Sensitive In Vivo Assay for Detection of Murine Leukemia Viruses

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A test is reported for the in vivo detection of the replicative ability of murine leukemia viruses (MLV) in the BALB/c mouse strain. Growth in the spleen was assayed by the complement-fixation assay for MLV group-specific antigen after injection of newborn mice. Low doses of laboratory-derived and wild-type MLV from cell-culture and animal-grown sources were detected as early as 7 to 14 days postinoculation. The sensitivity of this in vivo test compared favorably with in vitro assays for MLV. In vivo detection of MLV replication was correlated with long-term oncogenicity.

The use of in vivo tests for detection of infectious endogenous murine viruses has been described by Rowe (9). The test involved inoculation of newborn mice and harvesting of the appropriate target organs for serological assay of viral antigens. The target organs and the time period of the assay varied with the suspected agent. This report describes the development of a sensitive in vivo assay for murine leukemia viruses (MLV), the spleen antigen test (SPAT). Natural isolates of MLV from BALB/c (B/c) mice have been shown in neoplasia-induction studies to require a long latent period before the onset of leukemogenesis (7, 8). Groups of mice receiving these inocula were sampled and sacrificed at 63 days of age, and their spleens were tested by complement fixation for the presence of the group-specific (gs) antigen of the type-C viruses. There was a very good correlation in these groups of inoculated animals between MLV detection in a sample of the animals tested and subsequent leukemia incidence in the remainder of the animals receiving the same inocula. This led us to determine whether a standard, sensitive in vivo assay for MLV could be developed by using several strains of tissue culture-grown and animal-passaged MLV.

MATERIALS AND METHODS

Virus preparations from tissue culture. Stocks of tissue culture-grown Rauscher leukemia virus (R-MLV), Moloney leukemia virus (M-MLV), Gross leukemia virus (G-MLV), and AKR leukemia virus (AKR-MLV) were originally obtained from Janet Hartley, National Institute of Allergy and Infectious Diseases. Tissue culture-grown viruses were harvested from supernatant fluids of acutely infected secondary mouse embryo fibroblasts 14 days after infection. The fluid was clarified by centrifugation at 10,000 × g for 10 min and stored over liquid nitrogen. AKR-MLV and Gross-MLV were grown in NIH Swiss mouse embryo (NIH-E) fibroblasts. These and other MLV which grow to higher titer (100- to 1,000-fold) on NIH-E than on B/c embryo (B/c-E) fibroblasts are termed N-tropic. A wild-type virus (S138) from a spontaneous B/c leukemia was produced on B/c-E fibroblasts and is termed B-tropic because it propagates to higher titers on B/c-E fibroblasts (30- to 100-fold) than on NIH-E fibroblasts. R-MLV and M-MLV are termed NB-tropic because they propagate equally well on both B/c-E and NIH-E fibroblasts (3).

Virus preparations from animal passage. Animal-passaged M-MLV and Friend leukemia virus (F-MLV) were produced by intraperitoneal inoculation of newborn BALB/c mice, with subsequent harvest of the plasma at 28 and 14 days, respectively. The plasma was collected at a dilution of 1:2 in 0.153 M potassium citrate, clarified at 2,400 × g for 10 min and 10,000 × g for 10 min, pooled, and stored over liquid nitrogen. R-MLV was also produced by intraperitoneal inoculation of newborn B/c mice; spleens were harvested at 28 days and viral preparations were made by the Moloney procedure (4). Low-titered viral preparations obtained from naturally occurring tumors of the B/c mouse were also prepared by the Moloney procedure (4).

Assay for induction of MLV-gs antigen in spleen. Log dilutions (10⁻¹ to 10⁻⁴) of tissue culture grown and 10⁻³ to 10⁻⁴ of animal grown) of all of the virus preparations were made, and 0.1 ml of each was inoculated intraperitoneally into each of eight newborn B/c mice. At various time periods (up to 63 days), depending on the virus tested, each group and five uninoculated control mice were sacrificed, and 10% (wt/vol) spleen extracts were tested for MLV-gs antigen in complement fixation versus 4 U of antisera reactive against the MLV-gs antigen (6).

Assay for induction of MLV-gs antigen in vitro
(CoMuL test). Duplicate plates of secondary B/c-E and NIH-E fibroblasts, pretreated with 25 μg of diethylaminomethyl-dextran per ml, were inoculated with 0.1 ml of the virus preparations and maintained for 21 days. Cells were then harvested and tested for MLV-gs antigen as part of the standard complement fixation for murine leukemia (CoMuL) test (1). Complement fixation at antigen dilutions of 1:2 or greater was scored positive; the mean infectious dose per 0.1 ml was calculated for the SPAT assay, and the mean tissue culture infectious dose per 0.1 ml was calculated for the CoMuL assay.

RESULTS

Comparison of in vivo and in vitro titers.
Table 1 shows the comparison of infectious virus titers obtained by the SPAT and CoMuL procedures. It is seen that, without exception, the Friend, Moloney, and Rauscher viruses, whether grown in vitro or in vivo, gave higher titers in the in vivo test than in a single-passage CoMuL test on either of the two strains of mouse embryo fibroblasts. Of interest was the natural MLV isolate from a B/c tumor, a B-tropic virus (2) which gave high titers in the B/c spleen assay, with a slightly lower titer in B/c-E fibroblasts and, as expected, little or no activity at the dilution tested on NIH-E fibroblasts. The N-tropic AKR-MLV and G-MLV strains, conversely, gave no activity in B/c spleens or B/c-E fibroblasts, whereas they gave titers of at least 10⁴ mean tissue culture infectious doses per 0.1 ml on NIH-E fibroblasts.

Time course of spleen infection. To examine the time course of spleen infection, 0.1 ml of log dilutions of each of the viruses was inoculated into each of 30 to 32 newborn BALB/c mice, and five test animals along with five uninoculated controls were sacrificed at 1 week and at weekly intervals for 6 weeks thereafter. The spleens were tested in complement fixation as described. Table 2 shows these results. The B/c, F-, M-, and R-MLV gave titers at 14 days after inoculation which were generally within one-half log of the titer obtained at 42 days, suggest-

### Table 1. Comparison of SPAT and CoMuL assays for cell-culture and animal-grown murine leukemia viruses

| Virus         | Source          | Day postinoculation | SPAT assay (ID₅₀/0.1 ml ± SE)ᵃ | CoMuL assay (TCID₅₀/0.1 ml ± SE)ᵇ |
|---------------|-----------------|---------------------|---------------------------------|-----------------------------------|
| B/c-MLV-S138  | Cell culture    | 42                  | 4.91 ± .40                      | 4.1 ± .48                         |
| G-MLV-S1323   | Cell culture    | 42                  | <1                             | <1                                |
| AKR-E-MLV1290 | Cell culture    | 42                  | <3                             | <3                                |
| M-MLV-S1240   | Cell culture    | 42                  | 4.75 ± .48                     | 3.50 ± .36                       |
| R-MLV-S1197   | Cell culture    | 42                  | 5.17 ± .59                     | 3.50 ± .36                       |
| F-MLV-A00862  | Plasma pool     | 35                  | 6.75 ± .58                     | >5.0                              |
| R-MLV-A00590  | Spleen pool     | 35                  | 6.75 ± .34                     | 4.25 ± .48                       |
| M-MLV-A00717  | Plasma pool     | 21                  | 5.50 ± .36                     | 4.00 ± .59                       |

ᵃ See Materials and Methods for description of assay. ID₅₀, mean infectious dose; SE, standard error.
ᵇ Titer obtained at 21 days. TCID₅₀, mean tissue culture infectious dose; METC, secondary mouse embryo tissue culture.
ᶜ Log virus titer ± standard error in B/c mice.

### Table 2. Spleen antigen test of leukemia viruses from animal and cell culture sources

| Virus         | Source          | Mean infectious dose/0.1 ml |
|---------------|-----------------|----------------------------|
|               |                 | Days postinoculation       |
|               |                 | 7  | 14 | 21 | 28 | 35 | 42 |
| B/c-MLV-S138  | Cell culture    | -  | 4.50 ± .35*b               | 4.42 ± .46                      | -  | -  | 4.91 ± .40                      |
| G-MLV-S1323   | Cell culture    | -  | .1                           | <1                             | <1 | -  | -  | <1                           |
| AKR-E-MLV1290 | Cell culture    | -  | <3                           | <3                             | <3 | -  | -  | <3                           |
| M-MLV-S1240   | Cell culture    | -  | 4.38 ± .35                   | 4.50 ± .34                     | 4.75 ± .48                      | -  | 4.75 ± .48                      |
| R-MLV-S1197   | Cell culture    | -  | 4.17 ± .37                   | 4.50 ± .34                     | 4.66 ± .46                      | -  | 4.17 ± .59                      |
| F-MLV-A00862  | Plasma pool     | 6.10 ± .40                 | 6.10 ± .37                     | 6.90 ± .24                     | -  | 6.75 ± .58                      |
| R-MLV-A00590  | Spleen pool     | 4.84 ± .45                 | 6.50 ± .35                     | 6.37 ± .31                     | -  | 6.75 ± .34                      |
| M-MLV-A00717  | Plasma pool     | <4                         | 5.10 ± .24                     | 5.50 ± .36                     | -  | -  | -  | -  | -  | -  |
| Uninoculated Control | 0   | 0  | 0  | 0  | 0  | 0  | 0  |

ᵃ - , Not tested at this time interval.
ᵇ Log of virus titer ± standard error based on tests of 10% spleen extracts from five mice per dilution.
ing that this assay could be used at 14 days. The spleens from the uninoculated control animals were shown without exception to have no detectable MLV-gs antigen activity.

Correlation of SPAT assay with oncogenic potential. In separate experiments, low-titered viral preparations obtained from naturally occurring tumors of the B/c mouse were tested by the SPAT (63 days) and CoMuL assays and simultaneously tested for their oncogenic potential by inoculation into newborn B/c mice. Table 3 shows the characterization of 133 such inocula and their ability to subsequently induce lymphoreticular neoplasms. It is seen that a positive SPAT assay and, specifically, a concomitant positive SPAT and CoMuL assay (an indication of good titer) were good indicators of the inoculum's oncogenic potential. Because of the low titers of these preparations, the CoMuL plates were tested after each of three blind passages (total of 63 days in culture) before they were scored negative; this is known from previous experiments to increase the sensitivity of the CoMuL by about two logs. With this increased sensitivity there were some positive CoMuL-B/c-E tests that were SPAT negative.

Most of the inocula yielding concomitant SPAT- and B/c-E-CoMuL-positive assays gave highly significant rates of neoplasia induction. In the two instances where the SPAT assay was positive and the B/c-E-CoMuL assay was negative, the percentage of inocula giving significant induction rates was low (28% and 0%, respectively, for SPAT positive, NIH-E and B/c-E negative and CoMuL positive, B/c-E negative and NIH-E positive). In the case where only the SPAT assay was positive, only four of the 15 inocula gave significant induction rates even though the overall rate (22%) was high.

**DISCUSSION**

In conclusion, one can obtain a sensitive assay for the infectivity titer of B-tropic and NB(dual)-tropic MLV by the in vivo procedure described in this report. Although this study did not include an in vivo assay for N-tropic viruses, a suitable N-type mouse strain such as C57L/J(5) can no doubt be used to develop a similar SPAT assay for N-tropic viruses. It should be noted that other mouse strains with the FV-1a genotype tested by the authors have high gs-antigen expression naturally. It is felt that for routine titration of MLV the conventional in vitro procedures (1, 5, 10) offer many obvious advantages. The value of obtaining an in vivo titer is that the in vivo growth potential of the inocula is most highly correlated with its leukemogenic potential (7). Conversely, undetectable in vivo growth in the spleen at 6 weeks after inoculation only rarely resulted in subsequent leukemogenesis. This is of importance when studying the oncogenic potential of low-titered natural isolates of MLV where conclusive experiments have heretofore required up to 2 years. Although a positive SPAT assay does not guarantee ultimate leukemogenesis where titers are very low (<0.5 logs), the requisite for establishing in vivo growth potential is essential.

The SPAT assay has also been found by the authors to be useful in detecting small amounts of residual infectious virus in concentrated (10^{11} particles/ml) formalin-inactivated preparations. Two of these formalin-treated preparations were found to be negative in the XC assay (10) but positive by the SPAT assay for infectious MLV, probably because of interference by the high concentration of inactivated virus particles in these preparations and the limited number of susceptible cells in a given culture dish. Detection of infectious R-MLV by the SPAT procedure in the formalin-inactivated preparations was accomplished at 14 and 28 days, respectively, the latter being negative at 14 days.

**Table 3. In vivo (SPAT) and in vitro (CoMuL) MLV isolation as an indicator of leukemogenic potential**

| MLV isolation results by: | No. of inocula* | Incidence of LR neoplasms* | No. of inocula giving significant induction rates* |
|--------------------------|-----------------|---------------------------|-----------------------------------------------|
| B/c CoMuL               | B/c-E NIH-E     |                           |                                               |
| + + -                    | 32              | 191/546 (35%)             | 23 (73%)                                     |
| + - -                    | 15              | 51/236 (22%)              | 4 (29%)                                      |
| - + -                    | 8               | 22/138 (16%)              | 1 (12%)                                      |
| - - +                    | 5               | 13/120 (11%)              | 0 (0%)                                       |
| + + +                    | 63              | 30/87 (34%)               | 4 (80%)                                      |
| + - +                    | 2               | 81/869 (9%)               | 4 (6%)                                       |
| Controls (uninoculated)  | 23/230 (10%)    |                           |                                               |

* Cell-free preparation of spleens and tumors from BALB/cCr mice bearing spontaneous neoplasms (7).

* LR neoplasms, lymphoreticular neoplasms, including lymphocytic leukemia, lymphosarcoma, and reticulum cell neoplasms.

* Each individual inocula was evaluated by X^2 statistics for rates of lymphoreticular neoplasms, which exceeded the control rate (23/230, 10%). The number of inocula giving a P value of <0.05 is shown. Of the 36 inocula where the P value for significant induction was <0.05; 28 had values of <0.005.

* P < 0.005 when compared to control rate (23/230). Remaining incidence rates do not differ significantly from the control rate at P ≤ 0.05.
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