Murine Leukemia Virus Assay Techniques: A Comparative Study (36462)

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The purpose of this study was to compare the sensitivity of three different assay techniques for murine leukemia viruses: the mouse antibody production test (1), and two *in vitro* assays: (a) based on the appearance of a new surface antigen on JLS-V9 cells after infection (2); and (b) the plaque assay on D56 cells, recently worked out by Bassin, Tuttle, and Fischinger (3). This was motivated partly by the fact that in experiments concerning the relationship of virus production and the presence of viral specific cell surface antigen, we assayed infectious virus by the capacity of culture media to induce the specific antigen in the JLS-V9 cells. In a number of hybrids obtained after fusion of lymphoma cells with various partners, the two properties were not concordant. We have encountered lines which carried the cell surface antigen but did not release infectious virus (4). It seemed to be necessary, therefore, to ascertain whether the sensitivity of virus detection can be improved.

Materials and Methods. The virus source was a 50% homogenate prepared from the Moloney leukemia virus-induced lymphoma YAC (5). The YAC lymphoma was carried in syngeneic A/Sn mice in the ascitic form by weekly ip inoculations of 10^6 cells. Ascitic fluid was collected from 47 (A × C3H) F_1 hybrid female mice at day 8, postinoculation. The cells were washed twice with Hanks' balanced salt solution (BSS) and adjusted to give a 50% cell suspension. This mixture was then homogenized for 3 min with an Omni-Mixer at 16,000 rpm. The homogenate was cleared by centrifugation at 5000*g* for 20 min. The resulting supernatant fluid was further centrifuged at 10,000*g* for 60 min. All procedures were carried out at 4°. After the second centrifugation, aliquots of the supernatant were frozen in liquid nitrogen. Samples were diluted 1:10 and filtered through a Millipore filter (pore size 0.45 μm) before use.

Virus quantitation by the mouse antibody production test is based on the inverse relationship between the inoculated virus dose and the latency period of antibody appearance (1). In each experiment, the cytotoxic effect of sera from groups of 2 (A × C57 leaden) F_1 hybrid female mice were pooled and tested against YAC target cells (1).

The virus assay, based on the appearance of specific antigen on JLS-V9 cells detected by immunofluorescence, was performed as reported (2), except for a slight modification of the procedure. While the infection was previously performed 24 hr after seeding, it was carried out after 3–5 hr in the present experiments. This modification was introduced because we had found that susceptibility of the cells to the virus is higher during the G_1 and early S phase (6). The JLS-V9 line is rapidly growing and soon after seeding the majority of cells enter the S phase. The cultures were split 1:10 twice weekly and tested in the indirect membrane immunofluorescence test for the presence of the specific antigen.

Plaque formation on D56 cells after infection with Moloney leukemia virus was performed exactly as described by Bassin, Tuttle, and Fischinger (3).

Results. The results of 3 individual titrations in the mouse antibody production test are shown in Table I. Three weeks after virus inoculation, the mice receiving a 10^{-1} and 10^{-2} dilution developed cytotoxic antibodies. At 6 weeks, 3 additional groups infected

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TABLE I. Comparison of the Three Assay Techniques for Moloney Leukemia Virus.

Time after virus infection	Virus dilutions									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
	Mouse antibody production test[a] (cytotoxic index)									
3 weeks	*0.57,0.33*	*0.36*,0.27	0.11,	0.12,	0,0,0					
	0.19	0	0,0	0,0						
6 weeks	*0.88,0.66*	*0.79,0.88*	*0.55,0.63*	*0.64,0.70*	0.23,*0.52*	0.22,0.16	0.08			
	0.70	*0.42*	*0.75*	0.28	0.27					
9 weeks				*0.70,0.65*	*0.78,0.64*	*0.76,0.77*	*0.40,*	*0.33,*	*0.43,0.38*	0,0
				0.68	*0.81*		*0.69,0.46*	*0.52*	*0.40*	
	Antigen induction on JLS-V9 cells[b] (fluorescence index)									
A. 4 days		0	0	0	0	0				
7 days		0	0							
11 days		*1.0*	0.20	0.15	0.11	0.01				
14 days		*0.98*	*0.49*		0.20	0.04				
21 days			*0.65*	*0.69*	*0.44*	0.16				
28 days				*0.84*	*0.86*	*0.50*				
B. 4 days	*0.78*	*0.32*	0.15,*0.43*	0.29,0.24	0.08	0,0				
			0.24							
14 days	*0.71*	*0.47*	*0.71,0.63*	*0.73,0.39*	0,0.23	0.19,0	0,0.25	0,*0.43*	0,0	0,0
			0.74,0.49	*0.75*	*0.31,0.54*	*0.33,0.31*	*0.40*	0		
24 days			*0.88,0.81*	*0.65,0.72*	*0.61,0.67*	*0.70,0.43*	*0.40,0.62*	*0.54,0.35*	*0.39,0.33*	0.11,0.07
				0.81	*0.58*	*0.51*	*0.45*	*0.43*	*0.51*	0.15
	Plaque test on D56 cells[c] (no. of plaques/plate)									
5 days	Confl.	Confl.	82,77,113	28,20,57,	4,2,2,14	1,0,0,1	0,0,0,0	0		
	Confl.	Confl.	Confl.	90,109	5,21					
	Confl.	Confl.	Confl.							
		Confl.								

[a] The mice were injected with Moloney leukemia virus, bled weekly, and the sera were tested against Moloney lymphoma cells (YAC) in the cytotoxic test. Each number represents the mean cytotoxic index of sera collected from 2 (A × C57 leaden) F_1 hybrid female mice (cytotoxic index).

[b] Five titration experiments. (A) cells infected 24 hr after seeding (Expt. I); (B) cells infected 3–5 hr after seeding (Expt. II: 10^{-1}, -10^{-6}; Expt. III: 10^{-3}, 10^{-5}, -10^{-10}; Expts. IV and V: 10^{-3}, -10^{-10} dilutions of the virus were tested). In each fluorescence test one uninfected JLS-V9 line was included as control, and a fluorescence index was calculated as follows: The percentage of negative cells in the virus-infected samples was subtracted from the percentage of negative cells in the uninfected cell sample and divided by the latter figure.

[c] Plaques were counted macroscopically on plates fixed with methanol and stained with May-Grünwald and Giemsa. Each number represents the mean of 2 plates.

with virus dilutions (10^{-3}, 10^{-4} and 10^{-5}) had antibodies. At 9 weeks postinoculation, even a 10^{-9} dilution of virus gave positive results. Mice inoculated with the 10^{-10} dilution remained antibody-free for the 6-month observation period.

Five virus titrations were carried out on JLS-V9 cells. Parts A and B in Table I show the antigen appearance on JLS-V9 cells when virus infection is carried out 24 and 5 hr after seeding, respectively. It is evident that the latter condition increases sensitivity. Tested on day 4, the cells infected with the two highest concentrations (10^{-1} and 10^{-2} dilutions) expressed the new antigen. Fourteen days later, all cultures infected with virus dilutions up to 10^{-7} were positive. The virus in the 10^{-9} dilution was also detectable, but the antigen appeared only 24 days after infection. The cells infected with the 10^{-10} dilution remained negative during the 5-week observation period.

The results of 5 independent virus titrations on D56 cells are shown in Table I. Confluent plaques developed on the monolayers infected with either a 10^{-2} (2 experiments) or 10^{-3} (2 experiments) dilution of the virus. In 2 out of 4 experiments, no plaques were observed with the 10^{-6} dilution although in 2 cases, a single plaque was found in plates infected with this dilution. When compared on day 5, the plaque test is about 10 times more sensitive relative to the JLS-V9 test, since plaques can regularly be observed with a 10^{-5} virus dilution in the former, and the antigen is detected with a 10^{-4} dilution in the latter test. While the end point dilution of the same virus preparation was 10^{-6} in the D56 cells, a 10^{-9} dilution of the virus was still detectable in the JLS-V9 test, although detection of this low virus amount required considerable time (24 days).

The sensitivity of the two *in vitro* assays also compared after they had been similarly performed, *i.e.*, by propagation of the infected cultures. In three experiments, subsequent to plaque counts in virus infected plates, the D56 cultures were divided 1:10 and further passaged every 3–4th day. The subcultures were observed both for plaque formation and for the appearance of Moloney-type cell surface antigen by the mixed hemadsorption method (4, 7). The results of one typical experiment are shown in Table II. In the plates infected with 10^{-2} and 10^{-3} virus dilutions, plaques were confluent. Infection with a 10^{-4} dilution produced 90 and with 10^{-5}, 12 plaques were produced. Further virus dilutions did not yield plaques. In cultures infected with a 10^{-6} dilution of virus, plaques appeared during the second passage while in cultures infected with a 10^{-7} dilution, a few plaques were formed during the third passage. During further passages, the characteristics of these cultures were changed since the cells did not form a regular monolayer. They tended to pile up and were easily detached from the glass. In cultures infected with 10^{-8}, 10^{-9}, and 10^{-10} virus dilutions, no plaques could be observed even after prolonged passages.

The noninfected D56 cells did not react with anti-Moloney serum (Table II). The Moloney-type membrane antigen appeared, however, after infection with Moloney leukemia virus. Similar to the JLS-V9 system, the period of time for its appearance was dependent on the virus dose used. In cells infected with the highest virus doses (10^{-2}, 10^{-3}, and 10^{-4}), it was easily detected during the second passage. With higher dilutions (10^{-5} and 10^{-6}), the antigen appeared on the third to sixth passage. It is of interest that while the antigen was detectable on the ninth passage after infection with the 10^{-8} and 10^{-9} diluted virus, no plaques could be seen in these sublines nor were the growth characteristics of the cells changed. Cultures infected with a 10^{-10} virus dilution remained negative during the entire observation period. It must be established, through the dilution process, whether a virus variant which is lacking the mechanism for changing growth behavior but is still able to multiply in the cells and induce the antigen, has been selected.

Discussion. These experiments demonstrate that the Moloney-type antigen is absent but appears on D56 cells after superinfection with leukemia virus. Plaque formation normally precedes the detection of the cell membrane antigen, presumably reflecting the time

TABLE II. Appearance of Plaques and the Moloney Antigen in Superinfected D56 Cells during Serial Passage.

After virus infection (days) (no. of passages)		Uninfected cells	No. of plaques Infected cells virus dilution: 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
5	No. of plaques	0	Confl.[a]	Confl.	90	12	0	0	0	0	0
10	No. of plaques	0	Confl.	Confl.	Confl.	7	5	0	0	0	0
(2)	Antigen[b]	—	+	+	+	—	—	—	—	—	—
15	No. of plaques	0	Confl.	Confl.	Confl.	Confl.	8	4	0	0	0
(3)	Antigen	—	+	+	+	+	—	—	—	—	—
25	No. of plaques	0				Confl.	Confl.	Confl.	0	0	0
(6)	Antigen	—				+	+	+	—	—	—
35	No. of plaques	0						Confl.	0	0	0
(9)	Antigen	—						+	+	+	—

[a] The cells did not form a dense monolayer, but showed a tendency to pile up and detach from the glass.

[b] The antigen was assayed by the mixed hemadsorption test (4). The anti-Moloney serum was used in 1:3, 1:10, 1:30, 1:100 and 1:300 dilutions. When the lines became positive, they reacted with serum dilution up to 1:30, 1:100. Usually 50–100% of the cells had 3 or more erythrocytes attached.

necessary for virus spread with consequent antigenic conversion of the majority of the population. Also, the carrier cultures change their growth characteristics and this occurs when antigen is detectable.

On the basis of the present work, we can conclude that the sensitivity of the three assays in detection of mouse leukemia viruses is similar. The mouse antibody production test requires, however, a considerable period of time. In comparing the two *in vitro* systems, the advantage of the plaque assay is its higher sensitivity (10-fold) when read at day 5, the ease of scoring, and the correlation of the number of plaques with the virus concentration. The correlation of antigen positive cells with virus concentration is less exact in the JLS-V9 test due to sampling errors. This occurs because monolayers are not inspected *in situ*. Small virus amounts can be detected in both *in vitro* systems when the infected cultures are carried for a longer period of time.

The sensitivity of the two cell lines to the Moloney virus was found to be identical when they were scored for the appearance of the surface antigen. As mentioned earlier, the lack of plaques in the cultures infected with the two lowest doses may be due to a variant virus.

The JLS-V9 test may be useful for viruses with a varying host range; *i.e.*, the L-cell virus which neither forms plaques nor induces the membrane antigen on D56 cells, although it successfully infects JLS-V9 cells. It should be emphasized that the growth characteristics of the JLS-V9 cells are not affected by the virus infection. This suggests that the induced antigenic conversion is not sufficient to impose the change. Also, the kinetics of the change of growth pattern and antigen appearance on the D56 cells showed that the former parameter appears earlier, consequently one may consider the two as independent virus-induced characters having no casual relationship.

Summary. Sensitivity of three assay techniques for the detection of murine leukemia viruses were compared. These techniques were the mouse antibody production test, appearance of new surface antigen(s) on JLS-V9 cells, and production of plaques on D56 cells. The sensitivity of the three assays in the detection of murine leukemia virus is similar. The plaque assay on D56 cells offers certain advantages over the other tests since smaller quantities of leukemia virus could be detected on day 5 after infection by using this technique. The antibody production test requires 9 weeks for completion. For viruses with a differing host range which will induce neither plaque nor membrane antigens on D56 cells, the assay of JS-V9 cells may be the preferred method.

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