GENETIC INFLUENCE IN ROUS SARCOMA VIRUS INFECTION

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ABSTRACT

GENETIC INFLUENCE IN ROUS

SARCOMA VIRUS INFECTION

by Richard H. Reamer

Genetic relationships of three Rous sarcoma virus strains, Bryan standard (BS RSV), Harris (HA-RSV), and Schmidt-Ruppin (SR-RSV) were studied by comparing the response of individual backcross chicken embryos cell cultures to the three viruses. Cell cultures were prepared following modification of Rubin's technique (1960).

There were four patterns of response of the cells to BS-RSV and HA-RSV: (1) resistance to both, (2) sensitivity to both, (3) resistance to BS RSV only, and (4) resistance to HA RSV only.

Embryos of the original parent lines 6 and 7 responded differently. Line 6 was homozygous susceptible while line 7, though uniformly resistant to BS-RSV, produced embryos some of which were susceptible to HA-RSV,

and BS-RSV appeared to be quite different in their host range.

The Schmidt-Ruppin strain acted as a mixture of viruses, one causing cellular response similar to that by BS-RSV, the other similar to that of HA-RSV.

A cell phenotype was present which could have resulted only through genetic recombination of the two parent line chromosomes. This indicates that there are two separate loci, one controlling infection by BS-RSV and the other controlling infection by HA-RSV.

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by

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INTRODUCTION

The objective of the present investigation was to determine the relationships among three strains of the Rous sarcoma virus based on the response of cell cultures prepared from chicken embryos sensitive or resistant to Bryan standard Rous sarcoma virus (BS-RSV). The criterion of infection was the foci of transformed cells in response to the virus strains.

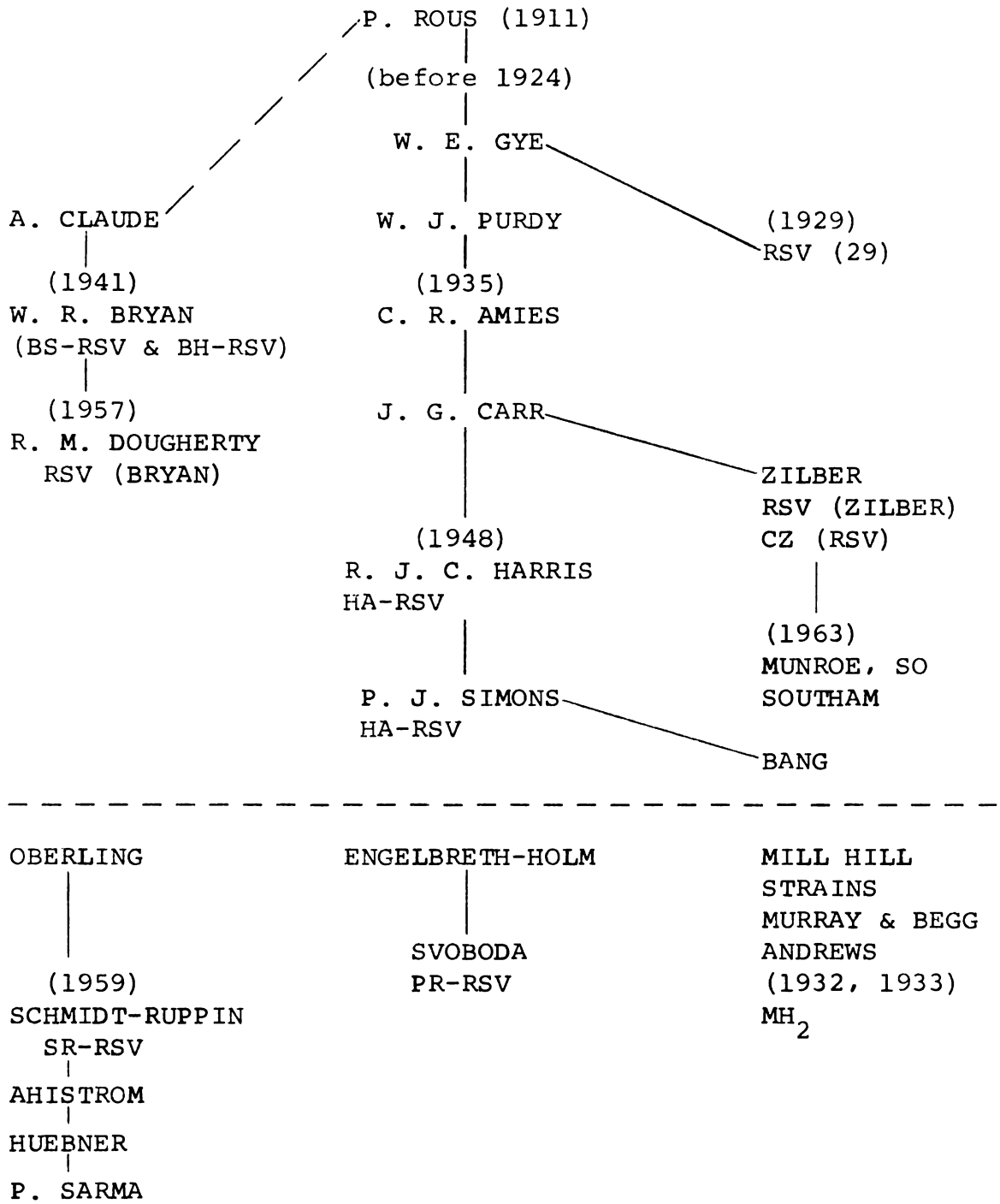
LITERATURE REVIEW

Rous in 1911 described a sarcoma in the subcutaneous tissue of the breast of an adult hen as clusters of spindle-shaped fibroblasts, with vacuoles at the periphery. The tumor was first transmitted with cellular suspensions and later with cell-free filtrates. Cell division was most frequently amitotic, but mitosis did occur (56).

The origin and history of Rous sarcoma virus (RSV) is presented in Figure 1. The term strain refers to the origin and passage history of the viruses. Many strains can be antigenically differentiated (67, 45, 66). Recent work indicates that some of these strains contain two or more antigenically different viruses (78). Some strains are infective for mammals (1). There are also differences in the morphological type of transformation induced by these viruses in cell cultures (54, 74).

The amount of infectious virus recoverable from Rous sarcomas is highly variable and at times no viruses

Figure 1.--Origin of Rous Sarcoma Virus Strains



Adapted from Simons P. J. and Dougherty R. M. (1963).

can be recovered even from highly malignant tumors (57, 64). The absence of virus in sarcomas is related to the dose of virus, the age of the tumor, and the age of the host (15, 18, 26, 50). Recent experiments have confirmed the dual origin of non-infective Rous sarcomas; (1) a low initiating dose results in the formation of antibodies, (2) in the case of the high initiating dose of RSV, the immunologically competent cells within the tumor suppress viral synthesis in the sarcoma cells (64). There is no correlation between neutralizing antibody and recovery of virus from a tumor (50).

Defectiveness of the Rous Virus

The Bryan high titer Rous sarcoma virus (BH-RSV) contains a Rous associated virus (RAV) which is several times the concentration of RSV and can induce a cellular resistance to the neoplastic transformation of RSV. The RAV is closely related antigenically to RSV and produces erythroblastosis in chickens when inoculated intravenously in embryos (63).

Single foci of transformed cells picked from RSV infected cell cultures containing anti-RAV sera, multiplied indefinitely without morphological differences and failed to produce either RSV or RAV. When RAV was added to such cells, they quickly produced large amounts of both RSV and RAV. It was concluded that this strain was a defective virus which could produce mature virus only in the presence of a helper virus such as RAV (35).

The failure of the replicating RSV genome to mature into infectious virus suggests that the RSV is defective and is not capable of stimulating the cells to synthesize the specific portion of the outer coat of the virus. Transformed cells which do not produce measurable virus are designated non-producer (NP) cells. The NP cells, when implanted in chicks, do not produce detectable neutralizing antibodies. The failure of chickens with NP tumors to resist RSV infection reinforces the conclusion of the absence of an outer coat of the virus (35).

Viruses of the leukosis group such as RAV, avian myeloblastosis, and Rubin's isolate designated Resistance Inducing Factor (RIF), can serve as helpers for activation of NP cells (35). Viruses which are structurally similar

but biologically distinct such as Newcastle Disease Virus (NDV) are ineffective as helpers (37).

There are numerous evidences of a serological relationship between RSV and viruses of the avian leukosis group (43, 11, 30, 27, 35, 63). The leukosis viruses cause a proliferation of blood-forming cells resulting in visceral lymphomatosis, erythroblastosis, myeloblastosis, and osteopetrosis. Neutralizing antibodies formed against myeloblastosis virus also neutralize erythroblastosis and visceral lymphomatosis viruses. These viruses are related to RSV virus because their antisera neutralize RSV. The RSV antiserum neutralizes visceral-lymphomatosis and myeloblastosis virus but not erythroblastosis virus (11). The RAV isolated by Rubin (63) is non-cytopathic microscopically but does produce leukosis in chickens. The RAV is indistinguishable from RSV in thermal stability, growth rate, site of cellular maturation and immunological specificity.

The RSV bears the antigenic imprint of the particular helper virus associated with it. When two antigenically distinguishable leukosis viruses, such as RIF (36) and RAV, are used for activation of NP cells, the resulting viruses are designated RSV(RIF) and RSV(RAV);

the RIF and RAV indicating the helper protein coat. When anti-RAV serum is mixed with RIF, all the neutralizing antibody against RSV(RIF) but not against RSV(RAV) is absorbed. When RSV(RAV) is mixed with anti-RAV serum, neutralizing antibody against both viruses is absorbed (36).

A second helper virus, RAV-2, has recently been isolated from BH-RSV (37). It is antigenically unrelated to RAV-1 although both are found in the same virus preparation. The RAV-2 does not grow in some embryos in which RAV-1 multiplies. The original studies were conducted with cell cultures prepared from embryos from Kimber Farms, Niles, California. The cells resistant to RAV-2 were designated K/2 cells. All the cell cultures from these embryos were sensitive to RAV-1. A RSV obtained by activating an NP with RAV-2 is insusceptible to interference by RAV-1. These experiments lead to the conclusion that the helper virus is responsible for two important characteristics of RSV: (1) the host range and (2) susceptibility to viral interference. These are properties conferred by the virus coat (37).

It is probable that all chickens reared under usual conditions become infected with avian leukosis viruses, and

when they are used as host for propagation of RSV strains many antigencially different progeny may result. This probably is the main reason for the evolution of antigenically distinct strains of RSV.

Properties of the RSV

According to electron microscopy particles 67-80 mu. in diameter are present in cytoplasmic vacuoles in tumor cells but not in normal cells (19). In cross section, the particles are round, contain a dense nucleoid about 34-40mu. and are surrounded by a thin, limiting membrane (31). The particles are released by a budding process at the cell membrane (40).

Filtration of RSV indicates it to be from 75-100mu. in diameter (29, 30). The specific gravity is 1.16-1.19 in rubidium chloride (20) and the sedimentation constant in sucrose is from 600-655S which indicates a molecular weight of about 10^7 (42).

The half-life of the Bryan standard Rous virus (BS-RSV) in 0.01M phosphate buffered saline containing

1% horse serum is 4 hours at 37°C. However, the half-life of the virus at 37°C varies from two to six hours depending on the strain, source of tumor, and the diluent (13, 51, 55). At -50 to -76°C. in potassium citrate, RSV remains infective for one to two years (14). The RSV can survive many years when dried by sublimation (25) and it is ten times more resistant to inactivation by ultraviolet light than NDV and animal virus of similar size and composition (58). The RSV is ether sensitive (30) and contains Ribonucleic acid (RNA) as determined by fluorescent microscopy, enzymatic digestion (48) and paper chromatography (9). Between 24-60% of the virus is lipid and 0.62-1.84% is RNA.

In turkeys, tolerance to RSV can be produced by inoculating turkey embryos or one-day-old poults intravenously with whole blood from the chicken in which the tumor was propagated (69). However, blood from different strains of chicken, pigeons, guinea pigs, sheep, and human group A (Rh+) also confer tolerance, thus indicating that the RSV tumor and its causative agent have Forssmann antigens in common (38, 39). This particular relationship is questionable. The ability of fresh anti-chicken embryo cell rabbit

antiserum to suppress neoplastic properties of RSV on susceptible cells is due to the anti-cell antibody which damages the cell and suppresses cell division so that tumors cannot form. About 40% of this cell division inhibition is due to the Forssmann type antibody as indicated by removal of that amount of activity by adsorption of the anti-cell serum with sheep red blood cells. However, the virus itself is not neutralized. All the apparent RSV antibody of the anti-cell sera can be removed by adsorption with chicken embryo cells (12, 61).

The Schmidt-Ruppin strain of RSV (SR-RSV) induces in hamsters a specific complement-fixing antibody which is reactive with the homologous virus and with the soluble antigens of the leukosis viruses (41). This seems to be a group specific antigen common to all the members of the avain sarcoma leukosis group (2, 53).

In Vitro Aspects of RSV Growth

Infection of chicken embryo cell cultures by RSV results in the production of discrete foci of neoplastic

cells, which provides a simple method for investigations using RSV and Rous sarcoma cells (46). During the replication cycle of the virus, there is an eclipse period of about two days. Although viral antigen can be detected by fluorescent antibody microscopy in 24 hours, virus cannot be detected by electron microscopy until the second day after infection of the cell. The number of fluorescent particles increases rapidly and by the fourth day they are concentrated in patches along the cell membrance (40, 75, 76). The number of sarcoma cells within a focus doubles every 15-20 hours. All cells release virus when there is a confluent layer of tumors. There is 40-70% more RNA in infected cells than in noninfected cells. Several morphological types of foci are produced by different strains of RSV (54). One strain produces cytopathic effects in rat, guinea pig, and mouse cell cultures (10).

Resistance of cell cultures from normal chicken embryos to infection with RSV is reported to be due to RIF. The RIF infected cell causes a reduction of the number of infected RSV viral receptor sites (60, 62, 70). Interferon can also account for an apparent interference

with RSV foci, but to be effective it must be available to the cells during the early stages of the cell virus interaction (7).

Recently it was reported that the group specific antigen of the sarcoma-leukosis viruses is synthesized in the nucleus, moves to the cytoplasm, and then can be detected on the cell surface (53).

Importance of the Genetic Character of the Host

The heritability of resistance to RSV in fowls has been demonstrated by the mating of an RSV resistant male to several close-relative females. Progeny showing resistance to RSV tumor growth were selected for mating and resistant offspring were consistently produced (33). Genetic resistance to RSV cultivation on the chorio-allantoic membrane (CAM) is controlled by a single pair of autosomal genes and susceptibility to the RSV is dominant (49). Intra-cranial inoculation of day old chicks confirmed the dominance of susceptibility and the control by a single pair of autosomal genes (79, 80).

Cell cultures from embryos of genetically resistant chickens resist transformation by RSV. This resistance is controlled by a single autosomal recessive gene pair (22, 21).

The susceptibility or resistance of an antigenically related avian leukosis virus designated RPL-12 is influenced by the same locus as that controlling BS-RSV resistance or susceptibility (17, 22, 11, 27). The RPL-12 virus causes no cytopathic changes in cell culture but does interfere with the transformation of the BS-RSV (60). An allel of the BS-RSV gene or an altogether different gene was suggested in recent work where a RSV(RAV-2) was used to challenge cells. There was no apparent effect of the gene controlling BS-RSV on the RSV(RAV-2). The results also indicated that susceptibility to RSV(RAV-2) was dominant. The expression of the gene as a component, or lack of a component, on the cell surface determines whether or not adsorption or penetration takes place (65).

Two subgroups of the avian tumor viruses are distinguished on the basis of their host range. The first, referred to as subgroup A, consists of RAV-1 and viruses having similar antigenic envelopes. The second group is designated subgroup B and is represented by RAV-2

and its immunological relatives. These A and B subgroup viruses react with different cellular receptors during the initiation of infection. Selective resistance of chicken embryo cultures to one subgroup is probably correlated with the absence of a corresponding cellular receptor site. Helper viruses of each subgroup will induce resistance only to the RSV strain which are within its group (78).

MATERIALS AND METHODS

Bryan Standard Rous Sarcoma Virus

The Bryan standard RSV (BS-RSV) was supplied by Dr. Ray Bryan, National Cancer Institute, and designated by him as C.T.-750. The BS-RSV used in cell culture was a 20% tumor suspension, twice clarified by centrifugation at 2,000g for 60 minutes at 4°C, and filtered through a 0.02 Selas candle. The virus was propagated by one wing web passage and two passages in the breast muscle of line 15I chickens.

Harris Rous Virus

This strain was obtained from Dr. F. Bang of Johns Hopkins University. The preparation was a 10% extract of 15I CAM pocks, twice clarified by centrifugation at 2,000g for 60 minutes at 4°C, and filtered through a 0.02 Selas candle.

Schmidt-Ruppin Rous Virus

This strain was obtained from Dr. Padman Sarma at the National Institutes of Health. The preparation was a 10% extract of line 7 CAM pocks. The extract was twice clarified by centrifugation at 2,000g for 60 minutes at 4°C and filtered through a 0.02 Selas candle.

Chicken Embryos

Since 1939, close inbred lines of Single Comb White Leghorn chickens have been separately maintained at the U.S.D.A. Regional Poultry Laboratory, East Lansing, Michigan (81). Embryos used were from chickens of the second back cross of line 6 by line 7. This means that the F_1 (6X7) was mated back to line 7; then the resulting progeny were mated to line 7 again. On the basis of intra-cranial inoculation of the second back cross (BX-2) one day old chicks, a random sample of progeny was available which had an equal probability of being either resistant or susceptible

to BS-RSV. The line 6 and line 7 progeny were also used. Line 6 chickens are susceptible to and line 7 are resistant to BS-RSV (22).

Preparation of Cell Cultures

Cell cultures were prepared from 9 day old embryos by a modification of the procedure described by Rubin (60). Decapitated embryos were dropped into 25 X 150 mm. test tubes containing approximately 20, 3/16 diameter perforated glass beads and 5ml of phosphate buffered saline (PBS). The embryos were fragmented when the tube was inserted in revolving rubber cup of a Vortex mixer. The fragments were washed with 20ml of PBS, and after the cells settled by gravity, the supernatant fluid was decanted. This procedure was then repeated. A 0.25% solution of trypsin (Nutritional Biochemical Company) was diluted 1/5 with PBS and 20ml was added to each tube.

The tubes were placed in a 37^{o}C water bath and shaken by hand every ten minutes. After one hour, the supernatant fluid was carefully removed by a pipette and

placed in similar tubes, and centrifuged at 2,000g for five minutes. The supernatant fluid was then poured off. The pellet was resuspended in growth medium to contain 1×10^6 cells per ml. Ten ml. of the cell suspension was added to 100mm diameter tissue culture petri plates (Falcon Plastics), and incubated at $37^{\circ}C$ in an atmosphere of 5% CO_2.

Growth Medium	ml.
10 X 100 (Microbiological Associates)	100
Tryptose Phosphate Broth (Difco)	100
Calf Serum (Colorado Serum Co.)	40
Sodium Bicarbonate 2.8% solution	20
Penicillin and Streptomycin (10,000 units/ml.)	10
Water (deionized and sterilized)	750
Phosphate Buffered Saline:	
NaCl	8.0gm
KCl	0.2gm
Na_2HPO_4	1.2gm
KH_2PO_4	0.2gm
H_2O	1 liter

After four days, the cells were treated with 5 ml. of a 0.05% trypsin solution for ten minutes at 37°C and then centrifuged and resuspended in growth medium to contain 2 X 10^5 cells per ml. Five ml. of the cell suspension was added to 60mm diameter petrie plates.

Each of the three viruses was appropriately diluted and 0.1 ml. inoculum containing 10^3 focus forming units was added to 2 plates of each individual embryo cell culture. After 24 hours, when the cells had formed a monolayer, the supernatant fluid was poured off and the cells were overlayed with 5 ml. of agar medium.

Agar Medium	ml.
2 X 199	50
Tryptose Phosphate Broth	14
1.8% Agar	50
Calf Serum	6
Sodium Bicarbonate (2.8% solution)	2.6
Stock Antibiotics	1.0

Three days later, 3 ml. of growth medium was added to enhance visual recognition of the foci. On the 4th or 5th day after infection, the number of foci on 1/10 of

the area of a plate was counted. If no foci were observed the entire plate was examined.

The transformed areas are made of round refractile cells in grape-like clusters while the normal cells are flat and diamond shaped. Idealy the foci are discrete and easily seen against the normal cell background (Fig.2).

Quantitative Methods

The criterion for resistance was the absense of foci on the cell monolayer in response to a standard challenge virus dose which would normally produce 1,000 foci. This is a relatively rigid criterion and has been previously used with the BS-RSV (22,21).

To test the significance of the data, an X^2 test, using the 2 X 2 table method, was calculated according to:

$$X^2 = \frac{n(ad-bc)^2}{k} \qquad k=(a+b)\ (c+d)\ (c+d)\ (b+d)$$

n = total number of observations.

		X Virus: Sensitive	X Virus: Resistant	
Y Virus	Sensitive	a	b	a+b
	Resistant	c	d	c+d
		a+c	b+d	n

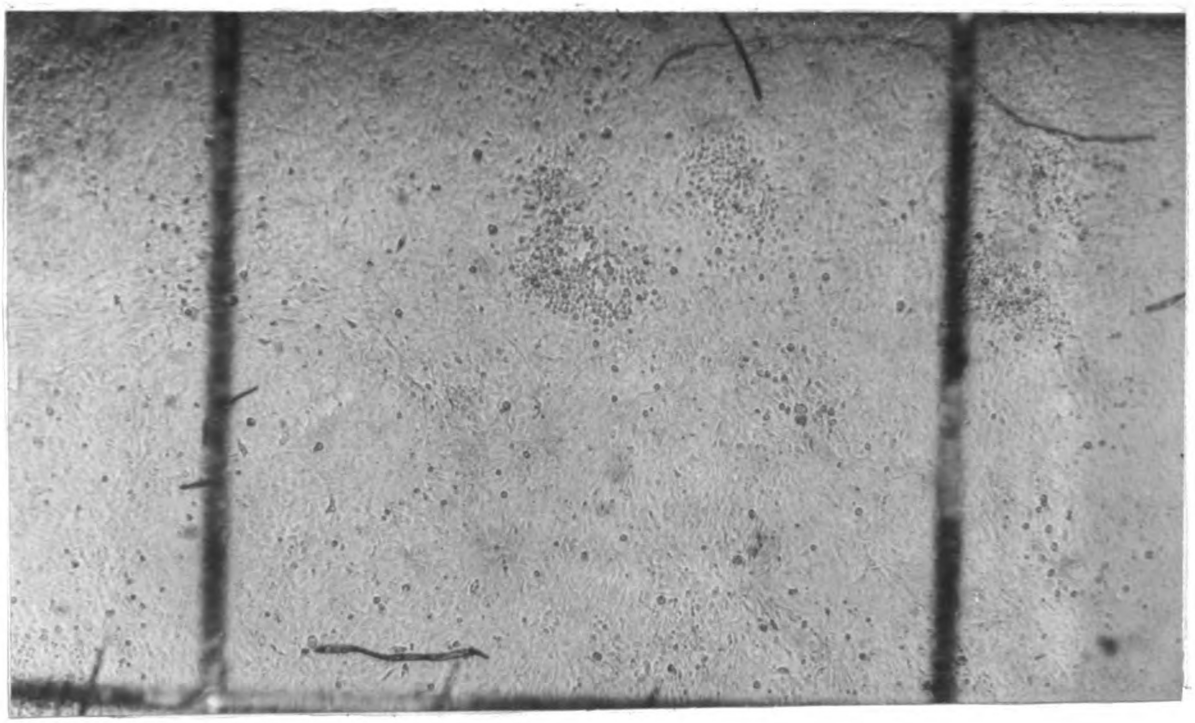


Figure 2.--Neoplastic foci in background of normal chicken embryo fibroblasts.

Resistance Inducing Factor

Supernatant fluids were collected from 12 day old primary cultures of individual embryos which were resistant to BS-RSV or HA-RSV or to both viruses. Two ml of these fluids were inoculated on line 6 cells and after 6 days the cells were trypsinized, replated, and challenged with BS-RSV to determine presence of RIF.

Absence of RSV in Resistant Challenged Cells

Supernatant fluids plus cell free extracts of resistant challenged cells were clarified by 2,000g for 60 minutes and 2 ml were inoculated on line 6 cell cultures to test for foci producing ability.

RESULTS

The response of secondary embryo cells of 10 different pedigreed matings to challenge by BS-RSV and HA-RSV places each BX embryo into one of four categories; (1) Resistant to HA-RSV and sensitive to BS-RSV, (2) Sensitive to both viruses, (3) Resistant to both viruses and, (4) Sensitive to HA-RSV and resistant to BS-RSV (Tables 1-3). One dam (111) supplied 23 embryos which provides a model for patterns of resistance.

The parent lines 6 and 7 produce progeny which respond differently to the virus challenges. Individual embryo cell cultures from line 7 were either in category 3 or 4 (Table 4 and 5). These data support the hypothesis that line 7 is homozygous resistant to BS-RSV and indicates that this line also is segregating genes for resistance to HA-RSV. All embryos from line 6 (Table 6) were in category 2 suggesting that this line is homozygous susceptible to both viruses.

Supernatant fluid from twelve day old primary cultures of cells, which were resistant to one of the viruses or to both of them, were inoculated on sensitive line 6 cultures. The RIF was apparently not responsible for the resistance because there was no difference in the virus titer of the control and that of the test challenge (Table 7).

Supernatant fluids and the cell free extracts of resistant cells were tested on line 6 for foci producing ability but no foci were observed (Table 8).

The response of secondary cells to SR-RSV can be defined in terms of their patterns of sensitivity or resistant to the BS-RSV (Table 9). When all the embryos of the BX are compared, it is apparent that none was sensitive to BS-RSV and resistant to SR-RSV.

The response of secondary cells of SR-RSV can also be defined in terms of the patterns of sensitivity or resistance to HA-RSV (Table 10). Of the embryos sensitive to HA-RSV, 97% were also sensitive to SR-RSV.

When the response of secondary cells to challenge with BS-RSV is defined in terms of response to HA-RSV, there is a great difference in host range (Table 11).

The X^2 test supports the relationship of SR-RSV to both BS-RSV and HA-RSV but an independence of the BS-RSV and the HA-RSV. The response of the BX embryo cells to different combinations of the three viruses can statistically be rated as follows.

VIRUS PAIR	X^2	SIGNIFICANCE
BS-RSV, HA-RSV	0.36	Independence of infective ability on same cells.
BS-RSV, SR-RSV	28.6	Dependence of infective ability on same cells.
HA-RSV, SR-RSV	14.25	Dependence of infective ability on same cells.

TABLE 1.--Challenge responses of embryos from several individual pedigreed backcross dams[a]

DAM NUMBER	NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
144	72	134	141
144	54	130	103
144	0	0	0
144	0	...	0
144	0	...	0
161	0	0	41
161	0	0	0
161	78	0	0
161	..	0	0
163	0	81	136
163	168	60	169
163	0	0	0
163	0	0	0
163	60	0	0
163	73	0	116
126	14	300	291
126	0	0	0
126	0	0	214
126	28	0	170
126	...	100	220
132	0	335	293
132	82	310	294
132	204	316	281
132	0	0	0
132	0	0	0
132	136	0	268
132	...	11	17
132	...	0	58
132	...	0	19
132	...	31	94
117	0	263	252
117	14	60	154
117	264	0	271
117	227	0	274
117	24	0	50

[a]All foci counts represent 1/10 of the total plate area, a zero represents a total plate area determination.

TABLE 2.--Challenge responses of embryos from several individual pedigreed backcross dams[a]

DAM NUMBER	NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
137	0	82	251
137	16	300	132
137	0	0	269
137	0	0	0
137	0	0	0
137	14	0	200
137	16	0	216
137	109	0	0
137	97	0	281
137	...	195	267
137	...	0	221
113	0	141	223
113	14	208	284
113	13	142	165
113	0	0	101
113	0	0	86
113	0	0	0
116	0	217	257
116	22	300	203
116	202	331	362
116	15	208	202
116	62	0	240
116	52	0	285
116	14	0	307
123	0	314	320
123	0	152	137
123	0	151	175
123	22	54	59
123	0	0	0
123	0	0	0
123	14	0	268
123	18	0	242
123	217	0	269
123	33	0	34
123	...	0	0

[a]All foci counts represent 1/10 of the total plate area, a zero represents a total plate area determination.

TABLE 3.--Challenge responses of embryos from a single backcross dam (111)[a]

NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
0	91	44
0	278	264
0	276	248
0	304	321
0	236	...
0	101	108
0	160	131
0	103	134
0	105	113
0	71	9
13	300	137
18	112	152
15	121	147
67	53	163
0	0	0
0	0	211
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
18	...	256
..	133	63
..	0	0
..	0	0
..	0	110
0	...	35

[a]All foci counts represent 1/10 of the total plate area, a zero represents total plate area determination.

TABLE 4.--Challenge responses of embryos from a single pen (pen 18) of Line 7 dams

NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
15	0	116
14	0	121
76	0	217
208	0	145
0	0	0

[a]All foci counts represent 1/10 of the total plate area, a zero represents a total plate area determination.

TABLE.5-Challenge responses of embryos from several pedigreed line 7 dams[a]

DAM NUMBER	NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
315	0	0	0
315	0	0	0
315	0	0	0
315	14	0	29
315	16	0	30
312	0	0	0
312	22	0	51
312	18	0	14
344	0	0	0
344	0	0	0
345	0	0	0
345	0	0	0
347	0	0	0
347	0	0	0
347	0	0	0
347	0	0	0
347	0	0	0
347	0	0	0
347	0	0	0

[a]All foci counts represent 1/10 of the total plate area, a zero represents a total plate area determination.

TABLE 6.--Challenge responses of embryos from several pedigreed dams and a single pen of line 6 dams[a]

DAM NUMBER	NUMBER of HA-RSV FOCI	NUMBER of BS-RSV FOCI	NUMBER of SR-RSV FOCI
236	44	170	243
251	37	261	248
231	61	144	170
231	40	251	76
231	38	73	72
251	72	202	212
251	68	314	314
251	59	219	192
236	47	137	87
236	29	311	310
PEN NUMBER			
32	22	300	298
32	224	312	346
32	115	321	357
32	166	290	291
32	270	281	248
32	215	308	336
32	117	316	341
32	237	287	263
32	213	314	321
32	167	306	287
32	89	291	264
32	216	314	280
32	188	287	310
32	260	311	318
32	234	267	296
32	163	258	318
32	80	309	328
32	129	310	266
32	212	277	259
32	136	294	302

[a]All foci counts represent 1/10 of the total plate area, a zero represents a total plate area determination.

TABLE 7.--Test for RIF activity of twelve day old supernatants from cells resistant to BS-RSV or HA-RSV[a]

SUPERNATANT from EMBRYO CELLS	RESISTANT TO	NUMBER of FOCI on BS-RSV CHALLENGE
18	BS-RSV & HA-RSV	106
18	BS-RSV & HA-RSV	99
123	BS-RSV	89
137	BS-RSV	70
123	HA-RSV	65
111	HA-RSV	117
116	NONE	92

[a]Six days were allowed for RIF induction on sensitive cells after inoculation of 2 ml per assay plate.

TABLE 8.--Test of supernatant and cell-free extract for the presence of virus in the challenged cells which were resistant to transformation.[a]

SOURCE of CELLS and SUPERNATANT	NUMBER of BS-RSV or HA-RSV FOCI
18	0
18	0
123	0
137	0
123	0
111	0

[a]Six days were allowed for the development of foci after inoculation of 2 ml per assay plate.

TABLE 9.--Frequency of backcross embryos falling in different categories as a result of BS-RSV and SR-RSV challenge

		BS-RSV	
		Sensitive	Resistant
SR-RSV	Sensitive	41	26
	Resistant	0	26

TABLE 10.--Frequency of backcross embryos falling into different categories as a result of reponse to HA-RSV and SR-RSV challenge

		HA-RSV	
		Sensitive	Resistant
SR-RSV	Sensitive	35	25
	Resistant	3	22

TABLE 11.--Frequency of backcross embryos falling into different categories as a result of response to BS-RSV and HA-RSV challenge

		BS-RSV	
		Sensitive	Resistant
HA-RSV	Sensitive	17	17
	Resistant	19	25

DISCUSSION

The results indicate that separate genes are responsible for the sensitivity or resistance of cells to BS-RSV and HA-RSV. Homozygous resistance of line 7 chicken embryo cells to BS-RSV and also the significant degree of resistance to HA-RSV is in contrast to line 6 chicken embryo cells which were homozygous susceptible to both viruses.

Quantitative differences in the number of foci in a given cell culture were within normal variation limits. The HA-RSV was responsible for the greatest variation in the number of foci formed. Similar results have been reported by other investigators (78). Dougherty et al. (25) indicated that his HA-RSV strain, in cell culture, produced some foci which were very diffuse and indistinct thus making the accurate counting of foci difficult. The latter situation may have been the reason for the variable counts encountered with this HA-RSV virus.

Many viruses have the property of inducing the formation of an antiviral substance *in vivo* and also *in vitro*. The RSV and leukosis viruses apparently have this ability (7, 71). If interferon were responsible for the resistance tested in the present study, there would have been no selective resistance to one virus and not the others. Evidence for the genetic nature of the resistance is manifested by the fact that the resistance to BS-RSV of progeny of line 7 females can be changed to sensitivity by mating with a sensitive male (21). The line 7 embryos, which are at times resistant to both HA-RSV and BS-RSV, are free of subgroup A or B viruses (78).

Extracts from representative resistant, challenged cells, failed to produce any foci when inoculated on sensitive cells, thus indicating that the virus did not multiply in these cells. Evidence has previously been presented which suggests that resistance of cell cultures to BS-RSV extends to viral synthesis as well (52).

The host range of the BS-RSV and the HA-RSV places them in different subgroups and X^2 analysis supports this interpretation. This means that the genetic control of infection is different for each of these viruses. When

SR-RSV and BS-RSV are compared, their host range is essentially the same but it is also true that the host range of SR-RSV and HA-RSV are fundamentally the same. The X^2 analysis suggests that BS-RSV and HA-RSV are related to SR-RSV. One possible explaination is that the SR-RSV used is a mixture of two or more viruses, one being similar to the HA-RSV while the other acts similar to the BS-RSV. The possibility of a mixture of viruses in the SR-RSV strain is supported by the recent isolation of two SR RSV strains on the basis of host range. One was designated SR-RSV-1 and belonged to the A subgroup like BS-RSV; the other was designated SR-RSV-2 and belonged to the B subgroup like HA-RSV. The A and B subgroups are characterized by two other criteria, (1) their antigenic character, because there is cross neutralization within the group and (2) the interference pattern i.e. foci inhibition, occurs only when the helper and the RSV belong to the same group.

Four cell phenotypes have been identified on the basis of host range (79).

C/O = cells resistant to neither A nor B subgroup

C/A = cells resistant to A subgroup viruses

C/B = cells resistant to B subgroup viruses

C/AB = cells resistant to both subgroup viruses

Embryo cell responses to challenge may be used to tentatively genotype as well as phenotype lines 6 and 7. The following is a theoretical model where small a represents the gene controlling the A subgroup and small b represents the gene controlling B subgroup. The superscripts s and r represent susceptibility and resistance respectively.

LINE	GENOTYPE	PHENOTYPE	POSSIBLE GAMETES
6	$\frac{a^s b^s}{a^s b^s}$	C/O	$a^s b^s$
7	$\frac{a^r b^s}{a^r b^s}$	C/A	$a^r b^s$
	$\frac{a^r b^s}{a^r b^r}$	C/A	$a^r b^s$, $a^r b^r$
	$\frac{a^r b^r}{a^r b^r}$	C/AB	$a^r b^r$

The cell culture data from the total backcrosses (Table 11), when presented in the following manner, reveal a new cell phenotype unlike either of the original parent produces.

BS-RSV	HA-RSV	OBSERVATIONS	PHENOTYPE
Sensitive	Sensitive	17	C/O
Sensitive	Resistant	19	C/B
Resistant	Resistant	17	C/A
Resistant	Resistant	25	C/AB

Recombination apparently has taken place in the 6 X 7 genetic material to lead to the formation of a C/B cell phenotype.

The following is a representative recombination in the 6 X 7 genetic material;

	Line 6	Line 7
Possible gametes	$a^{s}b^{s}$	$a^{r}b^{r}$, $a^{r}b^{s}$
Possible 6 X 7 individuals (F_1)	$\frac{a^{s}b^{s}}{a^{r}b^{r}}$	$\frac{a^{s}b^{s}}{a^{r}b^{s}}$
Possible (F_1) gametes	$a^{s}b^{s}$, $a^{s}b^{r}$, $a^{r}b^{r}$, $a^{r}b^{s}$	
(F_1) x line 7 could lead to a C/B cell type; (first backcross)		$\frac{a^{s}b^{r}}{a^{r}b^{r}}$

When the first backcross (BX-1) is mated to line 7 the progeny are referred to as the second backcross (BX-2). Gametes of the first and second backcross are the same except that there may be some C/B chickens in the matings of the second backcross. This would increase the number of C/B cells of the BX-2 over the BX-1.

The fact that crossover occured indicates that there are two separate loci involved, one controlling presence or absence of A subgroup attachment sites and one controlling the presence or absence of B subgroup sites. The crossover further suggests that these loci are not closely linked.

The cell types which were expressed here are in agreement with those described recently. (78) However, the pedigreed matings used in this study enabled us to show how these particular cells came about.

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