produced no infectious virus detectable on any cell lines, although the physical presence of virus particles was detected by \(^3\)H-uridine labeling and density gradient centrifugation. The addition of RAV-1 to these NP cells yielded infectious virus of two types according to their host range.

A different approach to the problem of defectiveness was taken using RSV-29, a strain of RSV that has undergone the least number of passages beyond its isolation from chicken tumor #1 among all of the current strains of RSV (61). Biological characterization indicated that it was defective in producing mature infectious virus. It had the physical properties of RSV, as well as all of the genes except that which was needed to produce the envelope protein needed for infection (62). That result suggested that the original RSV was a defective-transforming virus that gave rise to other defective viruses like the Bryan high-titer strain and to nondefective viruses like the Prague strain. It was also proposed that all transforming viruses began as replication-defective viruses and that replicative-competent strains were generated during subsequent recombination with helper virus (61, 63, 64). The significance of the replication defect only became apparent much later, when it was discovered that it resulted from the substitution of viral replicative genes by host-derived cellular sequences important for transformation (64). It is noteworthy that the Prague strain of RSV, which is competent for viral replication, transforms mammalian cells but does not produce the virus unless the cells are fused with chicken cells (65).

Genetics of Susceptibility and Resistance to RSV. Genetic studies of RSV injected into several highly inbred lines of chickens by various routes supported the results of Prince (17), that is, susceptibility is dominant to resistance and is dependent for expression upon a single pair of autosomal genes (66). Similar results were found in a different set of highly inbred lines of chickens in which RSV was applied on the CAM of chicken embryos or in cell culture and measured respectively by pocks on the CAM and/or transformed foci, as well as virus production by cell cultures (67). It had previously been shown that these assay methods gave the same results as tumor production after inoculation into hatched chickens (68).

The above studies established that resistance to variants of RSV is controlled by a single gene, but they could not consider the possibility that different virus-specific envelope proteins determine the host range of RSV, because neither the defectiveness of RSV nor the nature of its helper was known at that time (47). The host range also depends on cellular receptors determined by the genome of the cells. The major helper virus in the stock of the Bryan high titer of RSV is RAV-1, which provides the virus-specific envelope required for infectivity of the defective RSV designated RSV(RAV-1). It produces foci on cell cultures derived from the normal chicken embryos of all of the strains of Kimber Farms White Leghorn chickens tested in my laboratory. RSV (RAV-2), however, fails to produce foci in a small fraction of the embryos from these strains. RSV(RAV-2) was therefore used to determine the genetic basis for the resistance and susceptibility of cells to infection (69). The results of single pair matings tested for susceptibility to RSV (RAV-2) showed that it is determined by a single pair of autosomal genes and that susceptibility is dominant over resistance. The gene has no influence on the susceptibility of cells to infection by either RSV (RAV-1) or RAV-1 alone. Because the expression of the gene for cellular susceptibility is contingent on the nature of the outer coat of the challenging virus, it seems likely that the gene controls a component of the cell surface that is involved in virus adsorption or penetration. Similar results and conclusions about the relationship between cellular susceptibility to RSV and the viral envelope were obtained with other lines of chickens vs. pseudotyped RSV and nondefective RSV (70, 71). Five groups of susceptibility to different types of RSV have been previously described (72, 73), which should be consulted in the original articles for their detailed characterizations.

The Bryan strain of RSV does not ordinarily infect mammals, but the Schmidt-Ruppin strain of RSV does (74). It produces sarcomas in hamsters, rats, mice and guinea pigs, with sarcoma-like but regressive lesions in rabbits. It is antigenically distinct from the RSV(RAV-1) component of the Bryan strain. It is therefore likely that the capacity of the Schmidt-Ruppin strain to infect mammals is related to its particular coat and its binding to a mammalian membrane receptor (69). The capacity of the Schmidt-Ruppin and Prague strains of RSV derived from the original RSV probably reflects local selective conditions favoring certain serotypes (72). Important factors in this selection may include resistance of chicken lines to certain subgroups as well as the nature of the indigenous tumor virus flora of the animal host.

Transformation Mutants

Temin (75) reported the first mutation that altered the morphology of cells in transformed foci. Unlike the rounding of cells in almost all transformed foci produced by the original stock of the Bryan strain of RSV at 7 d, approximately 1% of the foci at 9 d consisted of long, fusiform or mixtures of round and short fusiform cells. Virus isolated from these foci transformed cells into the same morphological phenotype. The results showed that the morphology of transformed cells was dependent on the genome of the virus and was faithfully transmitted to progeny cells during their proliferation. Statistical analysis of viral mutant production during development of transformed clones showed that viral mutations occur during the multiplication of the virus in the cells and not at the time of infection. Another class of mutants was derived from replication-competent strains of RSV (76, 77). These mutants, which were completely defective for transformation, played a significant role in physical identification of the transforming src gene of RSV.

The first temperature sensitive mutants of an avian sarcoma virus were isolated from the B77 strain of the virus (78). Use of these mutants was limited, however,
because the cells bearing the temperature-sensitive mutant were unable to grow at the nonpermissive temperature. Steve Martin (79) then treated the Schmidt-Ruppin strain of RSV with the mutagen MNNG (nitrosoguanidine) and tested clones of the surviving infected cells for ability to produce transformation at 41 °C. Approximately 2% of the clones failed to transform cells at the elevated temperature but produced virus at the same rate as cells infected with the nonmutagenized virus. Cells transformed by the mutant virus at 36 °C returned to normal morphology within 4 h when shifted to 41 °C. They returned to the transformed state in 2 d when shifted back to 36 °C. The reversible loss of the transformed state at 41 °C indicated that the mutant function is required to maintain the morphological alterations characteristic of transformation but is not required for virus replication.

DNA Proovirus Hypothesis

For different reasons I and Fred Prince independently introduced the idea that RSV infection might be similar to temperate bacteriophage and lyogenic bacteria (12, 42). The results of radiation experiments on RSV and on the early stages of its infection of chicken embryo cells lent credence to the integration of the RSV genome into the DNA of the host cell (31, 33). I stuck with that view until the pattern of congenital transmission of ALV showed that all viremic hens transmitted the virus to their embryos but no viremic roosters did so (37). Five of the 10 roosters in this study were viremic, which indicated that several hundred of the embryos should be congenitally infected if the virus were transmitted into the DNA of the spermatozoa of the viremic roosters (38).

These results did not deter Temin from persisting in his conviction that the RSV genome was integrated into the host cell genome, based partly on the stable inheritance of the virus in transformed cells that were not producing infectious virus (63). This was originally a genetic hypothesis that contained no implication about the molecular nature of the provirus. However, studies with inhibitors of nucleic acid synthesis indicated that DNA synthesis was essential for the early stages of infection with RSV (80, 81) and its helper virus (82). The DNA provirus hypothesis was first proposed by Temin on the basis of the inhibitory effects of actinomycin D on production of RSV (63). He recognized that actinomycin D also inhibited the growth of influenza virus that is not integrated into host cell DNA (83). However, he claimed that influenza virus production was only inhibited by early treatment with actinomycin D, but RSV production was not so restricted. Examination of the article on which this statement was based (84) does not bear out the claim, nor does it take into account that the latent period of RSV is much longer than that of influenza virus. The inhibition of RSV production by actinomycin D was attributed by John Bader (82) to a general effect on DNA in virus synthesis, but a specific effect was demonstrated by the direct action of cytosine arabinoside on DNA synthesis.

There was hesitation in accepting the DNA provirus hypothesis (63), possibly for three reasons: (i) the central dogma of the biology postulated a one-way transfer of information from DNA into RNA; (ii) there was question about the interpretation of some of the inhibitor experiments; and (iii) there was no congenital transmission of RIF (which is closely related to RSV) by viremic roosters. This skepticism continued for several years despite the demonstration of the virus-specificity of the early DNA synthesis (82). However, the DNA provirus hypothesis became widely accepted with the demonstration by Temin and Mizutani (85) of a virion enzyme they called “endonuclease-dependent DNA synthesis” and the independent discovery by Baltimore (86) of the same enzyme he called “viral RNA-dependent DNA polymerase,” which later became “reverse transcriptase.” Those findings overcame the objection of a one-way transmission of information from DNA to RNA and clinched in the minds of all but a few contrarians the DNA provirus hypothesis (81). If there was any doubt left, it would have been purged by the demonstration of infectious DNA from RSV-transformed, non-virus-producing rat cells (87). The latter result clinched the view that the infection of newborn rats by the Prague strain of RSV propagated in Czechoslovakia (88).

With remarkable prescience Temin also proposed that “the Rous sarcoma appeared before the RSV. Other events not involving a virus led to the formation of genes for cancer and the chicken sarcoma. The sarcoma was presumably infected by an ALV, and RSV was formed by a rare combination” (63). That proposal was later bolstered by molecular evidence that the original RSV was replication-defective (61, 64), but elaborating on that is beyond the scope of this premolecular history of RSV biology. It should be noted, however, that the studies of RSV led to the identification of cellular oncogenes (89). Retrospective and Prospective. In the 5 y that followed the successful transplantation of chicken sarcomas and the recovery of RSV from the tumors, Rous carried out an intense study of several chicken sarcomas, as indicated in the numerous references to his work cited here. He published approximately as many subsidiary articles on chicken sarcomas in the same time span, indicating the breadth and productivity of his investigations. He later turned his interest to the Shope rabbit papilloma virus, the first well-studied agent known to cause epithelial tumors in mammals (90). He was particularly interested in the progression of the papillomas to carcinomas (91). He combined these studies with the effects of carcinogenic hydrocarbons, in which he discovered and named the processes of initiation and promotion (92). Initiation is generally accepted as a genetic event. As great a scientist as Rous was, he ardently opposed the generally accepted “somatic mutation hypothesis” of carcinogenesis (30). That is not too surprising considering that Albert Einstein, the architect of the special and general theories of relativity, rejected quantum mechanics, although it is the rigorously tested foundation of modern atomic physics (93). These two examples show that even the greatest scientists are not infallible.

I had the opportunity to visit Rous in 1953 at the Rockefeller Institute when I was embarking on my work with RSV. He was a cheerful, cordial host who invited me to lunch in the Institute’s august dining hall and then introduced me to Richard Shope in the latter’s laboratory. Shope was standing alone at a workbench; in those days biologists seemed to do their experiments with their own hands. Rous gave me a bound collection of his papers on the chicken sarcomas and their viruses. He also gave me two sealed ampoules of lyophilized tumor tissue containing virus from the osteochondrosarcoma (7). I used those to lure a graduate student, Howard Temin, away from embryology to join the effort to develop a practical cell culture assay of RSV, with the promise he could work on the osteochondrosarcoma virus from chicken tumor #7 when the assay of RSV was established (22). The retrospective hitch in that recruiting effort was that although the assay of RSV worked (23, 94), the viral contents of the osteochondrosarcoma ampoules were no longer active. Nevertheless, it has been said that “the focus assay revolutionized the study of RSV making it possible to study the interaction of a single cell with a single virus particle, and the development of this assay led to rapid advances in retrovirology that began in the 1960s” (95).

I quit working with RSV in approximately 1970 to study cell growth regulation, confident that my former associates Temin, Hanafusa, Vogt, and Martin would carry the work on RSV and ALV forward without me, which they did with a vengeance. My main insight into cell growth regulation was that the level of membrane-activated free magnesium plays the key role in the coordinate control of normal cell proliferation via its direct effect on the
overall rate of protein synthesis, which determines the rate of onset of DNA synthesis several hours later (96). Evidence from transformed human fibroblasts suggests they have lost their capacity to regulate magnesium (97). Depriving transformed cells of adequate external magnesium normalizes their morphology and growth behavior (98). It would be of interest to determine whether that is true in RSV-transformed chicken cells. There are several other questions about the biology of RSV and ALV infection that are still unresolved, such as the following. (i) Is any virus produced in naturally occurring chicken sarcomas before they have undergone serial transplants that increase the malignancy of the tumors? (ii) What is the material in the medium of cultures massively infected with RSV that inhibits the growth of normal and transformed cells? (iii) Given that retroviral genomes integrate into cellular DNA, why do highly viremic roosters fail to congenitally transmit RIF (i.e., ALV), whereas all viremic hens do so (38)?

It is clear that in vitro studies of the biology and molecular genetics of RSV have had enormous impact in our understanding of oncogenes and the neoplastic transformation of cells (95, 99, 100). Arriving at this conclusion was facilitated by the simplicity of the system in which a single gene of the virus determines the neoplastic transformation of a single cell and its descendents within a few days, without intermediate steps. However, this very simplicity raises questions about its adequacy for characterizing the complex development of human cancer. The natural history of solid human cancers spans several decades and involves progressive stages of preneoplastic and neoplastic development (101). Recent genomic studies indicate that there are between 1,000 and 10,000 somatic substitutions in most adult human cancers and as many as 100,000 in lung cancer and melanoma (102). There are in addition other types of genetic and epigenetic change that increase the complexity of the process. Just as was the case with RSV, the understanding of human cancer should be facilitated by a quantitative cell culture model that exhibits all stages of the process. Such a model is represented by the progressive spontaneous transformation of NIH 3T3 cells in serial passage at high population density in low serum concentration (103). Serial selection of cells with increasing capacity to escape contact inhibition as measured quantitatively by small but significant increments in saturation density illustrates the early field stages of cancerization that precede visual evidence of neoplastic transformation. There is heterogeneity in the starting population of cellular ability to overgrow confluent cultures. These preexisting, low fitness variants are the early objects of selection, giving rise to flat hyperplastic fields that spawn new mutations. The latter have the capacity to produce transformed foci of increasing size and density, leading to a capacity for tumorigenesis. The model gains credibility from recent evidence that the driving force in human cancer is selection (104, 105), just as it is in spontaneous transformation in culture. The cell culture models of RSV transformation and spontaneous transformation complement each other and provide a fuller understanding of human cancer. What Rous wanted a century ago has had, and will continue to have, reverberations in tumor biology for a long time to come.

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