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Determination of Subgroup-Specific Feline Oncornavirus-Neutralizing Antibody

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A microneutralization assay was developed for antibody-to-subgroup-specific feline oncornaviruses. This study combines the economic advantage of a microtiter system and the quantitative focus reduction method which permits construction of multiplicity curves for determination of virus-neutralizing titers. A twofold increase in Snyder-Theilen feline sarcoma virus (ST-FeSV) on feline embryo cells decreased by approximately twofold the titer of reference goat serum prepared against Kawakami-Theilen feline leukemia virus. Similar dose effects with FeLV serotype virus preparations were not observed. An assay system utilizing FeLV serotypes on sarcoma-positive leukemia-negative cells demonstrated slightly greater sensitivity than one employing ST-FeSV on FE cells. Differential antibody responses to the three subgroup-specific feline oncornaviruses (A, B, and C) were observed in reference goat sera. This test demonstrated good reproducibility as well as sensitivity and constitutes a significant improvement over end point dilution assay systems.

In vitro assays for determining virus-neutralizing (VN) antibody include infectivity (plaque or focus) reduction or end point dilution methods for assay of the surviving virus fraction after interaction of virus and serum (3, 17). Plaque or focus reduction methods depend upon accurate quantitation of viral infectivity as discrete focal areas of cytopathic effect, whereas end point dilution methods quantitate on "all-or-none" effect (scored as infected or not infected) of virus infectivity. End point methods requiring complete inhibition of viral infectivity were shown to be less sensitive than plaque or focus reduction methods (3, 17). Approximately sixfold higher antibody concentrations were required by end point dilution methods than were needed to produce a 50% fall in virus infectivity (17).

Previously described in vitro assay systems for measuring VN activity of sera against avian (12), murine (1, 2, 16), and feline (8, 15) oncornaviruses have utilized both end point (8, 12, 15) and focus reduction (1, 2, 16) methods, but used rather substantial amounts of virus infectivity. Microtiter methods for determining VN activity (8, 9, 11) have provided rapid assay procedures for titration of large numbers of sera with a minimum of material and expense.

The major objective of this study was to develop a sensitive VN assay for feline oncornavirus subgroups, by combining the advantages of the microtiter assay system with a focus reduction technique employing minimal input virus. The effect of input virus on the sensitivity of titer estimate determinations is also described.

MATERIALS AND METHODS

Feline oncornaviruses. Snyder-Theilen feline sarcoma virus (ST-FeSV) containing subgroups A plus B (14) was prepared as feline tumor homogenates and purified according to a modified Moloney (6) procedure or was used as cell-free filtrates. A 10 to 20% suspension of minced tumor tissue in Leibowitz medium (L-15) was homogenized for 3 min and centrifuged at 2,300 × *g* for 20 min. The supernatant was centrifuged as before and at 18,000 × *g* for 1 min. Supernatants were then either used as cell-free filtrates or cushioned over sucrose for further purification. In the latter case, supernatants were layered over 5 ml of 45% sucrose (density, 1.2 g/ml) and centrifuged at 40,000 × *g* for 3 h. The resulting virus band at the interface was dialyzed to remove sucrose. Virus preparations were then frozen in 2-ml volumes at −70 C.

Feline leukemia virus (FeLV) pseudotypes of murine sarcoma virus [Harvey-MuSV (FeLV), subgroup A; Harvey-MuSV (FeLV), subgroup B; and Moloney-MuSV (FeLV), subgroup C] (13) were kindly supplied by Padman Sarma of the National Cancer Institute as tissue culture supernatants and were used as sources of FeLV of different subgroup specificities. Stock pools of all three pseudotype viruses were prepared from infected feline embryo (FE) cell culture supernatants and frozen in 2-ml samples at −70 C.

Cell cultures. Primary FE cell cultures were prepared by standard tissue culture techniques and were grown and subcultured in L-15 medium (Flow Laboratories, Inc., Rockville, Md.), supplemented

with 10 to 20% fetal calf serum and 1% glutamine (200 mM). Media were supplemented with gentamicin (Shearing Corp., Kenilworth, N.J.) at 50 mg/ml or with penicillin (200 U/ml), streptomycin (100 μg/ml), and Mycostatin (12.5 μg/ml). Cell cultures were passed at 1:2 or 1:3 split ratios and were used for infectivity and VN assays between passages 2 and 15.

A sarcoma-positive, leukemia-negative (S+L−) MuSV-transformed cat cell line (4), kindly provided by Peter Fischinger of the National Cancer Institute, was used for assay of subgroup-specific FeLV infectivity. These cells were grown in McCoy 5A medium plus 15% fetal calf serum and antibiotics as indicated above. This cell line permits the direct infectivity determinaton of both xenotropic murine leukemia viruses and ecotropic FeLV (4).

Sera. Goat anti-Kawakami-Theilen feline leukemia virus (KT-FeLV) was prepared as reported earlier (10). Briefly, sucrose gradient-purified KT-FeLV (subgroups A + B + C) was ether-disrupted, emulsified in complete Freund adjuvant, and injected into a goat at five weekly intervals. Antiserum (used as reference serum in VN assays) was collected 1 month after final immunization. Goat antiserum to KT-FeLV was then diluted 1:10 with maintenance medium and stored at −20 C in 1-ml volumes. Nonimmune goat sera were stored undiluted. Cat sera tested were from cats prior to and after exposure to Gardner-Arnstein FeSV ST-FeSV, or KT-FeLV.

In vivo adsorption of hyperimmune sera. Specificity of goat antiserum to KT-FeLV was determined after an in vivo adsorption procedure according to Hardy et al. (5). Undiluted goat serum (3.5 ml) was injected intraperitoneally into a 6-lb (ca. 2.7 kg), 3- to 4-month-old cat. After 18 h the cat was exsanguinated. Serum was recovered, aliquoted, and frozen at −70 C.

Infectivity assay. Serial twofold dilutions of stock virus preparations were made in 0.1-ml volumes in microtiter plates. Indicator cells (FE or S+L−) were then added (5,000 to 10,000 cells/0.025 ml) to each well, and the plates were shaken briefly prior to incubation for 18 h at 37 C. Medium was changed after 18 h and every 3 to 4 days thereafter. Cells were then fixed in buffered formalin and stained with Giemsa when optimal viral infectivity was observed (7 to 8 days for ST-FeSV on FE and 10 to 12 days for FeLV on S+L− cells).

VN assays. Two assay systems were evaluated. One employed ST-FeSV assayed on actively growing FE cells, whereas the other used the FeLV's of different subgroup specificities assayed on S+L− cells. Feline or goat sera were diluted in maintenance medium and heated at 56 C for 30 min to inactivate complement. Additional twofold serial dilutions were prepared in microtiter plates using three wells per serum dilution (0.1 ml per well). Pretitrated stock virus suspension containing 3 to 8 focus-forming units per 0.025 ml was then added to each microtiter well except serum and cell control wells. After gentle agitation to mix virus and antibody, plates were incubated at 4 C for 60 min. Indicator cells (10,000) were then added to all test wells in a volume of 0.025 ml and incubated for 18 h at 37 C. Medium was changed every 3 to 4 days thereafter, subsequently fixed with buffered formalin, and stained with Giemsa after foci became apparent. The ST-FeSV/FE assays were fixed at 7 to 8 days, whereas the FeLV/S+L− assays were fixed at 10 to 12 days. Virus survivals (V/V_o) at each serum dilution were then determined and end point titers were determined at $V/V_o = 0.2$.

RESULTS

Infectivity assays in microtiter plates. Titration patterns for ST-FeSV and FeLV subgroups from MuSV (FeLV) virus pools were determined at several different dilutions (Table 1). Using preconfluent, actively metabolizing test cells, foci were clearly defined and easily quantitated at optimal virus concentrations, at which linear dose-response patterns of focus formation with virus dilution were observed. ST-FeSV and FeLV subgroups A, B, and C demonstrated linear dose-responses for dilutions 25:50, 10:80, 40:80, and 10:20, respectively. Optimal virus dilutions at which linearity of dose-response was observed were those resulting in approximately 7 to 20 foci per three micro-

TABLE 1. *Infectivity titration patterns of ST-FeSV and MuSV (FeLV) pseudotype viruses in microtiter plates*

Virus[a]	Reciprocal of virus dilution	Avg no. of foci per three microtiter wells	Estimated titer (FFU/ml × 10^{-3})[b]
ST-FeSV	10	>30.0	>4.00
	25	19.1	6.34
	40	12.2	7.00
	50	7.7	5.10
FeLV-A	10	17.6	2.34
	20	9.0	2.40
	40	6.9	3.60
	80	2.4	2.50
FeLV-B	10	>21.8	>2.90
	20	>20.0	>5.40
	40	16.2	8.64
	80	8.5	9.04
FeLV-C	10	19.5	2.60
	20	9.0	2.40
	40	2.4	1.26
	80	1.7	1.82

[a] ST-FeSV infectivity was assayed on low-passage FE cells, whereas FeLV-A, -B, and -C subgroup viruses from MuSV (FeLV) pools were assayed on feline S+L− indicator cells. All data represent pooled results from three or more replicate assays.

[b] FFU, Focus-forming units.

titer wells. Virus dilutions resulting in focus counts above 20 and below 7 provided inaccurate titer estimates (Table 1).

An inhibitory effect on focus formation at high virus concentrations was seen for subgroup B FeLV. Dose-responses at 1:40 and 1:80 virus dilutions (16.2 and 8.5 foci/two wells, respectively) show linearity, whereas at lower dilutions the titer estimate is reduced because of difficulty in accurately quantitating focus counts of >7 per well (Table 2).

Determination of VN titers. The assays for antiviral envelope antibody (VN) in goat and cat sera was performed by the constant virus-varying serum dilution method. Virus survivals (V/V_0) were obtained at each serum dilution (three wells per dilution) and represented the proportion of surviving virus infectivity at each serum dilution (V) divided by the mean virus concentration from the control assay (16 wells). Multiplicity neutralization curves were then plotted as in Fig. 1. Presented here are VN titer determinations for hyperimmune goat anti-KT-FeLV. Three or more survival points were used for the placement of a curve (slope = 1) passing through the equivalence region for antigen and antibody (antibody/virus ratio = 1) (3). This curve describing the transition between V/V_0 = 100% and <10% was routinely observed to approximate a straight line with a slope of 1. Titer estimates at V/V_0 = 0.20 were determined as serum dilution values extrapolated from the multiplicity neutralization curve.

Subgroup-specific antibody determination of hyperimmune anti-KT-FeLV goat sera. VN antibody titers in hyperimmune goat anti-KT-FeLV serum to the three FeLV subgroups were determined. Results of a single assay for determination of all three subgroup reactivities at virus inputs of approximately 10 and 20 foci per serum dilution are presented in Fig. 2. Titers derived from survival curves for each virus indicated an approximate threefold higher titer to B and C subgroups than to subgroup A. This same relative difference in anti-subgroup-specific antibody content for hyperimmune anti-KT-FeLV sera was observed in the analysis of multiple VN antibody determinations (Table 3).

Effect of virus concentration on VN titer. Higher concentrations of input ST-FeSV were found to decrease observed VN titer estimates. An input of 20 focus-forming units per dilution of goat anti-KT-FeLV yielded a VN titer of 600, whereas an input of 10 resulted in a VN titer of 1,200 (Fig. 1). Further evidence for this observed viral effect is presented in Table 2. Virus pools 1 and 2 gave similar infectivities at slightly different dilutions and yielded similar VN titer estimates. The 1:25 dilutions of pool 2 resulted in an observed VN titer estimate that

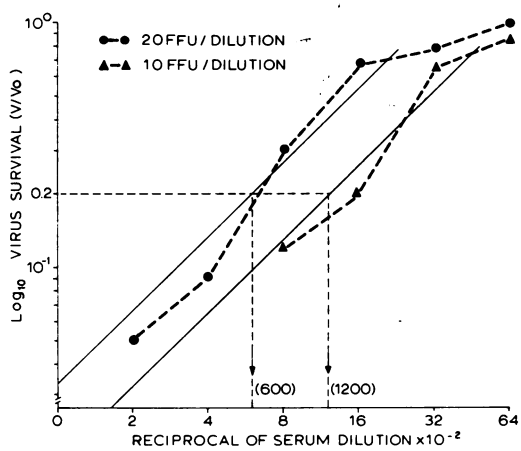

FIG. 1. *Determination of neutralizing titer of hyperimmune goat anti KT-FeLV for ST-FeSV (subgroups A + B) reactivity. Survival curves of ST-FeSV on FE cells at two different input virus concentrations are represented. End points were determined at V/V_0 = 0.20 after placement of the curve (slope = 1) along the abscissa based upon a minimum of three point estimates for virus survival. Each curve represents the mean of five determinations. FFU, Focus-forming units.*

TABLE 2. *Effect of ST-FeSV concentration on VN titer estimates using FE indicator cells*

Virus pool[a]	Virus dilution	Input foci (no. per serum dilution)	No. of assays	Mean VN titer at V/V_0 = 0.20[b]	P
1	1:20	19.1 ± 3.2	8	600 ± 0.08	
2	1:25	19.1 ± 3.6	9	560 ± 0.18	
3	1:10	9.0 ± 2.6	6	1,000 ± 0.17	
2	1:50	7.7 ± 2.5	7	1,000 ± 0.13	<0.01[c]
2	1:40	13.2 ± 3.0	11	1,100 ± 0.12	<0.001[c]

[a] Virus pools were prepared as cell-free 20% tumor homogenates.
[b] Geometric mean titer estimates using n + 1 procedure ± one standard error.
[c] Probability values in comparison with mean titer estimates obtained with 1:25 virus dilution.

was approximately one-half (1:560) that of VN titer estimates determined with 1:50 and 1:40 dilutions of pool 2, as well as with a 1:10 dilution of pool 3.

Similar virus concentration effects as described above for ST-FeSV were not as apparent for different concentrations of FeLV-A using the same antisera (Table 3). Virus from 7-day supernatants resulted in a mean VN titer of 740 at an input virus level of 5.6 foci, whereas FeLV-A derived from a 13-day viral harvest gave mean VN titer estimates of 1,200 and 1,400 at dilutions of 1:25 and 1:50, respectively. Similarly, different concentrations of FeLV-B resulting in 8.1 and 14.9 mean focus inputs gave identical geometric mean titer estimates.

Specificity of reference antiserum. Goat antiserum to KT-FeLV absorbed in vivo by injecting undiluted serum into a cat (Table 4) indicated an approximate 70- to 75-fold reduction in titer. The expected reduction in VN titer on the basis of dilution in total blood volume (50×) accounted for two-thirds of the observed titer reduction.

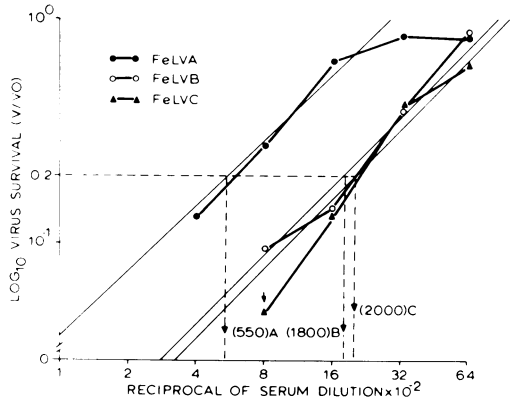

FIG. 2. *Determination of VN titer of hyperimmune goat anti-KT-FeLV for each of the three FeLV subgroups (FeLV-A, FeLV-B, and FeLV-C). Survival curves for each virus subgroup assayed on S+L− cells were obtained using input virus concentrations of approximately 10 and 20 focus-forming units (FFU) per dilution.*

Comparison of VN assay systems. The ability of both assay systems to detect antiviral (VN) antibody in hyperimmune goat sera showed variable results. Highest VN titers obtained for hyperimmune goat anti-KT-FeLV with the ST-FeSV on FE assay system were roughly 1:1,000 (Table 2). By contrast, highest titers achieved using the FeLV-B on S+L− assay system were approximately fourfold higher (1:4,000), whereas those using FeLV-A on similar cells were 1:1,200 and 1:1,400 (Table 3). Sera from cats exposed to Gardner-Arnstein FeSV and KT-FeLV were tested in both assay systems for comparison of relative sensitivities (Table 5). Sera either positive by both systems or negative by both systems (positive correlation) comprised 84% of 32 sera tested. The negative correlation observed favored the FeLV/S+L− assay system, indicating a slightly greater sensitivity.

DISCUSSION

Assay systems presented here to measure VN antibody to feline oncornaviruses were found to be both sensitive and reproducible. VN antibody titer estimates reflected antiviral activities to FeLV subgroups when present as single antigenic subgroups (FeLV; subgroups A, B, or C assayed on S+L− cells) or as mixtures (ST-FeSV; A + B assayed on FE cells). Survival curves constructed from the analysis of surviving virus over a range of several serum dilutions permitted an accurate estimation of VN potency within the region of antibody-virus equivalence (3).

The effect of increased virus concentration in lowering the VN antibody titer estimates was observed with ST-FeSV and was likely due to

TABLE. 3. *Effect of subgroup-specific FeLV concentration on VN titer estimates using S+L− (81-C) indicator cells*

Virus and pool[a]	Virus dilution	Input foci (no. per dilution)	No. of assays	Mean VN titer at $V/V_0 = 0.20$[b]	P
FeLV A-1	1:25	5.6 ± 2.6	8	740 ± 0.21	
FeLV A-2	1:25	8.7 ± 2.9	16	1,200 ± 0.15	
FeLV A-2	1:50	5.0 ± 1.7	6	1,400 ± 0.32	<0.05[c]
FeLV B-1	1:25	8.1 ± 2.8	11	4,000 ± 0.17	<0.001[d]
FeLV B-2	1:50	14.9 ± 2.2	5	4,000 ± 0.24	

[a] FeLV serotype pools were prepared from MuSV (FeLV) pseudotype virus preparations grown in FE cells and represent virus from either 7-day (A-1; B-1) or 13-day (A-2; B-2) supernatants.
[b] Geometric mean titer estimates using $n + 1$ procedure ± one standard error.
[c] Probability value obtained in comparison with MuSV (FeLV) pool A-1 at a 1:25 dilution.
[d] Probability value obtained in comparison with MuSV (FeLV) pool A-2 at a 1:25 dilution.

TABLE 4. *Comparison of in vivo adsorbed and nonadsorbed hyperimmune goat anti-KT-FeLV by VN*

Assay system	VN titer[a]		Titer reduction (fold)	Approximate expected reduction (fold) in titer[c]
	Nonadsorbed	Adsorbed[b]		
ST-FeSV/FE	1,200	17	70×	50×
MuSV (FeLV) B/S+L−FE	3,000	40	75×	50×

[a] Titer determined at virus survival of 0.20 for both virus systems.

[b] Undiluted goat hyperimmune KT-FeLV (3.5 ml) was injected intraperitoneally into a 6-lb cat, and the cat was exsanguinated after 18 h.

[c] Based on a blood volume of 174 ml.

TABLE 5. *Correlation between two virus-cell systems for VN antibody in cat sera*[a]

Correlation	Test system		Distribution of 32	
	ST-FSV/FE	FeLVB/S+L−	No. tested	%
Positive	+	+	5	16
	−	−	22	68
Negative	−	+	5	16
	+	−	0	0

[a] Positive sera represent those which reduce input viral infectivity by 80% at a dilution of ≥1:4. Sera were obtained from cats injected with either ST-FeSV or KT-FeLV.

the presence of substantial noninfectious virus or of virus envelope antigens not bound to intact virus. Similar virus dose effects were observed in other virus systems (3, 18). In the present study it was shown that optimal levels of infectious virus are required to permit accurate quantitation of focus-forming capacity. Optimal levels were from dilutions demonstrating linear dose-response infectivity with virus dilution. Tumor homogenates likely contain substantial amounts of noninfectious virus-associated viral antigen. It is therefore likely that those low dilutions of ST-FeSV prepared as cell-free filtrates from tumor tissue contain sufficient concentrations of viral antigen to effectively lower the observed titer estimates. By contrast FeLV serotype virus pools prepared from MuSV (FeLV)-infected FE cell supernatants failed to demonstrate a substantial lowering of titer estimates at the lower virus dilutions used. These infected cell supernatants do not likely contain sufficient quantities of noninfectious virion-associated antigens to effectively reduce VN titer estimate levels. Similar independence of focus-forming activity on VN antibody survival effects was observed using murine leukemia virus on S+L− murine cells (2).

Detection of feline oncornavirus antibody was apparently more efficient when assayed by the FeLV/S+L− system than with the ST-FeSV/FE system. The maximum titer observed for hyperimmune goat anti-KT-FeLV (subgroups A, B, and C) using ST-FeSV (A + B) on FE cells was 1:1,000, whereas the titers achieved using specific subgroup viruses on S+L− cells were 1:1,400 and 1:4,000 for anti-A and anti-B, respectively. Since the VN titer estimate of a serum containing more than one antiviral specificity will reflect the one present in least concentration, the former assay system had likely measured the anti-FeLV-A response. Additional evidence for the slightly greater sensitivity of the FeLV on the S+L− system was demonstrated by its ability to detect VN activity in several cat sera which were negative by the ST-FeSV on the FE cell system. The differences observed are likely due to variable dose effects which no doubt exist between the tumor tissue-derived ST-FeSV and the tissue culture-grown FeLV preparations. VN titer-estimate differences between assay systems are considered to be within acceptable limits in spite of these minor variations.

The variable response to the three feline subgroup specificities observed in hyperimmune goat sera may either reflect the virus subgroup content of the KT-FeLV used as immunogen or a variability in the antibody response of the goat to these virus antigens. A third possibility may be related to the observed restricted host range for subgroup A-FeLV (7), which apparently produced the weakest antibody response in the goat.

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