Replication of the Reticuloendotheliosis Virus (Strain T) in Chicken Embryo Cell Culture

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Investigations were conducted on the *in vitro* replication of the reticuloendotheliosis (RE) virus (strain T) in specific-pathogen-free chicken embryo fibroblast (CEF) cultures. Active virus production was detected in the tissue culture fluid 24 hr after infection. When injected into chickens, samples taken 42 hr after infection of the cell cultures killed approximately 50% of the birds at a 1:100 dilution. The RE virus titer remained at this level for 5 days before declining. Cell-free virus preparations from tissue cultures rarely resulted in 100% mortality of the assay birds. The level of cell-associated virus was very low. Evidence that the reticuloendotheliosis was not induced by a mycoplasma was indicated by failure to isolate an organism on PPLO Agar (Difco) and failure of kanamycin or amphotericin B to inhibit multiplication of RE virus *in vitro*. RE virus appeared to be unrelated to members of the avian leukosis and sarcoma complex. It did not induce resistance in CEF cultures to sarcoma viruses of the A or B subgroup of this complex. Similarly, preinfection of cell cultures with leukosis viruses of the A or B subgroup did not inhibit or reduce the replication of RE virus.

The reticuloendotheliosis (RE) virus (strain T) was isolated by Twiehaus and Robinson in 1958 from an adult turkey with leukosis-like lesions (*unpublished*). The virus was subsequently adapted to young chickens (7) and later to Japanese quail (9). This virus differs significantly from other avian leukosis viruses in its decreased length of incubation period, high mortality pattern, and ability to infect several different genetic lines of chickens.

Although the virus replicates in chicken embryo fibroblast (CEF) cultures, no evidence of a cytopathic alteration was observed (8). The virus grown in quail embryo fibroblasts did not interfere with the Bryan strain (9) of Rous sarcoma virus (RSV). Complement fixation tests for avian leukosis viruses were negative when tissue culture cells served as the source of antigen (10). The results were variable when diseased tissue was employed. Serum neutralization studies indicated that RE virus was not related to several common leukosis viruses (10). However, antisera prepared against the Bryan strain of RSV neutralized RE virus. Electron microscopic observations revealed that the virus shared morphological characteristics with both avian leukosis and murine leukemia viruses (12).

This report describes the *in vitro* replication of RE virus in CEF cultures. The relationship of the RE virus to members of the avian tumor

virus group was investigated by using interference tests against viruses of the A and B subgroups of this complex.

MATERIALS AND METHODS

Viruses. The original preparation of RE virus (strain T) was obtained from E. K. Russell of the National Cancer Institute through the courtesy of G. Theilen of the University of California at Davis. This preparation consisted of a pool of chicken liver and spleen tissue from an infected bird. The material was subsequently passed in specific-pathogen-free strain 813, white Leghorn chickens (Kimber Farms, Niles, Calif.) and the plasma was collected when the birds became moribund. The RAV-1, RAV-2, RSV (RAV-1), and RSV (RAV-2) preparations were kindly supplied by Peter Vogt, University of Colorado Medical Center, Denver.

Bioassay of infectivity. For assay, 1- to 3-day-old chickens were inoculated with 10-fold dilutions of tissue culture fluid. Each bird received 0.25 ml intra-abdominally. The birds used for assay were white Leghorn cockerels strain 60F, 60X, and 55X obtained from the Indiana Farm Bureau Cooperative, Indianapolis. These strains had been found equally sensitive to cell-free preparations of RE virus. Uninoculated birds were caged with the inoculated birds for the duration of the experiment. Mortality occurring 7 days after inoculation was considered specific, based on postmortem examination.

Infectious cycle tissue culture studies. Chicken embryo fibroblast cultures were prepared from 11-day-old embryonated, strain 813, white Leghorn chicken

TABLE 1. *Replication of reticuloendotheliosis virus (strain T) in chicken embryo fibroblasts*

Extracellular virus				
Time after infection	Virus dilution	No. dead ^a	Mortality	Average time to death
	log	total	%	days
Inoculum ^b	0	6/10	60	18
	-1	2/10	20	25
	-2	0/10	0	—
18 hr	0	0/10	0	—
24 hr	0	4/10	40	42
	-1	0/10	0	—
	-2	0/10	0	—
30 hr	0	5/10	50	34
	-1	1/10	10	48
	-2	0/10	0	—
36 hr	0	2/10	20	25
	-1	2/10	20	27
	-2	1/10	10	43
42 hr	0	6/10	60	18
	-1	4/10	40	25
	-2	4/10	40	40
5 days	-3	0/10	0	—
	-1	6/10	60	23
	-2	5/10	50	47
6 days	-3	0/10	0	—
	-4	0/10	0	—
	-1	4/10	40	27
7 days	-2	1/10	10	32
	-3	0/10	0	—
	-4	0/10	0	—
Control ^c	-1	1/10	10	35
	-2	0/10	0	—
	-3	0/10	0	—
	-4	0/10	0	—
	—	0/20	0	—

^a Virus was assayed by injection of 0.25 ml of tissue culture supernatant fluid intra-abdominally into 60F white Leghorn cockerels. The experiment was terminated 4 weeks after the last specific death.

^b First passage infectious tissue culture fluid (0.5 ml/plate).

^c Uninoculated birds were caged with the test birds as contact controls for the duration of the experiment.

eggs. The cells were grown in Difco 199 medium with 8% heat-inactivated newborn calf serum (Hyland) and 11% Tryptose Phosphate Broth. The cultures were incubated at 38 C in a CO₂ incubator (2% CO₂).

Secondary cultures containing 2.6×10^6 cells were plated in 60-mm plastic petri dishes. The virus was added to the cells in suspension. The virus inoculum (0.5 ml per plate) was undiluted second-passage tissue culture fluid or plasma collected from a moribund bird. The cells were incubated overnight and samples

were collected for 3 days at 6-hr intervals, beginning at 18 hr after infection. Samples were then collected daily for 7 days. The culture fluid was rendered cell-free by centrifugation at 2,000 rev/min for 20 min at 4 C. The cells were harvested with trypsin, were counted, and suspended to give a final concentration of 2.0×10^6 cells per sample. The material was stored at -70 C prior to titration in chickens. To determine the level of cell-associated virus, the cells were ruptured by three freeze-thaw cycles, and the supernatant fluid was titrated.

Interference studies. Procedures for the interference test were essentially those of Rubin (6) and Vogt and Ishizaki (10). Interference tests were conducted with viruses of the A and B subgroups of the avian tumor virus group. After four cell transfers, RE virus infected and control cultures were challenged with RSV(RAV-1) or RSV(RAV-2). The relative plating efficiency on control and RE virus infected cells was determined. RSV assay techniques were essentially those described by Rubin (5).

In the reciprocal experiment, cultures of CEF were infected with RAV-1 or RAV-2. These cultures, together with uninoculated control cells, were transferred on the third and sixth day after infection. After the final transfer, the cultures were challenged with RSV(RAV-1), RSV(RAV-2), or RE virus. The relative plating efficiency of the indicator viruses on cells preinfected with the different leukosis viruses was determined. The cultures superinfected with RE virus were allowed to incubate 3 days, and samples of the culture fluid were collected and titrated.

TABLE 2. *Replication of reticuloendotheliosis virus (strain T) in chicken embryo fibroblasts*

Cell-associated virus ^a				
Time after infection	Virus dilution	No. dead ^b	Mortality	Average time to death
hr	log	total	%	days
36	0	0/10	0	—
	-1	0/10	0	—
	-2	0/10	0	—
42	0	4/8	50	34
	-1	0/10	0	—
	-2	0/10	0	—
48	0	3/8	37	37
	-1	0/10	0	—
	-2	0/10	0	—
60	0	5/8	62	37
	-1	0/10	0	—
	-2	0/10	0	—
5 days	0	4/10	40	36
	-1	0/10	0	—
	-2	0/10	0	—

^a Cells (2.0×10^6 /sample) were ruptured by three freeze-thaw cycles.

^b Virus was assayed by injection of 0.25 ml intra-abdominally into strain 55X white Leghorn cockerels. The experiment was terminated 4 weeks after the last specific death.

TABLE 3. *Replication of reticuloendotheliosis virus in vitro in the presence of various antibiotics*

Treatment ^a	Concn	Virus dilution	No. dead ^b	Mortality	Average time to death
	mg/ml	log	total	%	days
None	—	0	6/10	60	20
		—1	5/10	50	24
		—2	4/10	40	26
		—3	0/10	—	—
Kanamycin	500	0	6/10	60	19
		—1	3/10	30	27
		—2	4/10	40	35
		—3	0/10	—	—
Amphotericin B	1	0	7/10	70	20
		—1	5/10	50	26
		—2	3/10	30	29
		—3	0/10	—	—

^a Antibiotics were present in the culture media throughout the entire course of the experiment. Samples of the culture fluid were harvested 3 days after infection.

^b Virus was assayed by injection of 0.25 ml intra-abdominally into line 60F white Leghorn cockerels. The experiment was terminated 4 weeks after the last specific death. Uninoculated birds caged with the test birds for the duration of the experiment were negative.

RESULTS

Replication of RE virus in vitro. Active virus production in CEF cultures was detected in the culture fluid on the first day after infection. As noted in Table 1, samples collected at 24 hr after infection produced a 40% mortality with an induction period of 42 days. Virus production and release into the culture fluid continued at a slow but measurable rate for the first 12 hr after the onset of synthesis. Between 36 and 42 hr after infection, there was a rapid rise in virus synthesis and release into the culture fluid. The percentage of mortality was significantly increased at all dilutions, with a corresponding decrease in the incubation period of the disease. Samples of culture fluid collected at 42 hr were infectious for approximately 40% of the birds at a 1:100 dilution. The maximal virus titer in CEF cultures infected with RE virus was reached at this time. The titer remained essentially constant through the fifth day.

Tissue culture fluid from infected cultures rarely produced 100% mortality. A variable proportion of chickens was resistant to the highest dose of RE virus. During the period of maximal virus synthesis, the average time of death was 20 days with undiluted culture fluid, 23 days with a 1:10 dilution, and approximately 1 month with a 1:100 dilution. The range in the

incubation period of the disease was 5 days for samples collected during the period of maximal virus synthesis. Uninoculated birds, caged with the test birds for the duration of the experiment, remained negative. When infectious plasma of the same titer was used as a source of virus inoculum, a similar growth pattern in vitro was observed.

The amount of cell-associated virus was found to be extremely low. As noted in Table 2, cell-associated virus was detected only in samples taken during the period of maximal virus synthesis. The freeze-thaw process was not responsible for the low virus in the intracellular state.

Effect of antibiotics on replication of RE virus. The possibility of this agent being a member of the order *Mycoplasmatales* was considered. Amphotericin B or kanamycin was added to the culture medium at levels known to interfere with mycoplasma replication in vitro (4). These antibiotics were present throughout the entire course of the experiment (Table 3). The replication of RE virus in vitro was not inhibited or reduced by these antibiotics. Similarly, the administration of oxytetracycline or tetracycline, during the course of infection with RE virus in chickens, did not alter either the percentage of mortality or the latent period of the disease. An attempt to isolate an agent on PPLO Agar (2) from diseased tissue or infectious plasma was also negative.

Interference studies. Experiments were conducted to establish whether cells infected with RE virus become resistant to superinfection with A or B subgroup of the sarcoma viruses. The indicator viruses were added to the cultures 12 days after infection with RE virus. As noted in Table 4, preinfection of cells with RE virus did not reduce their susceptibility to infection with viruses of either subgroup. RSV(RAV-1), at a 1:100 dilution, produced 125 foci on the control plate and 100 foci on the plate containing cells infected with RE virus. The indicator virus of the B subgroup [RSV(RAV-2)] produced 115 foci on the control plate and 150 foci on the RE virus infected cells.

As noted in Table 5, cultures infected with avian leukosis viruses of both subgroups were equally sensitive to infection with RE virus. Culture fluid collected with uninoculated cells and cells infected with RAV-1 or RAV-2 6 days prior to challenge with RE virus produced the same mortality and incubation period in chickens. Specific interference against the respective subgroups had been established in the preinfected cultures prior to the addition of RE virus.

DISCUSSION

The biological properties of the replication of RE virus in CEF cultures have many character-

TABLE 4. *Susceptibility of reticuloendotheliosis virus infected cells to infection with sarcoma viruses of the A and B subgroups*

Virus ^a	Subgroup	Dilution	Foci plate		Relative sensitivity ^b
			Control cells	RE virus infected cells	
RSV(RAV) ^c -1	A	<i>log</i>			
		-2	TNTC ^d	TNTC	—
		-3	125	100	0.80
		-4	10	15	1.50
RSV(RAV)-2	B	-5	0	0	—
		-2	TNTC	TNTC	—
		-3	115	150	1.30
		-4	10	15	1.50
		-5	0	0	—

^a Fourth-passage chicken embryo fibroblast cultures containing 2.5×10^6 cells were infected with 0.2 ml of RSV(RAV-1) or RSV(RAV-2); (5.5×10^6 foci-forming units/ml) at the above dilutions.

^b Relative sensitivity = average number of foci on test plate divided by average number of foci on the control plate.

^c RSV = Rous sarcoma virus.

^d Too numerous to count.

TABLE 5. *Replication of reticuloendotheliosis (RE) virus in cells infected with avian leukosis viruses of the A and B subgroups*

Interfering virus	Subgroup	Preinfection	Relative plating efficiencies ^a of challenge viruses		RE virus ^b dilution	No. dead ^c	Mortality	Avg time to death
			RSV(RAV-1) ^d	RSV(RAV-2)				
None	—	<i>days</i>			<i>log</i>	<i>total</i>	<i>%</i>	<i>days</i>
		—	1.0	1.0	0	5/10	50	19
					-1	4/10	40	23
					-2	3/10	30	27
RAV-1	A	6	0.04	0.89	-3	0/10		
					0	4/10	40	19
					-1	5/10	50	22
					-2	2/10	20	29
RAV-2	B	6	1.05	0.032	-3	0/10		
					0	7/10	70	20
					-1	4/10	40	23
					-2	5/10	50	29
					-3	0/10		

^a Relative sensitivity = average number of foci on the test plate divided by average number of foci on the control plate.

^b Culture fluid was harvested 3 days after infection with RE virus.

^c Virus was assayed by injection of 0.25 ml of tissue culture supernatant fluid intra-abdominally into strain 60X white Leghorn cockerels. The experiment was terminated 4 weeks after the last specific death. Uninoculated birds caged with the test birds for the duration of the experiment remained negative.

^d RSV = Rous sarcoma virus.

istics in common with the avian tumor virus group. The comparatively long incubation period, the time at which the maximal rate of virus synthesis is reached, and the low level of cell-associated virus are similar for viruses of the avian leukosis and sarcoma complex. Virus production in infected cell cultures is detected 24 hr after infection with RE virus. The subsequent rise in virus synthesis may be due to an increase

in the number of cells synthesizing virus or to an increase in the number of cells synthesizing virus or to an increase in the rate of synthesis by the individual cells, or to both. The time at which the maximal rate of virus synthesis is reached in the RE virus infected culture is approximately 42 hr. At this time, an equilibrium between virus synthesis and thermal inactivation appears to be established. The decline in virus titer detected on

the sixth day after infection is probably due to exhaustion of nutrients in the culture medium. The major difference in the multiplication cycle of RE virus and other avian tumor viruses is quantitative. Perhaps the reason for the lower titer is that only a specific cell type is capable of replicating RE virus.

Tissue culture fluid collected from RE virus infected cultures rarely produced 100% mortality. Theilen et al. (10) also did not observe 100% mortality in cell-free preparations from plasma or tissue culture fluid in spite of 50-fold concentration. A variable proportion of chickens is resistant to the highest dose of RE virus. Whether this is characteristic of the strain of birds used in the assay remains unknown. Serum neutralization studies, conducted with normal sera collected from 1-day-old cockerels of each of the strains used in these studies, were negative. The variable response does not appear to be due to maternal antibodies present in a portion of the birds used in the assay. The variability in susceptibility to RE virus may be due to an immune reaction developing in the hatched birds after inoculation. This suggestion, however, is not consistent with Theilen's (10) observation that RE virus infected embryonated chicken eggs also show this variability. Genetically determined resistance of chickens to RE virus has not been investigated.

Efforts to relate RE virus to members of the avian leukosis and sarcoma complex were negative. Members of the avian tumor virus group contain a group-specific antigen which can be detected in cells infected with viruses belonging to this group (3). An attempt to detect the group-specific antigen of the avian tumor virus group in RE virus infected cells has been negative (10). Failure to detect the group-specific antigen, however, does not constitute conclusive evidence for its absence. Since RE virus attains a much lower titer than other avian tumor viruses, the antigen could be present at levels below detection.

Another important characteristic of the avian leukosis viruses is the ability to induce resistance to RSV *in vitro*. Vogt and Ishizaki (11) have demonstrated that a distinct interference pattern exists between members of the avian leukosis and sarcoma virus complex. Leukosis and sarcoma viruses of the same subgroup show strong interference, whereas no interference is observed between viruses of different subgroups. Consequently, interference tests must be conducted separately to detect viruses of both subgroups (1). Interference studies conducted with viruses of the A and B subgroups indicate that RE virus

is antigenically unrelated to this group. Our present knowledge of RE virus does not permit a decision as to which existing viral group this agent may be related to.

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