PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 515–520 (1980)

Chicken Sarcomas and Expression of Chicken Endogenous Virus after Transplantation of Virus-Free Rous Hamster Tumor Cells (40807)

PADMAN S. SARMA,* FRED EDWARDS, MONA J. LAW,*
SUZANNE F. SKUNTZ,* AND LYMAN B. CRITTENDEN†

Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, and †U.S. Department of Agriculture, SEA, FR, Regional Poultry Research Laboratory, East Lansing, Michigan 48823

Introduction. The Bryan strain of Rous sarcoma virus (B-RSV) is a defective avian sarcoma virus (1, 2) which needs a helper avian leukosis virus (ALV) for the synthesis of its viral envelope glycoproteins and thus infectious progeny sarcoma virus (1–5). The sarcoma viral genome is not defective for the cell transformation event or the intracellular synthesis of *gag* gene-coded gs antigen (2, 5, 6). Thus, avian cells transformed by B-RSV in the absence of ALV and hamster or other mammalian cells transformed *in vivo* or *in vitro* contain no detectable infectious B-RSV or its associated helper ALV, but do however contain the avian leukosis viral gs antigens and B-RSV genome rescuable as infectious B-RSV with helper ALV (6–9). This mode of interaction of B-RSV genome with hamster cells delineated in our previous studies (8) also showed that the hamster tumor cells were malignant and were thus transplantable in syngeneic hamster hosts which developed rapidly fatal sarcomas. On the other hand, the hamster tumor cells failed to elicit a tumor response in normal chicks unless a helper ALV was also included with the hamster tumor cell inoculum, or was already present in the chick as a consequence of congenital infection (9). The induced tumors were of chicken cell karyotype and contained high titers of B-RSV with the viral envelope antigenic characteristics of helper ALV.

Our more recent studies have shown that the hamster tumor cell line 3292 used in our original studies (8) has undergone a change in its properties on prolonged passage of the cells *in vitro*. Whereas the cells used in our previous studies within *in vitro* passage level of 19 failed to induce sarcomas in recipient normal chicks, the higher passage line of this tumor cell culture (passage 35 and above) induced sarcomas of chicken cell karyotype in chicks of diverse genetic strains which differ in the expression of the vertically transmitted avian endogenous RNA tumor virus of subgroup E. Chicks congenitally infected with the infectious form of this endogenous virus, RAV-O (chicken genetic lines 7, 100, and C) (10–13) and those containing the endogenous virus genome as rescuable virus "chick helper factor" (chf) (14–16) as in chicken genetic line 6, develop sarcomas which contain the RAV-O pseudotype of B-RSV, B-RSV (RAV-O) [also referred to in this paper as B-RSV (chf)]. In genetic line 15_B which contains no infectious RAV-O, demonstrable chf or avian leukosis gs antigens, sarcomas of chicken cell karyotype are induced which contain no demonstrable infectious B-RSV or RAV-O, but do contain high titers of the avian leukosis gs antigens and B-RSV genome rescuable as infectious RSV with helper ALV.

Our studies provide an animal model which shows that upon inoculation of avian sarcoma virus-transformed, but infectious virus-free, hamster tumor cells into chicks, B-RSV genome responsible for the original sarcomagenic transformation of these hamster tumor cells and for the synthesis of the gs antigen in these cells is transferred to supporting stromal chicken cells resulting in the sarcomagenic transformation of the chicken cells and the subsequent expression of the avian sarcoma viral genome in these cells. We successfully used the

0037-9727/80/050515-06$01.00/0

method to rescue and thus demonstrate covert avian endogenous RNA tumor virus in chicks.

Materials and methods. B-RSV(−) Hamster tumor cell line 3292. This culture was established as a monolayer culture in 1965 from a brain tumor (glioma) which we induced in a Golden Syrian hamster (8, 17). Although the cells contained avian leukosis-sarcoma viral gs antigen (CF titer of 20% cell pack antigens, 1:16), concentrated culture fluids as well as cell extracts of this culture contained no infectious B-RSV or the Rous associated virus (RAV). The cells contained the B-RSV genome rescuable as infectious B-RSV with helper ALV (8). The hamster tumor cells are referred to in this paper as B-RSV(−) cells to denote the presence of rescuable B-RSV genome and the absence of demonstrable helper ALV (18). The cell line was propagated *in vitro* with intermittent storage in a liquid nitrogen (−190°C) storage tank at passage levels between 19 and 35. Studies reported herein were performed with passage levels above 39. Electron microscopic examination of thin sections of the cells revealed the presence of budding type C viral particles. Cell antigens (20% cell pack) contained low titers of the gs antigen (p-30) of the endogenous hamster type C RNA virus (19), but our repeated efforts to isolate an infectious hamster type C virus from this hamster tumor cell line were not successful.

Cell cultures. Cell cultures of B-RSV(−) hamster tumor cell line 3292 were propagated and maintained in a medium consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. Monolayer cultures of chicken embryo fibroblasts (CEF), quail embryo fibroblasts (QEF), and turkey embryo fibroblasts (TEF) were prepared by the trypsinization technique (20). The embryonated chicken eggs used, free of demonstrable infectious ALV, including infectious endogenous virus RAV-O, were obtained from SPAFAS, Inc., Norwich, Connecticut. The turkey and quail embryonated eggs were obtained from Truslow Farms, Inc., Chestertown, Maryland.

Chicks. The chicks which were inoculated with B-RSV(−) hamster tumor cells were obtained from the following sources: (a) 1-day-old White Leghorn chicks from SPAFAS, Inc., a commercial farm specializing in the supply of chicks and embryonated chicken eggs free of demonstrable infectious ALV, including infectious endogenous virus RAV-O (however, the chicks have not been shown to be free of chf (14, 21)); (b) 1-day-old White Leghorn chicks from Truslow Farms Inc., Chestertown, Maryland (the chicks from this source were free of infectious ALV but were positive for gs antigen expression and rescuable chf); (c) White Leghorn chicks of defined genetic lines of chickens developed and/or maintained at the Regional Poultry Research Laboratory (RPRL), East Lansing, Michigan. The virus and viral gs antigen expression in these lines described in detail elsewhere (12) are designated and shown as follows: gs = group specific antigen of the leukosis-sarcoma viruses; chf = chicken helper factor (15, 16); V-E = infectious avian endogenous virus of subgroup E. Line 6_3 is gs+, chf+, and V-E$^-$; Line 7_2 and Line 100_B and Line C (21) gs-, chf-, and V-E$^+$; Line 15_B is gs-, chf- and V-E$^-$.

Inoculation of chicks with B-RSV(−) hamster tumor cells. The hamster tumor cells were inoculated intramuscularly on the ventral aspect of the wing in doses of 10^6 or 2×10^6 cells per inoculation site in chicks 2 or 3 days old. Before and after inoculation each group of genetically defined chicks as well as chicks received from two commercial sources were separately housed in brooder cages inside plastic isolators.

Processing of tumors for further studies. The tumors that developed in the inoculated chicks were processed in one or more of the following manners: (i) They were examined for the gs antigens of ALV and for infectious RSV and RAV. (ii) Monolayer cell cultures were prepared from selected tumors by the trypsinization technique. The cells were examined for karyotype through the courtesy of Dr. C. S. Stulberg of the Child Research Center of Michigan, Detroit, Michigan. Clarified culture fluids of these cultures were examined for infectious RSV and RAV. (iii) Pieces of tumors were stored in 10% formalin; thin sections were

prepared, stained, and examined histologically for pathology. Cultures free of demonstrable infectious B-RSV or RAV were tested for the presence of rescuable B-RSV. The cultures were superinfected with RAV-1. Culture fluids were collected at intervals of 3 days, clarified, and tested for the presence of focus-forming RSV by inoculation into normal CEF.

Complement fixation (CF) test. A CF test (COFAL test) we have described (23) was used to detect the presence of avian leukosis sarcoma viral gs antigens in the following preparations: cell pack antigens (20% by volume frozen and thawed three times) of cell cultures such as B-RSV(−) hamster tumor cells, chicken sarcoma tumor cell cultures, control and inoculated avian cell cultures used for the detection of cell transformation and viral replication, and extracts (20%, clarified) of sarcomas induced in chicks by inoculation of B-RSV(−) hamster tumor cells.

Test for infectious avian leukosis and sarcoma viruses. Cultures of QEF and TEF were inoculated with test material using 0.2 ml per culture dish. The cultures were maintained and examined periodically for foci of cell transformation characteristic of B-RSV. The presence of nontransforming viruses in the cultures was determined by testing cell pack antigen in the CF test for the presence of gs antigens (COFAL test) (23). Isolated cell-transforming viruses were tested to determine if they belonged to the avian leukosis viral subgroup E (endogenous virus) by inoculation of CEF cultures selectively genetically resistant to viruses of subgroup E (C/E) and by viral neutralization tests (20) using a potent quail antiserum against subgroup E virus.

Results. Although the 3292 line of B-RSV(−) hamster tumor cells contained no demonstrable infectious B-RSV or its associated helper ALV, sarcomas of chicken cell karyotype were induced in every genetic line of chicks we examined, including genetically undefined White Leghorn chicks of two commercial sources (Table 1). The proportion of chicks developing tumors were the highest in the test groups of chicks in which the tumors contained infectious B-RSV(RAV-O) and were the lowest in line 15_B chicks, the tumors of which failed to yield demonstrable infectious B-RSV(RAV-O) or RAV-O. The induced tumors were histologically classified as sarcomas. Cultured tumor cells from every line of chicks were tested and found to be of chicken cell karyotype. There were no persistent hamster chromosomes in the karyotypes of chick sarcomas we examined.

Clarified cell-free 20% extracts of all of

TABLE I. INDUCTION OF CHICKEN SARCOMA IN DIVERSE GENETIC LINES OF CHICKS WITH VIRUS-FREE B-RSV HAMSTER TUMOR CELLS AND THE EXPRESSION OF B-RSV GENOME AND ENDOGENOUS AVIAN LEUKOSIS VIRUS IN THE INDUCED TUMORS

Chicks used[a]	Proportion developing tumor[b]			Average CF titer of gs antigen	Recovery of RAV-O pseudotype of B-RSV (proportion positive)[e]
	Expt 1[c]	Expt 2[d]	Expt 3[d]		
Truslow Farms	10/12	16/18	17/20	≥64	8/10
SPAFAS	8/11	9/10	10/12	≥64	4/9
Line 6_3	7/14	14/15		≥64	7/8
Line 7_2	12/12	6/6		≥64	8/8
Line 15_B	4/9	18/49	7/35	≥64	0/17
Line 100_B		20/20		≥64	8/9
Line C	11/24			≥64	7/8

[a] See Materials and methods (chicks) for viral and *gs* antigen expression in these chicks.

[b] Average size of tumors at 30 days after inoculation, 2 × 3 cm; numerator shown is number of developing tumors, denominator shown is number inoculated.

[c] In this experiment the chicks were each inoculated with 10^6 cells.

[d] In these experiments the chicks were inoculated with 2 × 10^6 cells.

[e] Numerator, number of tumor extracts producing cell transformation in quail and/or turkey cultures; denominator, total number of extracts inoculated into quail and/or turkey cultures.

the tumors contained high titers of the gs antigens of the avian leukosis-sarcoma viruses. Infectious focus-forming B-RSV(RAV-O) was present in all or a proportion of the extracts derived from chicks other than those of line 15_B. In every case, the gs antigen of avian leukosis-sarcoma viruses was detectable by the CF test (COFAL test) (23) in transformed QEF and/or TEF cultures, but not in parallel control uninoculated cultures. The virus was identified as a member of avian leukosis virus subgroup E by viral neutralization tests and by inoculation of chicken cells susceptible or resistant to productive infection with this virus (C/E cells). Sarcomas of line 15_B chicks as well as cultured cells derived from these tumors failed to yield infectious B-RSV(RAV-O) or RAV-O, although they contained high titers of the avian leukosis viral gs antigens. Superinfection of these nonproducer (NP) cell cultures with an avian leukosis virus, RAV-1, resulted in the rescue of the B-RSV genome and the production of B-RSV(RAV-1). The cell-transforming virus was identified as B-RSV(RAV-1) by type specific viral interference induced against the focus-forming effects of this virus by RAV-1, and not by RAV-2.

All of the induced sarcomas in various lines of chicks were serially transplantable in less than 1-week-old SPAFAS chicks which developed identical sarcomas of 3 to 4 cm (diameter) within 2 to 3 weeks. The sarcomas invariably grew in size, ultimately resulting in the death of the hosts.

Discussion. Huebner *et al.* (24) have recently found that high passage cell lines of human tumors regularly induce sarcomas of rat cell karyotype in immunosuppressed rats. The rat tumor induction failed to occur when low passage human tumor cell lines were used. They have postulated that human *src* gene sequences might be transferred to rat cell chromosomes by the *in vivo* inoculation technique. In our studies, we induced sarcomas of chicken cell karyotype in chicks with infectious virus-free hamster tumor cells with two markers, the *src* gene and the *gag* gene of B-RSV(−). Our experiments using this virus-induced model suggest that the B-RSV(−) genome is transferred from these hamster tumor cells to supporting stromal chicken cells *in vivo*, as evidenced by sarcoma induction and the subsequent expression of the gs antigen in the induced chicken tumors and tumor cells we cultured *in vitro* from these tumors.

It has been postulated that injection of chicks with B-RSV(−) particles resulted in sarcoma formation (25). Since heterokaryons which are presumably formed in chickens are considered to produce B-RSV(−), it is reasonable to assume that the chicken tumors can be generated by infection of chicken cells with B-RSV(−) or B-RSV(chf) released by the heterokaryons. The difference between the hamster tumor cells at early and late cell passage levels may be due to the rate of spontaneous formation of heterokaryons *in vivo* with chicken cells or the survival rate of these heterokaryons *in vivo*. In our previously reported studies (8, 9), although the early passage line of the same hamster tumor cells failed to elicit a similar tumor response in chicks other than those naturally or articifially infected with infectious ALV, parallel *in vitro* cell cocultivation experiments we then performed using mixed cultures of hamster tumor cells and normal chicken cells mimicked the present *in vivo* chicken NP tumor induction experiments with the late passage line. Inoculation of cocultivated mixed cultures, but not parallel unmixed cultures into normal chicks led to the development of chicken NP cell tumors in these chicks, suggesting that chicken NP cells were presumably generated *in vitro* through cell to cell contact by similar mechanisms prior to inoculation into chicks. A side-by-side comparison of the high and low passage hamster tumor cell lines, to observe similarities and differences in the previously described *in vitro* (8) and the present *in vivo* methods, is not possible since the earlier passage line was lost in a liquid nitrogen storage accident in 1969. Recent *in vitro* cell cocultivation experiments using sendai cell fusion factor to achieve heterokaryon formation have confirmed that demonstrable quantities of infectious B-RSV(chf) can be generated *in vitro* using our present passage line of B-

RSV(−) hamster tumor cells and chf$^+$ chicken cells (26). Such a virus is not recovered in experiments using line 15_B cells which are negative for chf.

The avian endogenous type C RNA tumor virus is classified as a member of subgroup E (10, 13, 15). The viral sequences of this virus are present in most (12, 13, 27–30), but not all (31, 32) genetic lines of chickens. The virus is expressed in varying degrees; as infectious virus resulting in congenital infection and life-long viremia as in lines 7, 100, and C (10, 13), as rescuable chick helper factor sequences as in genetic line 6, and nonrescuable endogenous viral sequences (gs−, chf−, and V-E$^-$) as in line 15_B chicks and some SPAFAS chicks. The inoculation of sufficient numbers of high passage B-RSV hamster tumor cells into chf$^+$ chicks resulted in the rescue of the chick helper viral sequences and the formation of pseudotype of B-RSV with the viral envelope antigenic characteristics of subgroup E endogenous virus, B-RSV(chf). Thus, the chick inoculation technique could be used not only to identify chicks with congenital viremia induced by natural infection with infectious ALV or infectious subgroup E virus (RAV-O), but also for the demonstration of the presence of rescuable chf in apparently normal chicks. In line 15_B chicks which contain nonrescuable endogenous viral sequences, inoculation of the late passage hamster tumor cells led to the development of chicken NP tumors which contained the genome of B-RSV(−).

The type C virus particles we observed in the B-RSV(−) hamster tumor cell line could be B-RSV(−) particles (i.e., B-RSV without helper ALV) (18) and/or the endogenous type C RNA virus of the hamster (19). Thus, it is possible that the described transfer of B-RSV(−) genome from hamster cells to chicken cells could have been due to direct infection of chicken cells with particulate B-RSV devoid of glycoproteins, i.e., B-RSV(−) released by the hamster cells.

Summary. Inoculation of B-RSV(−) avian sarcoma virus transformed, but infectious virus-free hamster tumor cells into diverse strains of chicks resulted in the induction of sarcomas at the site of inoculation in the inoculated chicks. The induced sarcoma cells were of chicken karyotype. They contained high titers of the group specific (gs) antigens of the viruses of the avian leukosis-sarcoma virus group. Sarcomas induced in chicks with no demonstrable avian leukosis gs antigen or rescuable chicken endogenous virus (chick helper factor) contained no demonstrable infectious virus, whereas, those induced in genetic lines of chickens which express the chicken endogenous type C RNA virus either as infectious virus or as rescuable chick helper factor contained the chicken endogenous virus pseudotype of the defective avian sarcoma virus (Bryan Rous sarcoma virus). Our studies suggested that B-RSV(−) viral gene sequences responsible for the sarcomagenic transformation of the chick cells and *gag* gene sequences responsible for the expression of the avian leukosis-sarcoma gs antigens in the induced chick sarcomas occurred by the transfer of the genetic information of B-RSV(−) from the hamster tumor cells to supporting stromal chick cells in contact with the hamster tumor cells.

We are indebted to the late Dr. C. S. Stulberg and his colleagues at the Child Research Center of Michigan, Detroit, Michigan, for karyological and immunological studies to determine the species of cells we cultured *in vitro* from the chick tumors we induced by the transplantation of B-RSV(−) hamster tumor cells. We are also indebted to Dr. Mina L. Vernon of the National Cancer Institute for electron microscopic studies of the hamster tumor cells.

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Received October 15, 1979. P.S.E.B.M. 1980, Vol. 163.