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Assays for Endogenous and Exogenous Lymphoid Leukosis Viruses and Chick Helper Factor with RSV(−) Cell Lines

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Japanese quail cells transformed by the envelope-defective Bryan high-titer strain of Rous sarcoma virus [R(−)Q] were used as a source of the Rous sarcoma virus genome in three kinds of assays. (i) The simplest and most sensitive assay for infectious, endogenous viruses of the chicken belonging to subgroup E involved infection of a mixture of R(−)Q cells and turkey cells with the sample and assay of supernatants of these cells for focus formation on subgroup E susceptible cells. (ii) Inactivated Sendai virus-induced fusion of R(−)Q cells with live test cells was found to be a specific method for detection of chick helper factor. Focus formation by supernatant of the fused cells on subgroup E susceptible cells was correlated with the presence of subgroup E envelope glycoprotein on the plasma membranes of test cells. Whole blood cells as well as fibroblasts could be used in this assay. (iii) A method of assay for exogenous lymphoid leukosis viruses in which mixed cultures of R(−)Q cells and C/E cells and assay of supernatants for focus formation on C/E cells was as sensitive as assays presently used for exogenous lymphoid leukosis virus. Because no infectious Rous sarcoma virus was used as part of the procedure, the assays for infectious virus described here yielded pure pseudotypes of the input virus, an advantage for determining purity and subgroup of the input virus.

Genetic studies of the inheritance of endogenous virus expression require simple, sensitive methods of assay for detection and titration of endogenous subgroup E lymphoid leukosis viruses (LLV) in chickens (18, 28). Equally efficient assays for chick helper factor (chf), which is the endogenous expression of subgroup E envelope glycoprotein in the cell membrane, are needed (7, 24). chf is usually detected by its ability to phenotypically mix with the envelope glycoprotein of viruses that exogenously infect the chf-positive cell (8, 30). Genetic mixing with the endogenous genome controlling chf expression has also been observed (32). Methods developed in these studies were also applied to the assay and titration of exogenous LLV.

Rous-associated virus type O (RAV-O) and other subgroup E LLV have been detected and titrated by a variety of methods, including the activation of Japanese quail cells transformed by the defective Rous sarcoma virus genome of the Bryan high-titer strain RSV(−) called R(−)Q cells (1); by interference (17, 28); by infectious center methods (31); and by a phenotypic mixing assay (3). We have used a complement fixation avian leukosis (COFAL) procedure (20) on line 15_B C/C chicken embryo fibroblasts (CEF) that are unique in their ability to replicate RAV-O to high titers (17). We have also used turkey embryo fibroblasts (TEF) in similar procedures (2). The assay with 15_B C/C cells was very sensitive, but these cells often produced subgroup E virus spontaneously. Some sources of TEF were also very sensitive in this assay but could not be obtained on a regular basis. In this report, we describe a very sensitive routine assay in which TEF mixed with continuous lines of R(−)Q cells are used.

Most biological assays for chf rely on the susceptibility of the cells to be tested to either subgroup B or C sarcoma viruses and an assay for phenotypically mixed viruses on Japanese quail fibroblasts (QEF) that are resistant to subgroup B and C but susceptible to subgroup E (8, 18, 33). We sought an assay procedure that did not depend on the susceptibility of the host cell to specific viral subgroups. Hanafusa et al. (8) developed a method of introducing cell-free RSV(−) into any phenotype of host cells with inactivated Sendai virus and assaying for a subgroup E virus on QEF. We describe here a similar method in which inactivated Sendai virus is used to fuse R(−)Q cells to the test cells.

Many cell culture assays have been developed for the exogenous leukosis viruses. These include an interference assay called the resistance-in-

ducing factor (RIF) test (19), fluorescent-antibody assays (26), the COFAL test (20), the nonproducing (NP) cell activation assay (16), the plaque assay (6), and the phenotypic mixing (PM) test (15). We describe here a modification of the NP cell activation assay in which continuous lines of R(−)Q cells are used, and the need for developing new NP cells is thus eliminated. This assay produces a pure RSV pseudotype of the input LLV.

The infectious virus assays described here take advantage of the fact that RSV(−) cells do not produce infectious RSV when cocultivated with uninfected CEF but do produce infectious RSV when the CEF are infected with an LLV (22). These assays expand the usefulness of RSV(−) cells in LLV research.

MATERIALS AND METHODS

Cells. Continuous lines of R(−)Q cells, transformed by the Bryan high-titer strain of RSV (BH-RSV) but producing particles that lack the envelope glycoprotein gp-85, were those developed originally by Friss (4) and Murphy (12), called 3Q and 16Q, respectively. Continuous lines of hamster cells transformed by BH-RSV were obtained from Sarma (21) and from Okazaki and Payne (unpublished data) called 3292 and 339, respectively.

TEF were made by standard methods (27) from 13- to 15-day-old turkey embryos from hatching eggs provided by Orlopp Turkeys, Orosi, Calif. or the Department of Poultry Science, Michigan State University. Primary cells of pooled embryos were plated and, when confluent, were trypsinized and resuspended in ice-cold freezing medium consisting of our standard tissue culture medium supplemented with 10% dimethylsulfoxide (DMSO) and 15% calf serum. Vials of cells were frozen slowly and stored in the vapor phase of liquid nitrogen. After fast thawing, they were plated in 4% medium and used for assays when confluent. Addition of 1% chicken serum accelerated growth. We prepared 15_B C/C CEF from 10- to 11-day embryos from our specific-pathogen-free (SPF) flocks. C/E chf$^-$, group specific, antigen-negative (gs$^-$) CEF were prepared from embryos obtained from SPAFAS, Inc., Norwich, Conn. QEF were prepared from 7- to 9-day embryos obtained from the Poultry Science Department, Michigan State University. Line 100_B embryos from our own SPF flocks were prepared by standard methods and assayed for susceptibility to subgroup A, B, and E sarcoma virus pseudotypes as primary cells (27). Cells of each susceptibility phenotype, C/O, C/A, C/BE, and C/ABE, were frozen for future use

Viruses. RAV-0 was supernatant fluid from line 100_B C/O or C/A embryos that spontaneously produce high titers. RAV-1 (subgroup A), RAV-2 (subgroup B, RAV-49 (subgroup C), and RAV-50 (subgroup D) were LLV originally obtained from P. K. Vogt, Department of Microbiology, University of Southern California School of Medicine, and were propagated on C/E cells. RSV (RAV-0) was originally obtained from H. Hanafusa, Rockefeller University, and was propagated on C/O or C/A line 100_B cells. RSV pseudotypes of each LLV listed above were originally obtained from P. K. Vogt as stocks prepared by the activation of NP cells with cloned helper LLV.

Cell culture procedures. Cells were cultivated in a mixture of equal parts of F10 and 199 medium supplemented with 5% tryptose phosphate broth, 100,000 U of penicillin, and 100 mg of streptomycin per liter. Mycostatin and amphotericin B were sometimes added at the rate of 15,000 U and 1 mg/liter, respectively. Medium for growth of primaries and plating of other cells was supplemented with 4% calf serum. Maintenance medium for normal and transformed cells was supplemented with 2% calf serum, 5% bovine amniotic fluid, and 1% DMSO. RSV(−) hamster cells were maintained in regular growth medium. R(−)Q cells were maintained in growth medium supplemented with 1% DMSO and 1% chicken serum.

Cells were maintained and passaged by routine procedures. We incorporated 2 μg of diethylaminoethyl-dextran per ml into the medium for about 24 h at the time of virus infection (25). Except where otherwise stated, normal cells were plated at the rate of 1.2×10^6 cells in 60-mm plates and 0.5×10^6 in 35-mm plates and maintained in a humidified incubator at 38°C with an atmosphere of about 5% CO_2. RSV assays followed published procedures (27). Agar overlay also consisted of the F10-199 mixture with 5% calf serum and 0.9% purified agar.

Standard LLV assays. Assays for LLV were conducted by the COFAL method of Sarma et al. (20) as modified by Smith (22), and by the PM test developed by Okazaki et al. (15). Briefly, the COFAL test was conducted by infecting gs$^-$ cells in 60-mm plates, passaging them at weekly intervals for 14 to 21 days, and assaying for gs antigen in cell extracts by the complement fixation (CF) test. The phenotypic mixing test was conducted by infecting cells susceptible to all subgroups in 35-mm plates with RSV (RAV-0) and the test sample. After 7 days, cell-free fluids were assayed for focus formation on C/E cells. Foci on C/E cells indicated that a non-subgroup E virus was present. These assays are considered to be equally sensitive for detection and titration of exogenous LLV (15).

Preparation of inactivated Sendai virus. Stock Sendai virus was obtained from H. Hanafusa. We inoculated 0.1 to 1.0 hemagglutinating units of the virus into the allantoic cavity of 11- to 12-day-old embryos that were returned to the incubator for 3 days. Eggs were then placed in the refrigerator for 2 to 4 h. The allantoic fluid was harvested and clarified in a Sorvall RC2-B centrifuge at 8,000 rpm for 10 min. The supernatant was centrifuged, either undiluted or diluted 1:2 with phosphate-buffered saline, at 17,000 rpm in the Sorvall for 1 h. The pellet was suspended in cell culture medium without serum to 1/10 the original volume of allantoic fluid. Ice-cold β-propriolactone was diluted 1:10 in ice-cold distilled water and diluted again to a final dilution of 1:2,000 in the cold virus suspension. (Because β-propiolactone is a regulated carcinogen, it was kept ice-cold at all times, handled with gloves and diluted in a fume hood until the final dilution was 1:2,000.) It was kept refrigerated overnight and put in a 37°C water bath for 2 h before it was placed in ampoules and stored at −70°C for future use.

Sendai virus fusion of cells. Cell fusion methods were adapted from those of Okada (13, 14), Yamanaka et al. (34), and Fujita et al. (5). For the suspension method, 2 ml of serum-free medium was put into tubes (12 by 75 mm), and then the RSV(−) cells and the cells to be tested were added. The tubes were centrifuged at about 500 rpm in an International PRC-2 refrigerated centrifuge. The supernatant was removed carefully, and 0.1 ml of the appropriate dilution of inactivated Sendai virus, diluted in ice-cold, serum-free medium, was added. The tubes were kept refrigerated or on ice for 20 min with periodic mixing. They were then placed in a 37°C water bath for 20 min with periodic mixing. A 2-ml amount of cell culture medium with 4% calf serum was added to the tubes, and the medium and cells were placed in 35-mm cell culture dishes. The next day the medium was changed to maintenance medium.

For the monolayer method, RSV(−) cells were plated on 35-mm plates and washed twice the next day with ice-cold, serum-free medium (washing was omitted in some experiments without effect on the results), and 0.1 ml of the appropriate inactivated Sendai virus dilution was added to the plates on ice. After 20 min, the test cells were added in ice-cold medium supplemented with 4% calf serum. The plates were put in the cell culture incubator 20 min later. The medium was changed to maintenance medium the next day. The dilution of Sendai virus for use was chosen be testing dilutions with standard chf$^+$ cells and picking the highest dilution that gave the maximum yield of subgroup E RSV.

RESULTS

RAV-0 assay. Three samples of RAV-0 were assayed by five methods. One sample was supernatant from line 100 C/O cells and another from C/BE cells that were expected to have titers between 10^5 and 10^7, and 10^1 and 10^5 per ml, respectively (23). The third sample was a frozen stock of RAV-0. Tenfold dilutions were made, and 0.1 ml of each dilution was added to TEF in 60-mm plates, and to 16Q cells in 35-mm plates. Supernatants of the TEF were transferred to new cells at 7 days, and these cells were maintained for another 7 days; then the cells were collected for a CF test for gs antigen. Cell-free fluids from the TEF were added to 16Q cells at 7 and 14 days after infection of the TEF, and supernatants were assayed 3 days later for focus formation on 15_B C/C cells, indicating RSV rescue from the 16Q cells. Supernatants from the 16Q cells alone were collected at day 9 and assayed on 15_B C/C cells. Supernatants from the mixture of 16Q cells and TEF were assayed at day 9 on 15_B C/C cells. Results of earlier trials had shown that the endpoint was reached at 7 to 9 days of cocultivation.

Results in Table 1 show that the 14-day propagation of TEF with the 3-day rescue phase on 16Q cells and the method of cocultivation were about equally sensitive. We have adopted the TEF-16Q cocultivation method as standard because of its sensitivity and simplicity.

TABLE 1. *Comparison of several methods of RAV-0 assay with TEF and 16Q cells*

Source of RAV-0	Infectious U/ml of stocks				
	TEF, CF: 14 days[a]	16Q alone[b]	TEF 7-day fluids: 16Q[c]	TEF 14-day fluids: 16Q[c]	TEF-16Q: 9-day fluids[d]
L-100 C/BE	$<10^1$	$<10^1$	$<10^1$	10^4	10^5
L-100 C/O	10^5	10^2	10^5	10^7	10^7
Stock	10^5	10^2	10^6	10^7	10^7

[a] Portions (0.1 ml each) of 10-fold dilutions were put on 1.3×10^6 TEF in 60-mm plates, and the medium was changed to maintenance medium the next day. At 7 days, 2 ml of fluids was transferred to new turkey cells seeded as described in the text. Seven days later, the cells were collected and assayed for gs antigen by the complement fixation test.

[b] The same dilutions were put on 1×10^5 16Q cells in 35-mm plates in medium supplemented with 2% calf serum 1% DMSO and 5% bovine amniotic fluid. Medium was changed the next day and on day 4. Fluids were collected 9 days after infection and centrifuged for 5 min at 2,000 rpm to remove cells, and 0.5 ml of the supernatant was assayed for foci on 15_B C/C cells.

[c] Fluids were collected at 7 and 14 days after infection of the turkey cells alone, centrifuged for 5 min at 2,000 rpm, and 0.5 ml of the supernatant was put on 5×10^5 16Q cells in medium supplemented with 4% calf serum and 1% DMSO. The medium was changed the next day. On day 3, 0.5 ml of cell-free supernatant was assayed on 15_B C/C cells.

[d] The same dilutions were put on a mixture of 2×10^5 16Q cells and 3×10^5 TEF in medium supplemented with 4% calf serum. Medium was changed to maintenance medium 1 and 5 days after infection. On day 9, 0.5 ml of cell-free supernatant was assayed for foci on 15_B C/C cells.

Modified NP assay. Because the RAV-0 assay with a mixture of TEF and 16Q cells was simple and sensitive, we explored the possibility that the same assay with SPAFAS C/E cells for cocultivation would provide a sensitive assay for RAV-2, even though the 16Q cells alone were resistant to subgroup B. Preliminary studies with RAV-2 suggested that the assay would be more sensitive if the 16Q cells were added several days after the C/E cells were infected with RAV-2.

Table 2 shows the results of using inactivated Sendai virus fusion to enhance the rescue of the RSV(−) genome from the 16Q cells by RAV-2 and varying the time of adding the 16Q cells, the number of 16Q cells added, and the time after infection of collecting the supernatants. Sendai virus fusion was not required to reach maximum sensitivity if the 16Q cells were added at 3 days

TABLE 2. *Comparison of NP cell activation assay systems, using 16Q cells with the PM test for titration of RAV-2*[a]

Inactivated Sendai virus treatment	No. of 16Q cells added	Infectious U/ml				
		Cells added on day 3		Cells added on day 6		PM control
		7-day fluid	9-day fluid	7-day fluid	9-day fluid	
Yes	2×10^5	10^5	10^5	10^5	10^5	10^5
Yes	3×10^5	10^5	10^5	10^5	10^5	
No	2×10^5	10^5	10^5	10^4	10^5	
No	3×10^5	10^5	10^5	10^3	10^4	

[a] A total of 5×10^5 SPAFAS C/E secondary cells were plated in growth medium with 2 μg of diethylaminoethyl-dextran per ml and infected with 10-fold dilutions of RAV-2. The same dilutions were placed on a PM assay. The next day, the medium was changed to maintenance medium. At 3 and 6 days, the medium was removed completely, and the stated number of 16Q cells were fused to the SPAFAS C/E cells with the monolayer method or were added to the plates after a change of maintenance medium. At 7 and 9 days, 0.5 ml of cell-free supernatant was assayed for focus formation on SPAFAS C/E cells.

after RAV-2 infection, rather than at 6 days. This small variation in the number of 16Q cells was not important, and we now use 2×10^5 regularly. Even though the end points were equal at 7 and 9 days after infection with RAV-2, when the 16Q cells were added at 3 days in this experiment, other experiments have given higher focus counts and even higher end points at 9 days. Therefore, we routinely use 9-day supernatants.

Table 2 also shows that our selected routine procedure was equal in sensitivity to that in the PM test. Subsequent assays comparing the end points for RAV-1, RAV-2, RAV-49, and RAV-50 with the modified NP test, showed that it was as sensitive as the PM (15) and COFAL tests to all viral subgroups.

Assays for subgroup and purity of LLV. Because the pseudotypes produced in the RAV-0 and modified NP assays are not contaminated with subgroup E viruses as they would be in the PM test, these pseudotypes could be assayed for subgroup and contamination with other subgroups by standard methods of interference or host-range. Table 3 shows the host-range method of assay with our standard RAV-1 and RAV-2 stocks. Supernatants from the modified NP test conducted on chf$^-$ C/E CEF were assayed on C/E CEF for titer, on C/A CEF for purity of RAV-1, and on C/BE CEF for purity of RAV-2. Both RAV-1 and RAV-2 were very pure in this assay, which is sensitive to exogenous LLV. The lower dilutions of these same stocks were also assayed on the RAV-0 assay, which is sensitive to subgroup E viruses, and the supernatants were assayed on C/A CEF and QEF, which are Q/BC. Results show some contamination with subgroup E virus in the RAV-2. Therefore, the combination of these two assay methods provides a very sensitive system for assessing subgroup purity of LLV.

TABLE 3. *Assay for purity of RAV-1 and RAV-2 stocks by the modified NP and RAV-0 assays and focus assay on selectively resistant cells*

Assay method	Cells for focus assay	Infectious U/ml of stocks[a]			
		RAV-1		RAV-2	
Modified NP	C/E	10^6	10^5	10^7	10^7
	C/A	$<10^1$	$<10^1$	ND	ND
	C/BE	ND	ND	$<10^1$	$<10^1$
RAV-0	C/A	$<2 \times 10^{0}$[b]	$<2 \times 10^{0}$[b]	ND	ND
	Q/BC	ND	ND	2×10^0	10^1

[a] Replicate titrations; ND, Not done.

[b] Because the C/A cells produced RAV-0, this assay may not have been as sensitive for the detection of subgroup E virus as an assay with C/A cells derived from a non-RAV-0-producing line would be.

chf Assays. We chose to evaluate several methods of using 16Q cells in chf assays because cocultivation with these cells would be a simple method of introducing the RSV genome into test cells which are not necessarily susceptible to RSV of subgroup B or C (1). Our test material was whole blood cells (H. Hanafusa, personal communication), CEF and embryo mashes frozen in DMSO medium from Regional Poultry Research Laboratory (RPRL) line 6, which is known to be chf$^+$ and negative for RAV-0 production (L. B. Crittenden et al., manuscript in preparation).

A comparison of the monolayer and suspension methods with three dilutions of inactivated Sendai virus and with no Sendai virus is shown in Table 4. The suspension method yielded higher counts when fluids were collected 3 days and 5 or 6 days later and therefore was considered to be more sensitive than the monolayer method. Inactivated Sendai virus slightly increased the sensitivity of the assay. Sendai virus fusion had its most important effect when the suspension method was conducted for 3 days.

Concentration of line 6 CEF had little effect on the sensitivity.

Table 5 shows the results of an assay comparing 16Q cell density and treatment of the plated 16Q overnight with 2 μg of mitomycin C per ml to stop cell division (32) with cells untreated with mitomycin C when the monolayer method of assay was used. The idea was to stop 16Q cell growth so that the cells would not overgrow the plate. Whole blood cells and CEF with and without Sendai virus were used. Clearly, both the lower cell density and the mitomycin C treatment increased sensitivity, perhaps because both treatments reduced the number of 16Q cells actually fused to the CEF so that the ratio of 16Q and test cells was optimum. Clearly, Sendai virus must be used to detect chf in whole blood cells from line 6.

Table 6 shows results of an assay comparing 16Q cell number and mitomycin C treatment when assaying for chf in line 6 embryo mashes and whole blood cells in suspension. Pretreatment of the cells overnight with 2 μg of mitomycin C per ml before trypsinization and fusion by the suspension method did not appear to affect the sensitivity of the assay, in contrast to

TABLE 4. *Focus counts from chf assays, comparing the effect of Sendai virus fusion, the monolayer and suspension methods, and the number of days after fusion the fluids were collected*

Sendai virus dilution	Line 6 CEF (count × 10^5)	Focus counts/plate			
		Monolayer[a]		Suspension[b]	
		day 3	day 5	day 3	day 6
Undiluted	3	145	340	<500	438
	4	93	232	<500	<500
1:5	3	59	220	<500	<500
	4	88	323	476	<500
1:10	3	22	162	246	460
	4	31	169	262	296
No virus	3	75	264	126	330
	4	77	229	79	200

[a] The day before fusion, 16Q cells were seeded at a density of 5×10^5.

[b] A total of 1×10^6 16Q cells were used per tube.

TABLE 5. *Focus counts from chf assays with fusion in monolayers, comparing the effect of Sendai virus fusion, treatment of 16Q cells with mitomycin C before fusion, density of quail cells, and the number of days after fusion the supernatants were collected*[a]

Cell type	Sendai virus dilution	Collection day	Focus counts/plate[b]			
			Mitomycin C		No mitomycin C	
			3×10^5	5×10^5	3×10^5	5×10^5
CEF (4×10^5)	1/5	2	230	74	54	0
	1/5	4	>500	326	226	12
	None	2	62	50	36	0
	None	4	>500	424	10	3
Whole blood cells (1×10^8)	1/5	2	175	100	10	0
	1/5	4	>500	376	110	42
	None	2	0	0	0	0
	None	4	0	0	0	0

[a] Cells were CEF and whole blood cells of line 6.

[b] With indicated number of R(−)Q cells.

TABLE 6. *Focus counts from chf assays with fusion in suspension, comparing the effect of treatment of 16Q cells with mitomycin C before fusion, density of quail cells, and the number of days after fusion the supernatants were collected*

Cell type[a]	Collection day	Focus counts/plate[b]					
		Mitomycin C			No mitomycin C		
		3×10^5	6×10^5	1×10^6	3×10^5	6×10^5	1×10^6
EM	2	212	324	176	402	352	214
	4	>500	>500	>500	>500	>500	250
Whole blood cells	2	300	306	44[c]	>500	>500	410
	4	500	>500	262	>500	>500	>500

[a] A 0.4-ml amount of an embryo mash (EM) was prepared from 9- to 11-day line 6 embryos and frozen slowly in freezing medium. This sample represented cells from about 5% of an embryo. Whole blood cells were 1×10^8 whole erythrocytes of line 6.

[b] With indicated number of R(−)Q cells.

[c] Some cells were lost from this plate when the medium was changed.

the effect on the plate method. From 3×10^5 to 6×10^5 16Q cells per tube appeared to give optimum sensitivity. We now routinely use the tube method with 6×10^5 16Q cells, 1×10^8 whole blood cells, 0.4 ml of embryo mash, 1×10^6 secondary CEF, and 2×10^6 primary CEF. Supernatant is collected for focus assay after 3 days.

Comparison of RSV(−) cell types. The RSV(−) hamster cell lines, 339 and 3292, were compared in their ability to detect RAV-1 or RAV-0 through activation of the RSV(−) genome by LLV and detection by focus assay. The 3292 cells were clearly more sensitive than the 339 cells. Therefore, further comparisons were conducted only among 3292, 3Q, and 16Q cells.

Table 7 gives the results of comparing these 3 cell lines in a chf assay conducted by the monolayer method. The 3292 cells appeared to be more sensitive in the detection of chf in CEF at 2 days after cell fusion; this high sensitivity was dependent upon the use of Sendai virus fusion. Both 3Q and 16Q cells gave high focus counts after 4 days. The R(−)Q cells appeared to be sensitive in the detection of chf in whole blood cells when fused with Sendai virus, whereas chf was hardly detectable by the 3292 cells.

We have now chosen to use R(−)Q cells for our assays, and results in Table 7 suggest that the 3Q cells were more sensitive than the 16Q cells. At the time of the assay, the 16Q cells had been passaged for about 250 times twice weekly, whereas the 3Q cells had been passaged fewer than 60 times. Later assays with the suspension method, when the cells were at comparable passage numbers, showed that their sensitivities were about the same. We have also compared these 3Q and 16Q in the RAV-0 and modified NP assays and found the end points comparable in all experiments. We have chosen the 16Q cells as our standard because they generally grow faster and, at least at low passage levels, are comparable in sensitivity with the 3Q cells.

DISCUSSION

We have described simple, sensitive assays for RAV-0, chf, and exogenous LLV. The RAV-0 assay is very sensitive for the detection and assay of subgroup E LLV. However, because both QEF and TEF are sensitive to infection by subgroup A LLV and by some subgroup C and D LLV but not by subgroup B, this assay is not specific for RAV-0. If exogenous LLV are suspected, the supernatant should be assayed on both C/O and C/E cells. Samples that plate on C/O cells but not on C/E cells can be assumed to contain only subgroup E virus. Mixtures of exogenous and endogenous viruses would be more difficult to classify by host range than pure viruses. Cloning may be necessary if the components of the mixture must be determined.

The modified NP assay for exogenous LLV is as sensitive as the phenotypic mixing and COFAL assays. Its major advantage is that the supernatants from the assay are RSV pseudotypes free of subgroup E viruses put into the assay system because the RSV genome comes from R(−)Q cells rather than from RSV (RAV-0) as in the PM test. The modified NP assay is simpler to use than the original NP test because the NP R(−)Q cells are maintained as continuous cell lines and are readily available. The modified NP test assay is simpler than the PM test because true C/O cells are not always readily available, and large amounts of a subgroup E sarcoma virus are not required.

Both assay systems require adequate positive and negative controls to verify the freedom of the R(−)Q cells from exogenous infection with LLVs and to verify the phenotype of the cells

TABLE 7. *Focus counts from chf assays with the suspension method of cell fusion comparing three RSV(−) cell types after mitomycin C treatment*[a]

Cell type	Sendai virus dilution	Collection day	Focus counts/plate					
			No. of 3292 cells		No. of 3Q cells		No. of 16Q cells	
			3×10^5	5×10^5	3×10^5	5×10^5	3×10^5	5×10^5
CEF (4×10^5)	1/5	2	>500	>500	216	116	230	74
	1/5	4	>500	>500	>500	>500	>500	326
	No virus	2	30	14	4	0	62	50
	No virus	4	110	64	>500	>500	>500	424
Whole blood cells (1×10^8)	1/5	2	0	0	176	100	14	14
	1/5	4	8	6	>500	376	288	360
	No virus	2	0	0	0	0	0	0
	No virus	4	0	0	0	0	0	0

[a] RSV(−) cell densities were compared and the effects of Sendai virus presence or absence were compared. Cells were CEF and erythrocytes of line 6.

used for focus assay. We also titrate a standard stock of LLV or RAV-O to assess the sensitivity of each assay.

These assays are particularly useful for subgroup classification of LLV and the detection of small amounts of endogenous subgroup E viruses in LLV stocks or samples. We have shown the host-range method of checking purity, but the pseudotypes so derived could also be classified by interference, or by neutralization criteria (10, 29). In the system described here, only the modified NP assay is required to classify exogenous viruses. If pseudotypes that are free of subgroup E virus are to be obtained from the C/E cells used in the modified NP assay, the C/E cells must be chf negative. Pseudotypes obtained from the limiting dilution of an exogenous virus should also be a good source of seed stock for producing stocks of RSV.

The RAV-0 assay is useful for quantitating small amounts of subgroup E virus contaminating stocks of exogenous virus that are considered to be pure for subgroup. The supernatants from this assay can be assayed on C/A and C/C CEF for subgroup E virus contaminating stocks of LLV of subgroups A and C, respectively, and on Q/BC cells for subgroup E virus contaminating subgroup B stocks. Our present source of C/A cells is line 100, which is not highly sensitive to subgroup E virus, probably because spontaneous RAV-0 production interferes with infection by subgroup E sarcoma viruses (2).

Although we have not had access to large numbers of CEF that are C/O, that support the growth of RAV-0 to a high titer, and that are negative for endogenous virus expression (17a) even when treated with bromodeoxyuridine, such embryos should be appropriate for both the RAV-0 assay and the modified NP system. Thus, with such cells, the two assay systems could become one appropriate for all viruses of subgroups A through E.

Although the chf assay described here has not been compared directly with other published assays for sensitivity, it is useful for direct screening of large numbers of embryos or whole blood samples for chf activity, regardless of the cell susceptibility phenotype (Crittenden et al., manuscript in preparation). This method is highly correlated with radioimmunoassay for the envelope glycoprotein of subgroup E (24).

We do not know the mechanism of activation RSV(chf) after fusion with cells from whole blood. It may be due to fusion with erythrocytes or the blood leukocytes. We know that chicken erythrocytes can fuse with continuous cell lines and that the chicken chromosomes can be activated to be transcribed and produce chicken proteins (11). Thus, we are not sure whether the production of RSV(chf) is a genetic event or simply phenotypic mixing, with envelope antigen incorporated in the erythrocyte or leukocyte cell membrane. Clearly, the assay is correlated with chf assays conducted on CEF of the same types.

Two RSV(−) quail cell lines are efficient in all these assays, and an RSV(−) hamster (3292) cell line is good in some of them. We think that any RSV(−) cell line has potential for these assays, but the sensitivity of the assays may depend more on the individual conditions of the laboratory than on the specific origin of the cell line. Therefore, those using these methods should try more than one cell line under their own conditions before choosing an assay for routine use.

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LITERATURE CITED

1. **Calnek, B. W., and L. N. Payne.** 1976. Lack of correlation between Marek's disease tumor induction and expression of endogenous avian tumor virus genome. Int. J. Cancer **17**:235–244.
2. **Crittenden, L. B., J. V. Motta, and E. J. Smith.** 1977. Genetic control of RAV-O production in chickens. Virology **76**:90–97.
3. **Crittenden, L. B., E. J. Smith, R. A. Weiss, and P. S. Sarma.** 1974. Host gene control of endogenous avian leukosis virus production. Virology **57**:128–138.
4. **Friis, R. R.** 1972. Abortive infection of Japanese quail cells with avian sarcoma viruses. Virology **50**:701–712.
5. **Fujita, D., Y. C. Chen, R. R. Friis, and P. K. Vogt.** 1974. RNA tumor viruses of pheasants: characterization of avian leukosis subgroups F and G. Virology **60**:558–571.
6. **Graf, T.** 1972. A plaque assay for avian RNA tumor viruses. Virology **50**:567–578.
7. **Halpern, M. S., D. P. Bolognesi, R. R. Friis, and W. S. Mason.** 1975. Expression of the major glycoprotein of avian tumor virus in cells of chf(+) chicken embryos. J. Virol. **15**:1131–1140.
8. **Hanafusa, H., T. Miyamoto, and T. Hanafusa.** 1970. A cell-associated factor essential for formation of an infectious form of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **66**:314–321.
9. **Hanafusa, T., H. Hanafusa, T. Miyamoto, and E. Fleissner.** 1972. Existence and expression of tumor virus genes in chick embryo cells. Virology **47**:475–482.
10. **Ishizaki, R., and P. K. Vogt.** 1966. Immunological relationships among envelope antigens of avian tumor viruses. Virology **30**:375–387.
11. **Kao, F.** 1973. Indentification of chick chromosomes in cell hybrids formed between chick erythocytes and adenine requiring mutants of Chinese hamster cells. Proc. Natl. Acad. Sci. U.S.A. **70**:2893–2898.
12. **Murphy, H. M.** 1977. A new replication defective variant of the Bryan high titer strain of Rous sarcoma virus. Virology **77**:705–721.

13. **Okada, Y.** 1958. The fusion of Ehrlich's tumor cells caused by H.V.J. virus *in vitro*. Biken J. **1**:103–110.
14. **Okada, Y.** 1962. Analysis of giant polynuclear cell formation caused by H.V.J. virus from Ehrlich's ascites tumor cells. Exp. Cell Res. **26**:98–107.
15. **Okazaki, W., H. G. Purchase, and B. R. Burmester.** 1975. Phenotypic mixing test to detect and assay avian leukosis viruses. Avian Dis. **19**:311–317.
16. **Rispens, B. H., P. A. Long, W. Okazaki, and B. R. Burmester.** 1970. The NP test for assay of avian leukosis/sarcoma viruses. Avian Dis. **4**:738–751.
17. **Robinson, H. L.** 1976. Intracellular restriction on the growth of induced subgroup E avian type C viruses in chicken cells. J. Virol. **18**:856–866.
17a. **Robinson, H. L.** 1978. Inheritance and expression of chicken genes that are related to avian leukosis sarcoma virus genes. Curr. Top. Microbiol. Immunol. **83**:1–36.
18. **Robinson, H. L., and W. F. Lamoreux.** 1976. Expression of endogenous ALV antigens and susceptibility to subgroup E ALV in three strains of chickens (endogenous avian C-type virus). Virology **69**:50–62.
19. **Rubin, H.** 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **46**:1105–1119.
20. **Sarma, P. S., H. C. Turner, and R. J. Huebner.** 1964. An avian leucosis group-specific complement fixation reaction. Application for the detection and assay of noncytopathogenic leucosis viruses. Virology **23**:313–321.
21. **Sarma, P. S., W. Vass, and R. J. Huebner.** 1966. Evidence for the *in vitro* transfer of defective Rous sarcoma virus genome from hamster tumor cells to chick cells. Proc. Natl. Acad. Sci. U.S.A. **55**:1435–1422.
22. **Smith, E. J.** 1977. Preparation of antisera to group-specific antigens of avian leukosis-sarcoma viruses: an alternate approach. Avian. Dis. **21**:290–299.
23. **Smith, E. J., L. B. Crittenden, and T. H. Brinsfield.** 1974. Status of the endogenous avian leukosis virus in resistant cells from a producing line. Virology **61**:594–596.
24. **Smith, E. J., L. B. Crittenden, and A. K. Whitson.** 1978. Radioimmunoassay for the envelope glycoprotein of subgroup E avian leukosis-sarcoma viruses. Virology **84**:331–340.
25. **Toyoshima, K., and P. K. Vogt.** 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. Virology **38**:414–426.
26. **Vogt, P. K.** 1964. Fluoresence microscopic observations on the defectiveness of Rous sarcoma virus. Natl. Cancer Inst. Monogr. **17**:523–541.
27. **Vogt, P. K.** 1969. Focus assay of Rous sarcoma virus, p. 198–211. *In* K. Habel and N. Salzman (ed.), Fundamental technique in virology. Academic Press Inc., New York.
28. **Vogt, P. K., and R. R. Friis.** 1971. An avian leukosis virus related to RSV(O): properties and evidence for helper activity. Virology **43**:223–234.
29. **Vogt, P. K., and R. Ishizaki.** 1966. Patterns of viral interference in the avian leukosis and sarcoma complex. Virology **30**:368–374.
30. **Weiss, R. A.** 1969. Host range of Bryan strain Rous sarcoma virus synthesized in the absence of helper virus. J. Gen. Virol. **5**:511–528.
31. **Weiss, R. A., R. R. Friis, E. Katz, and P. K. Vogt.** 1971. Induction of avian tumor viruses in normal cells by physical and chemical carcinogens. Virology **46**:920–938.
32. **Weiss, R. A., W. S. Mason, and P. K. Vogt.** 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. Virology **52**:535–552.
33. **Weiss, R. A., and L. N. Payne.** 1971. The heritable nature of the factor in chicken cells which acts as a helper virus for Rous sarcoma virus. Virology **45**:508–515.
34. **Yamanake, T., and Okada, Y.** 1966. Cultivation of fused cells resulting from treatment of cells with HVJ. I. Synchronization of the states of DNA synthesis of nuclei involved in fused multinucleated cells. Biken J. **9**: 159–175.