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Splenocyte Plaque Assay for the Detection of Murine Leukemia Virus (38946)

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The development of the XC plaque assay has been a major advance in the detection of infectious murine leukemia virus (MuLV) (1, 2). In this method, tissue extracts or cell free tissue culture media are generally employed as the assay material. It has been reported that assay of tail extracts is particularly sensitive for the detection of N-tropic virus in AKR mice (3) and in offspring of crosses of AKR mice with low leukemia incidence strains (4, 5). We now report that assaying suspensions of spleen cells taken directly from the animal can be orders of magnitude more sensitive than testing either tail extracts or spleen extracts for the detection of virus other than that of AKR mice. The assay permits quantitation of spleen cells producing infectious MuLV. Further application of this technique permits identification of MuLV production by morphologically and functionally defined populations of lymphoid cells (6).

Materials and Methods. Animals. AKR/J (hereafter referred to as AKR), DBA/2J (hereafter referred to as DBA/2) and B10.A Sq Sn (hereafter referred to as B10.A) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. B10.A mice were originally developed from the C57Bl/10 Sc Sn line (7). Spleen cell suspensions or tissue extracts were prepared at the age indicated in the legend to tables.

Preparation of spleen cell suspensions. Spleens were aseptically removed from mice, minced in MEM 4X[4] (Grand Island Biological Company) supplemented with 5% unheated fetal calf serum and pressed through tantalum gauze with a small test tube. The cells were then centrifuged at 250*g* for 10 min at 20°, washed once and resuspended in the same medium supplemented with 10% unheated fetal calf serum. Clumps were eliminated by filtration through a plug of nylon wool (Leukopak, Fenwal Co., Morton Grove, Illinois) packed loosely in a Pasteur pipette. Cell viability was determined by the trypan blue exclusion test and the concentration of cells was adjusted as required.

Preparation of tissue extracts. Extracts were prepared from fragments of spleens and tails as described by Rowe and Pincus (3) by grinding the weighed tissues in a Ten Broeck apparatus after addition of enough MEM 4X with 10% unheated fetal calf serum to yield a 5% extract (w/v). The extracts were clarified by centrifugation at 1000*g* and assayed in the XC test within 4 hr of preparation.

Virus assay. The uv–XC procedure described by Rowe *et al.* (2, 3) was used. All cultures were grown and maintained in MEM 4X supplemented with 10% unheated fetal calf serum. Mouse embryo (ME) cell cultures were prepared from 14- to 17-day-old embryos (8). After treatment of the primary cultures with trypsin, the cells were seeded into 60 × 15 mm plastic Petri dishes (Falcon Plastics, Los Angeles, California) at a concentration of 4×10^5 cells/4 ml medium/dish. The next day the cultures were treated for 1 hr with 25 μg/ml diethylaminoethyl (DEAE)-dextran (Sigma Chemical Co., St. Louis). The cultures were then rinsed and inoculated with serial dilutions of lymphocytes or tissue extracts. In the latter case, an inoculum of 0.4 ml was plated on the embryo cells, then the dishes were rocked after 15 min and fed with complete medium after 30 min. The absorption method was not required when living cells were assayed; instead, the cells were plated

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[4] Eagle's Minimal Essential Medium with 1 × Earle's salts, 4 × amino acids, 2 mM L-glutamine, 4 × vitamins and $NaHCO_3$, supplemented with 120 U penicillin and 120 μg streptomycin/ml.

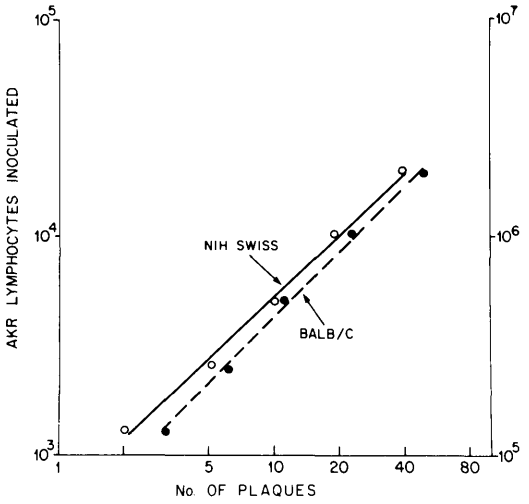

FIG. 1. Kinetics of XC plaque assay when AKR lymphocytes were assayed on NIH Swiss (solid line) or BALB/c (dashed line) embryo cells. Left hand ordinate: Number of spleen cells plated on NIH embryo cells. Right-hand ordinate: Number of spleen cells plated on BALB/c embryo cells. For each dilution of spleen cells, the ratio N/B = number of plaques on NIH ME cells/number of plaques on BALB/c ME cells = 80.

in 4.0 ml of complete medium. The remainder of the assay, including exposure to uv light, addition of XC cells, fixation and staining of the cultures and enumeration of plaques was carried out as previously described (3). The plaques produced by lymphocytes or other cells were morphologically identical to those produced by the extracts.

Results and Discussion. Figure 1 shows titration curves obtained by inoculating twofold serial dilutions of AKR (Fv-1^n) spleen cells on NIH and BALB/c ME cells. Upon inoculation on both types of embryo cells linear curves were obtained when the results were plotted on a log/log scale. The N/B ratio (see legend to Fig. 1) was consistent with the known N-tropism of AKR MuLV (9). Linear curves were also obtained with B10.A spleen cells that produced a B-tropic virus. The kinetics of the assay allowed quantitation of the number of plaque-forming units in spleen cell suspensions by simple extrapolation of the results obtained in two- or tenfold serial dilutions.

In B10.A (Fv-1^b) mice B-tropic MuLV is highly prevalent (10). Table I shows that the B-tropism of this virus was expressed

TABLE I. TROPISM OF MuLV FROM A B10.A MOUSE IN THE XC TEST

Inoculum					
Spleen cells[a]			Cell free tissue[a] culture fluid		
B[c]	N[d]	B/N[e]	B	N	B/N
18×10^{3b}	14×10	129	23×10^{2b}	15	165

[a] Inoculation with spleen cells or cell free tissue culture fluid from BALB/c embryo cells infected by spleen cells from the same mouse (male, 48 wk of age).

[b] Results expressed as plaques per 10^7 viable spleen cells inoculated or plaques per 0.4 ml undiluted tissue culture fluid inoculated.

[c] B = number of plaques on BALB/c embryo cells.

[d] N = number of plaques on NIH Swiss embryo cells.

[e] Ratio B/N = number of plaques on BALB/c embryo cells/number of plaques on NIH Swiss embryo cells.

whether the inoculum consisted of spleen cells or of cell-free tissue culture fluid (Table I). In both cases more than 100 times more plaques were detected on BALB/c than on NIH embryo cells. The ability to detect tropism was further tested in XC assays of pooled spleen cells from AKR, B10.A, and DBA/2 mice (Table II). In the case of AKR spleen cells the ratio N/B ranged from 40 to 260, consistent with the N-tropism of AKR virus (9). XC assay of pooled B10.A spleen cells yielded a B/N ratio that ranged from 50 to 280, consistent with B-tropism of the virus in these inocula. In three pools of spleen cells from DBA/2 mice N-tropic virus was detected (N/B range 30–50). The technique thus allows accurate identification of viral tropism. This indicates that the plaques found in the XC assay reflected replication of MuLV in the embryo cells and not direct interaction of MuLV in spleen cells with the XC cells.

Table III gives the results of XC tests with inocula of spleen cells, spleen extracts or tail extracts from four AKR and 13 B10.A mice. In order to compare the relative sensitivity of the XC test when spleen cells or spleen extracts were used as inocula, the recovery of cells from the spleen fragments was determined. The initial 0.4 ml of un-

TABLE II. TROPISM OF MuLV AS DETECTED BY XC ASSAY OF POOLED[a] SPLEEN CELLS FROM AKR, B10.A OR DBA/2 MICE.

		N[b]	B[c]	N/B[d]			B	N	B/N[e]
AKR pool	1	55 × 10	5	110	B10.A pool	1	25 × 10	4	63
	2	35 × 10	2	175		2	20 × 10	1	200
	3	5 × 10^2	4	125		3	16 × 10	1	160
	4	13 × 10^2	5	260		4	25 × 10	2	125
	5	55 × 10	5	110		5	20 × 10	4	50
	6	75 × 10	10	75		6	10 × 10	1	100
	7	5 × 10^2	8	63		7	7 × 10^2	5	140
	8	4 × 10^2	10	40		8	28 × 10	1	280
DBA/2 pool	1	20 × 10	5	40					
	2	15 × 10	5	30					
	3	50	1	50					

[a] Each pool consisted of cells from 5 to 12 animals of a single strain. The ages of the animals were 6–8 wk (AKR, B10.A) or 4–6 wk (DBA/2).

[b] N = number of plaques on NIH Swiss ME cells per 10^6 (AKR) or per 10^7 (B10.A, DBA/2) inoculated spleen cells.

[c] B = same on BALB/c ME cells.

[d] Ratio N/B = number of plaques on NIH Swiss embryo cells/number of plaques on BALB/c embryo cells.

[e] Ratio B/N = See legend to Table II.

TABLE III. COMPARATIVE SENSITIVITY OF THE XC TEST USING SPLEEN CELLS, SPLEEN EXTRACTS OR TAIL EXTRACTS AS THE INOCULUM[a].

Strain	Spleen cells	Spleen cells mitomycin treated[c]	Spleen extract	Tail extract
AKR				
1	14 × 10^2[b]	11 × 10^2	17 × 10	34 × 10^2
2	18 × 10^2	17 × 10^2	19 × 10	26 × 10^2
3	20 × 10^2	22 × 10^2	39 × 10	13 × 10^2
4	26 × 10^2	27 × 10^2	47 × 10	22 × 10^2
B10.A				
1	29	25	0	0
2	7 × 10^2	8 × 10^2	0	3
3	36 × 10^2	32 × 10^2	3	51
4	40 × 10^2	43 × 10^2	1	0
5	6 × 10^3		1	0
6	8 × 10^3		1	4
7	10 × 10^3		2	0
8	14 × 10^3		0	0
9	18 × 10^3		2	0
10	22 × 10^3		0	0
11	33 × 10^3		1	0
12	36 × 10^3		3	1
13	36 × 10^3		5	0

[a] All inocula were prepared from individual mice of strains AKR (male, 20 wk of age) and B10.A (male, 40–48 wk of age).

[b] The results are expressed as plaques per 10^7 spleen cells or as plaques per 0.4 ml of undiluted 5% (w/V) tissue extract. Since virus in AKR mice was N-tropic and that in B10.A tissue was B-tropic, only the results obtained by inoculation of NIH Swiss ME cells or BALB/c ME cells, respectively, are shown.

[c] Mitomycin-C (Sigma Chemical Co., St. Louis, Mo.) treatment for 30 min at 37°C, followed by two washings.

diluted spleen extract (5% w/v) was prepared from a 20-mg fragment of spleen. In the case of AKR mice, this amount of tissue contained at least 18×10^6 viable cells (range $18–30 \times 10^6$) and, in the case of B10.A mice, at least 28×10^6 viable cells (range $28–46 \times 10^6$). The largest amount of spleen extract inoculated was therefore derived from two to three times the maximum number of cells contained in inocula of viable spleen cells. It can be seen that in the case of AKR mice, similar titers of N-tropic virus were detected when either spleen cells or tail extracts were tested. Lower (five- to tenfold) titers were found in spleen extracts. By contrast, in the case of the B-tropic virus of B10.A mice, spleen cell inocula yielded far higher titers of virus than either spleen extracts or tail extracts. This may be due to the manipulations involved in preparing extracts. However, regardless of the mechanism, it is clear that if the extracts had been relied upon a substantial number of false negative results would have been scored.

It may be argued that the high titers of infectious virus seen with spleen cells as the inoculum could be due to *in vitro* activation and/or replication of MuLV in these cells. However, when spleen cells from four AKR and four B10.A mice were treated with mitomycin C at a concentration (25 μg/ml) that completely blocks DNA synthesis (11, 12) and tested immediately, no reduction of the amount of virus was seen (Table III). It has been shown that treatment of cells with mitomycin C (13, 14) or with other inhibitors of DNA synthesis (14) prevents replication of RNA tumor virus (for review see (15)). *In vitro* initiation of replication of MuLV in the inoculated spleen cells can therefore be excluded, especially since the effect of mitomycin C on DNA synthesis is irreversible (11).

The tail extract method has been used successfully in studies of the AKR-virus (4, 5). Since it can be carried out on biopsy specimens, this technique permits consecutive assays of individual animals. However, both this method and the assay of splenic extracts gave misleading results in B10.A mice (Table III). Only when direct assay of lymphocytes was done was the prevalence and titer of MuLV in these mice realized. It was also of interest that N-tropic virus was detected in young DBA/2 mice (Table II). This was not found by others who used extracts as assay material (4). The advantages of a highly sensitive assay for infectious MuLV using defined populations of viable lymphoid cells hardly needs emphasis. We suggest that the technique we have described may find numerous applications in the study of MuLV.

Summary. A modified XC assay for murine leukemia virus (MuLV) employing splenocytes taken directly from the animal is described. This modification can be more than 1000 times more sensitive than XC plaque assays employing tissue extracts. This technique should lend itself readily to the quantitation of infectious MuLV in defined populations of lymphoid cells.

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