JOURNAL OF VIROLOGY, Apr. 1970, p. 437-445

Vol. 5, No. 4
Printed in U.S.A.

Focus Formation by a Murine Sarcoma-Leukemia Virus Complex

II. Quantitative Aspects of the Interaction Between Radiation Leukemia Virus and Its Murine Sarcoma Virus Pseudotype in Strain C57BL Mouse Embryo Cells

A. DECLÈVE,[1] M. LIEBERMAN, G. M. HAHN,[2] AND H. S. KAPLAN

Department of Radiology, Stanford University School of Medicine, Stanford, California 94305

Received for publication 8 December 1969

A quantitative study has been made of the interactions between radiation leukemia virus (RadLV), its murine sarcoma virus pseudotype, and their C57BL host cells. The elimination of interference phenomena by delayed infection of cells with RadLV made possible the quantitative determination of the pseudotype in terms of defective sarcoma and endogenous RadLV particles. This in turn permitted the quantitative assessment of RadLV helper activity and of the various factors which influence the accuracy and sensitivity of the helper assay.

Research in the field of murine leukemia has long been hampered by the lack of a rapid and precise in vitro procedure for the titration of the corresponding viruses. An indirect approach to such an assay finally became possible with the discovery of murine sarcoma virus (MSV) (6, 10), which can be assayed in vitro by the formation of foci of morphologically altered cells (5, 11). This virus undergoes loss of infectivity in the hamster and can be rescued from the defective state with the help of murine leukemia viruses (7).

By studying titration patterns of MSV, O'Connor and Fischinger (12, 13) showed that three viral components are involved in focus formation: defective MSV particles, leukemia virus, and fully competent MSV particles, which appear to consist of aggregates of the first two. With this knowledge, it became possible to titrate murine leukemia viruses in terms of their helper activity.

Radiation leukemia virus (RadLV) was first tested for helper activity by Igel et al. (8), who established that it does behave as a helper virus, and that a RadLV pseudotype of MSV can be obtained. Later, Fischinger and O'Connor (3) adapted the quantitative helper titration to RadLV, using as indicator the Moloney leukemia virus (MLV) pseudotype of MSV, MSV(MLV), and Swiss mouse embryo fibroblasts as host cells. Attempts to use cells from C57BL mice, the host of origin of RadLV, resulted in reduced focus formation.

It appeared of interest to us to investigate RadLV helper activity, using the RadLV pseudotype of MSV and C57BL cells. From a practical standpoint, it would avoid the introduction into the laboratory of an extraneous virus, MLV. Titration of RadLV would thus take place entirely within its own system, and might result in a more sensitive assay. Furthermore, the characterization of our MSV(RadLV) stocks in terms of their viral components should be possible in such a system.

MATERIALS AND METHODS

Equations and symbols used. The equations used in the text are fully explained and justified in the accompanying article (4); we summarize them here for the convenience of the reader: equation 6, $P_T \cong dV/NK^i$; equation 5, $l_{ind} \times d = N[NP_T(0)]/V^2$; equation 9, $l(\text{assay}) = N\Delta F(0)/[V(\text{assay})V_{ind}\, d]K^{-i}$. P_T = probability of transformation; N = number of cells; K^i = dilution factor; $NP_T(0)$ = extrapolation to zero dilution of foci per dish; $\Delta F(0)$ = extrapolation to zero dilution of Δ foci; V = volume of fluid containing virus particles; C, D, L = competent, defective, leukemogenic particles; c, d, l = concentrations of above (in particles per milliliter); L_{ind} = leukemogenic particles in indicator; L(assay) = leukemogenic particles in helper.

Accuracy considerations. Error in estimate of cell number, ±5%; error in volume accuracy, ±5%; error in extrapolations, ±5%; error in dilutions (at

[1] Present address: Centre d'Etude de l'Energie Nucléaire, Mol-Donk, Belgium.

[2] Holder of a Dernham Senior Fellowship in Oncology from the American Cancer Society.

1:160), ±3%; standard error of the mean as indicated in the figures.

Derivation of virus stocks. RadLV was used either as a cell-free extract in phosphate-buffered saline (PBS) of RadLV-induced lymphoid tumors of C57BL/Ka mice (9) or as plasma of W/Fu rats with RadLV-induced autochthonous or isologously transplanted lymphomas (1). For some experiments, the plasma-derived virus was purified by banding on PBS-sucrose density gradients and by using the active fraction at density 1.16 g/cm^3. The virus-containing rat plasma and the density gradient bands were obtained through the courtesy of E. F. Walker of this department.

MSV(RadLV) was obtained by a modification of the method of Igel et al. (8) for the rescue of the defective MSV genome by RadLV; the original procedure was not successful in our hands. The modification (M. Lieberman, A. Declève, and H. S. Kaplan, Proc. Amer. Ass. Cancer Res., p. 51, 1969) consisted of (i) exposing the hamster tumor line HT-1, which is known to bear the defective MSV genome, to X-ray irradiation in a single dose of 5,000 r, to counteract its tendency to overgrow the normal cells; and (ii) substituting RadLV-infected cultures of infant C57BL/Ka spleen or thymus for the embryonic fibroblasts used by Igel et al. (8). The HT-1 cell line was obtained through the courtesy of H. Igel, National Institutes of Health, Bethesda, Maryland. After co-cultivation for 1 week, the mixed cell population was injected into neonatal C57BL recipients, both intramuscularly and intraperitoneally. In 7 of 12 animals, localized tumors in the thigh and on the diaphragm developed after about six weeks and were found on histologic examination to be fibromyosarcomas. Similar tumors, which grew progressively and killed nearly 100% of the recipients in 2 to 3 weeks, were produced by injection of coarse cell suspensions from the primary tumors, and of cell-free extracts of such transplant tumors into neonatal C57BL hosts. Of the other strains tested, only BALB/c mice and their F_1 hybrids with C57BL were found susceptible to sarcoma induction. The rescued MSV genome thus appears to have the sarcomagenic host range that would be expected of the RadLV pseudotype (8). In addition, recent work indicates that it is neutralized by anti-RadLV serum. For these reasons, we designated it MSV (RadLV); subsequent cell-free passages have been numbered P_1, P_2 .... In the studies reported here, we used the sixth passage of the pseudotype and refer to it as P_6 indicator virus.

Preparation of virus-containing extracts. The same procedure was used in the preparation of RadLV and of the MSV(RadLV) pseudotype. The source of material was, in the case of RadLV, lymphoid organs from leukemic C57BL mice and, in the case of MSV(RadLV), the sarcomatous growth at the site of virus injection, usually the thigh of mice of the same strain. The entire procedure was carried out at 4 C. The tissue excised from the donor animals was ground in a mortar with sand, and the resulting homogenate brought to 20% with PBS. This suspension was then clarified by four cycles of centrifugation of 10 min each at ca. 2,000 × *g*, in the multispeed attachment of the International refrigerated centrifuge. The final supernatant fraction served as virus stock and was stored frozen in liquid nitrogen.

In the case of MSV(RadLV) extracts, an additional step, designed to remove competent aggregates (13), was introduced. The 20% extract was diluted 1:10 in PBS and subjected to centrifugation at ca. 3,000 × *g* for 15 min in the SW 39 rotor of the Beckman-Spinco preparative ultracentrifuge. The upper 3/4 of the centrifugate was carefully aspirated, and the bottom layer discarded. The supernatant fluid was shaken on a Vortex mixer, at the time dilutions were made for inoculation of cell cultures and just before inoculation of the cultures.

Cell culture. Strain C57BL/Ka mouse embryo fibroblasts (MEF) were used for the focus assay of MSV(RadLV) and for the assay of RadLV helper activity.

Cells (12×10^6) from embryos aged 12 to 14 days were plated in 75-cm^2 plastic culture flasks (Falcon Plastic, Los Angeles, Calif.) in a growth medium consisting of McCoy's medium (Grand Island Biological Co.) with 15% fetal bovine serum (heated 30 min at 56 C) and 100 μg/ml of penicillin and streptomycin. The cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2 in air.

Secondary cultures were prepared 5 days later by trypsinization. Suspensions of 4×10^5 cells in 5 ml of growth medium were plated in integrid culture dishes (60 by 15 mm; Falcon Plastic, Los Angeles, Calif.) and incubated for 24 hr as above. At that time, the cultures were ready for infection. One or 2 dishes per tray of 12 were trypsinized for a direct cell count; cell density was usually 1.5×10^5 to 3.5×10^5 per dish. In each experiment, the actual cell number found was used for the calculation of virus titer.

MVS(RadLV) focus assay. The focus assay procedure was essentially that of Hartley and Rowe (5) as modified by Fischinger and O'Connor (2). In each titration, the secondary MEF cultures were infected with at least seven serial dilutions of virus stock. At the lower dilutions, 6 dishes were used per dilution and up to 12 at the higher dilutions to decrease the standard error. Growth medium was removed from the dishes, and 0.2 ml of the appropriate MSV(RadLV) dilution was added. The dishes were returned to the incubator for 1 hr, and then received either 0.1 ml of medium or 0.1 ml of a saturating dilution of RadLV (*see* below for definition). After additional incubation for 1 hr, the cultures were fed with 5 ml of maintenance medium, consisting of McCoy's medium with 5% agamma calf serum (heated for 30 min at 56 C), antibiotics, and 50 μg of mycostatin per ml. They were then incubated for 5 days to permit development of foci.

Counting of foci. To avoid the necessity of scanning all dishes on the same day, the cultures were permanently fixed, and foci were counted when it was convenient. The fixation procedure consisted of removing the medium from the cultures, washing with PBS, fixing for 10 min with 10% glutaraldehyde in PBS, washing with distilled water, and inverting to dry. At the time foci were to be counted, a small amount of water was added to the dishes, to facilitate

viewing. The foci were counted microscopically by two readers and by a third person whenever there was a discrepancy of more than 15%.

Assay of RadLV helper activity. The procedure used was similar to that just described for focus assay with the following exceptions: the secondary cultures were infected with 0.2 ml of a predetermined dilution of MSV(RadLV) indicator, and, after 1 hr of incubation at 37 C, 0.1 ml of serial, 1.5-fold dilutions of RadLV was added.

RESULTS

Effects of interference and their elimination. The focus assay of MSV(RadLV) yields the results shown in Fig. 1. When the indicator stock obtained after centrifugation and shaking is used, the titration curve is a straight line with a 45° slope, indicating that few, if any, competent particles remain. If, however, the final shaking step is omitted, the curve flattens out at the higher virus dilutions, indicating that competent virus is present. This confirms the earlier suggestion (13) that competent particles consist of aggregates of leukemia and defective sarcoma virions. The tendency to aggregate appears to be strong: after centrifugation to remove existing aggregates, competent particles are still evident, presumably formed by reaggregation. However, such aggregates must be loosely bound, since they can be dispersed by mere shaking.

By extrapolation to zero dilution of the straight line in Fig. 1, it is possible to determine the titer of the preparation expressed as focus-forming units per milliliter ($4.4 \pm 0.2 \times 10^6$). This value, however, gives no indication of the actual composition of the pseudotype in terms of defective (D) and leukemogenic (L) virions; it merely reflects the combined effect of these two types of particles on the host cell. By use of equation 5, the additional information may be gained from the focus-forming units per milliliter value, that the product of $I_{ind} \times d$ is

$$\frac{N[NP_T(0)]}{V_2} = \frac{2.5 \times 10^5(4.4 \pm 0.2 \times 10^6)}{4 \times 10^{-2}}$$

$$= 2.8 \pm 0.1 \times 10^{13} \text{ (particles/ml)}^2$$

To obtain an independent estimate of the value of d, a titration must be performed with sufficient helper virus (L particles) present to permit essentially every D particle to express itself. Only under such conditions can equation 6 be applied.

We performed a helper assay by the procedure of Fischinger and O'Connor (2) to establish the amount of RadLV which must be added to achieve helper saturation. The results, plotted as Δ foci versus dilution of RadLV, are shown in Fig. 2. The general appearance of the curve can be described as follows: a first region, at virus dilutions <10-fold, showing diminished focus formation, consistent with the finding of Fischinger and O'Connor, which has been attributed to interference; a second region (at

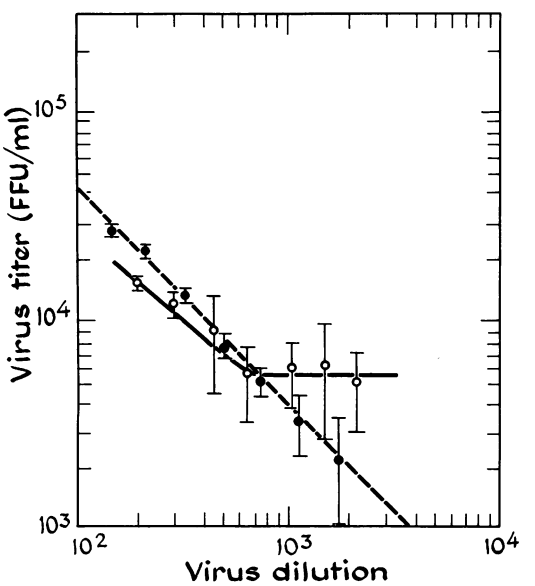

FIG. 1. *Focus titration pattern of P_6 indicator on C57BL MEF. Symbols: ○, virus preparation centrifuged; ●, virus preparation centrifuged and shaken.*

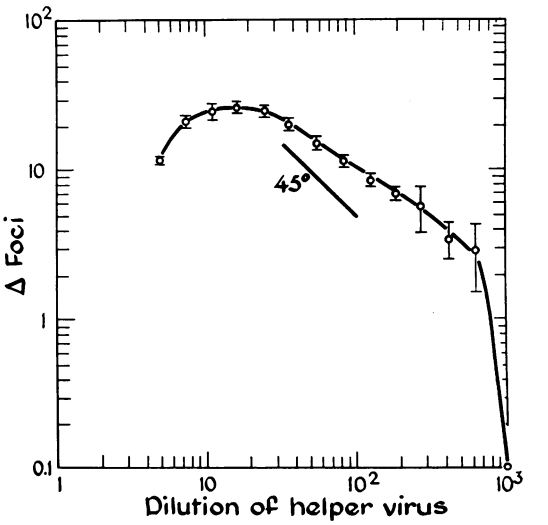

FIG. 2. *Effect of interference on helper assay of RadLV with P_6 indicator. Increasing dilutions of RadLV were added to cultures, together with a dilution of indicator which by itself yielded 6.7 ± 0.5 foci.*

10- to 30-fold virus dilutions) featuring an apparent plateau ("pseudo-plateau"); and a third region (at dilutions of 30- to 650-fold) which is more or less linear, but with a slope of less than 45°. The sharply descending last portion of the curve, at higher dilutions, is an artifact of the plotting procedure. The Δ foci here approach zero (and the log of 0 approaches minus infinity). From the overall appearance of the curve, one may suspect that interference is still effective at helper dilutions well beyond the pseudo-plateau. Clearly this experiment cannot yield data useful for the calculation of helper activity. The helper assay procedure requires a true plateau followed by a linear region of 45° slope.

We therefore investigated the possibility that sequential application of the indicator and RadLV might significantly reduce interference. Prior infection with indicator should permit the defective particles, which, as will be shown later, are relatively few in number, to attach to sites on the cell membrane but leave additional sites vacant for subsequent infection with RadLV. Conversely, prior infection with RadLV might permit the leukemia virions to occupy most sites, thereby reducing the probability of coinfection with defective sarcoma virus particles. To test this possibility, cultures were infected by three procedures: T0 procedure, in which both virus preparations were added to the culture dishes within 5 min of each other; T+ procedures, in which helper virus was added 1, 2, or 3 hr after infection with indicator; and T− procedures, in which helper virus was added 1, 2, or 3 hr before infection with indicator. Control cultures received an equivalent volume (0.1 ml) of medium in the place of helper virus preparation. In Fig. 3, the upper curves are numbers of foci obtained with indicator alone (curve b) and indicator plus RadLV (curve a). The increase in number of foci seen in curve b for the T+ procedures is probably a result of the increased time interval between infection by indicator and dilution of the virus by feeding, allowing for improved contact between the host cell and both L_{ind} and D virions. Decreases in number of foci for the T − 3 procedure may be partially due to the fact that 0.2 ml of medium was added to the cultures 1 hr before the indicator. This was done to keep the cells moist and may have diluted the virus somewhat, thereby reducing contact with the cells.

From curve 3a, it is difficult to estimate accurately the extent of interference and the time intervals during which it is operative. For this reason we have calculated curve 3c which normalizes curve 3a to take into account the differences in numbers of foci obtained with the different T procedures, relative to the results with indicator alone (curve 3b). Since two types of particles are involved in focus titration, we have subtracted the square roots rather than the actual focus numbers.

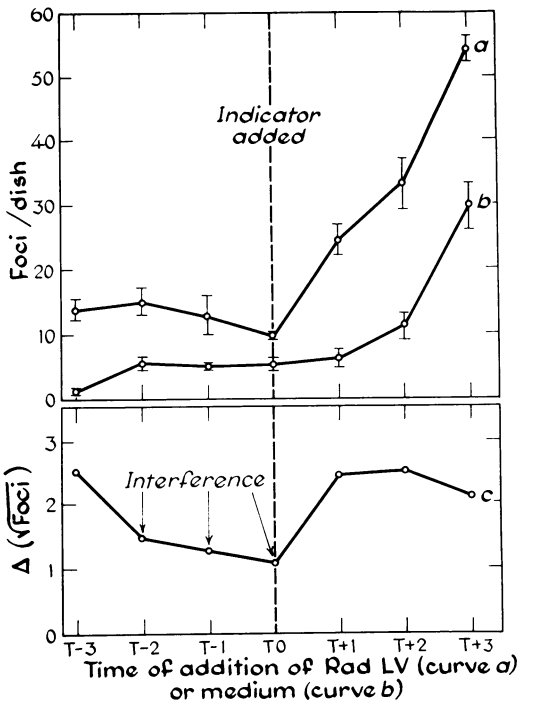

FIG. 3. *Results of timing experiments. P_6 indicator diluted 1:380; RadLV stock diluted 1:5. Curve a, infection with both indicator and RadLV. Curve b, infection with indicator only. Curve c, data of curve a normalized to accommodate variations in the number of foci obtained with indicator alone (curve b).*

Curve 3c shows that interference occurs with the T0, T − 1, and T − 2 procedures. It appears to lessen from T0 to T − 3, at which point it is no longer observable, for reasons which are not clear at present. It is evident that the T+ procedures effectively abolish interference.

Focus titrations were performed with the indicator alone by the use of procedures T − 3, T0, and T + 3 to ascertain that the differences observed in Fig. 3, curve b, would be maintained at other virus dilutions. The results plotted in Fig. 4 yield three parallel straight lines of 45° slope. Other experiments, however, have revealed that the displacement of the parallel lines is not constant from one experiment to the other. The number of foci to be expected from a given indicator with the T + 3 procedure is thus not predictable; hence, the T + 3 procedure is not appropriate for routine use. The method of

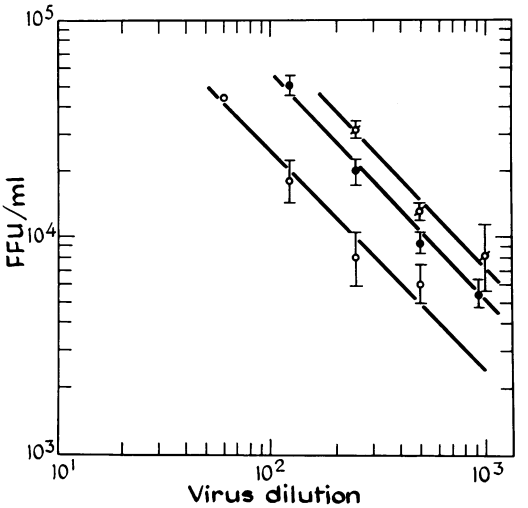

FIG. 4. *Titration patterns of P_6 indicator for T0, T − 3, and T + 3 procedures. The fact that these lines are essentially parallel justifies the procedure used to obtain Fig. 3c. The slopes (least squares) are −0.98, −1.06, and −0.99 for T + 3 (Ø), T0 (●), and T − 3(○), respectively. Corresponding residual variance values are 0.005, 0.005, and 0.011.*

choice appears to be the T + 1 procedure, since (i) there is no interference at this interval; (ii) the level of foci produced by indicator alone is predictable, i.e., it is the same as that found at T0; and (iii) the procedure is more rapid, so there is less chance of damage to the host cells. The T + 1 procedure was therefore routinely employed for helper assays in all subsequent studies.

It may be pointed out that interference depends not only on the time interval between inoculation of indicator and of helper, but also on their respective concentrations. Thus it was found that a RadLV dilution which produces interference with a fairly concentrated indicator preparation will not do so when used with a less concentrated indicator. However, any advantage derived from the use of a less concentrated indicator is nullified by the resulting loss in sensitivity.

Composition of indicator and assay of RadLV. A series of experiments was performed to study the composition of MSV(RadLV) and to measure the helper activity of RadLV. The T + 1 procedure was used and control assays were run simultaneously with the same ingredients, but following the T0 procedure (Fig. 5). It is evident that the curve depicting the T0 assay has essentially the same features as already described for Fig. 2; a few additional comments are in order. (i) The curve appears to have a linear segment with a 45° slope. That part of the curve could be used for extrapolation to zero dilution, but it is located at low Δ foci values. Here the standard errors become quite large, a necessary consequence of Poisson statistics and experimental limitations. This portion of the curve is, therefore, unsuitable for precise determinations. (ii) It will be noted that the standard errors are also quite large in the region of the pseudo-plateau, although for a different reason: there is considerable variation in numbers of foci in replicate dishes at this level. In addition, the foci are not well defined, leading to difficulty in counting.

Elimination of interference by the use of the T + 1 procedure results in the titration pattern also shown in Fig. 5. This curve differs from the preceding in the following respects.

(i) The 45° slope extends to higher values of Δ foci with resultant improvement in standard error and suitability of this portion of the curve for extrapolation.

(ii) A true plateau is reached, at higher Δ foci levels than could be obtained by the T0 procedure, indicating that saturation of the system with helper has been achieved. Note the small standard error in this zone, due to the greater constancy in numbers of foci and their more characteristic appearance, hence, ease of counting.

It seemed advisable to repeat the helper assays just described with a more active RadLV prepa-

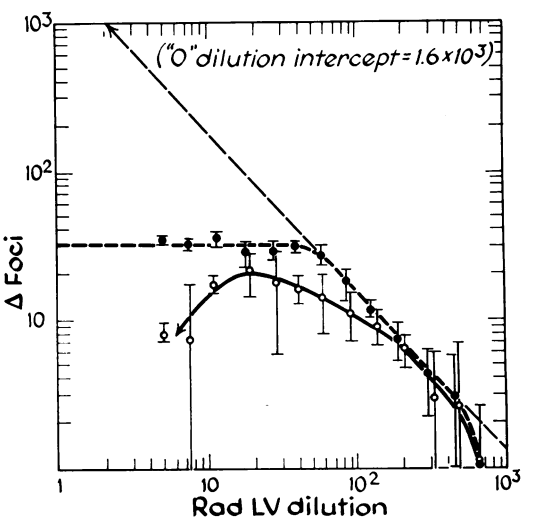

FIG. 5. *Comparison of T0 and T + 1 procedures for helper assay. The P_6 indicator alone, diluted 1:160, yielded 32.1 + 2.2 foci. Symbols: ○, T0 procedure; ●, T + 1 procedure.*

ration, so that it might be possible to extend the saturation plateau beyond the 10-fold dilution feasible with mouse-derived virus. A highly viremic rat plasma was used for this purpose (Fig. 6). It is evident that, provided the plasma virus has been purified by banding on a density gradient, the T + 1 procedure eliminates interference even at high RadLV concentrations, whereas the T0 procedure leads to the previously described interference effects. When nonpurified rat plasma is used, there appears to be inhibition of focus formation at low dilutions. This effect gradually subsides with dilution and the titration curve reaches the same plateau level as that observed for the purified preparation. The mechanism of this inhibition is not understood; immunological factors may be involved.

The T + 1 curve can be used for determination of the viral components of the system. The concentration of defective particles can be derived from the plateau level by use of equation 6. As an example, one may use the information obtained in Fig. 5. In the experiment in Fig. 5, 2.5×10^5 cells/dish were counted at the time of infection. The indicator, P_6, yielded an average of 32.1 foci by itself at the 1:160 dilution used. The observed value for Δ foci was 31; hence, the total number of foci at the plateau level was $32.1 + 31 = 63.1$. The concentration of defective particles in the diluted indicator is given by equation 6 for the 1:160 dilution: $d = P_T N/V = 63 \pm 2/(0.2 \pm 0.01) = 315 \pm 18$ defective particles/ml; the concentration in the undiluted indicator stock is then $315 \times 160 \cong 5.0 \pm 0.35 \times 10^4$ particles/ml. It was pointed out earlier, in the text discussion of the data of Fig. 1, that, for the indicator, $l_{ind} \times d = 2.8 \pm 0.2 \times 10^{13}$ (particles/ml)[2]. Hence there are in undiluted P_6 stock $2.8 \pm 0.2 \times 10^{13}/(5.0 \pm 0.35 \times 10^4) = 5.5 \pm 0.5 \times 10^8$ L_{ind} particles/ml.

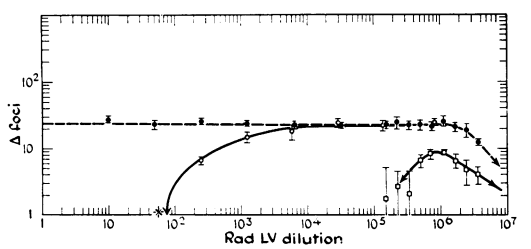

FIG. 6. *Helper assay with rat-derived RadLV. The P_6 indicator alone, diluted 1:250, yielded 13.0 ± 0.9 foci. Symbols: □, T0 procedure—RadLV purified by density gradient banding; ●, T + 1 procedure—RadLV purified by density gradient banding; ○, T + 1 procedure—non-purified RadLV. (*) The ΔF values for RadLV dilutions 1:10 and 1:15 are not shown because they represent numbers of foci lower than those given by indicator alone; such negative numbers would be meaningless in the graph.*

Figure 5 can also be used to calculate the number of virions [L(assay)] in the RadLV helper virus preparation, using either the procedure of Fischinger and O'Connor (2) or equation 9:

$$l(\text{assay}) = \frac{N\Delta F(0)}{d \times V(\text{assay}) \times V_{ind}} K^j$$

$$= \frac{2.5 \times 10^5 \times 1.6 \times 10^3 \times 160}{5 \times 10^4 \times 0.1 \times 0.2}$$

$$\cong 6.3 \pm 0.8 \times 10^7 \text{ particles/ml}$$

Importance of endogenous leukemia virus. If every defective particle in an indicator stock is to be expressed, sufficient helper must be available to saturate the system, i.e., to ensure that every host cell is infected by at least one leukemia virion. According to Poisson statistics, this means that there must be on the average five virions per cell; at this concentration, the probability that a given cell contains no virion is about 0.01.

The very high concentration of leukemia virions (L_{ind}) found in the P_6 indicator (5.5×10^8 particles/ml of undiluted stock) suggests that they may well contribute to saturation in assays of exogenous RadLV.

To test this hypothesis, we performed a focus titration of indicator in the presence of a constant amount of rat-derived RadLV. The numbers of reacting particles at each indicator dilution are given in Table 1, and the results of the experiment are expressed in Fig. 7 as foci versus dilution (A) and as focus-forming units per milliliter versus dilution (B). Under the conditions of this experiment, the system is saturated with helper virus when there are a total of approximately 1.5×10^6 leukemia virions present. The column showing total number of leukemia particles in Table 1 shows that the sum of $l_{ind} + l(\text{assay})$ falls below saturation level at indicator dilutions greater than about 1:150. The expected break in the titration curve is indeed observed in this region, verifying the hypothesis. From this point on, the number of foci becomes a function of the dilution of l_{ind} as well as of d; hence the 45° angle of the slope in curve 7b (corresponding to 63.5° in curve 7a).

This experiment illustrates the importance of specifying with accuracy the composition of the indicator to achieve a better understanding of the interactions between the defective sarcoma and helper viruses.

Maximum values of Δ foci at various indicator dilutions and their effect on assay accuracy and

TABLE 1. *Titration of P_6 indicator with constant amount of RadLV*[a]

Indicator dilution	P_6 indicator, 0.2 ml		Rat RadLV, 0.1 ml	
	No. of defective particles in indicator	No. of leukemia particles in indicator	No. of leukemia particles in assay material	Total no. of leukemia particles
1/100	1×10^2 [b]	1.1×10^6	9×10^5	2.0×10^6
1/150	0.66×10^2	7.3×10^5	9×10^5	1.6×10^6
1/225	0.44×10^2	4.8×10^5	9×10^5	1.3×10^6
1/337.5	0.29×10^2	3.2×10^5	9×10^5	1.2×10^6
1/506.25	0.19×10^2	2.0×10^5	9×10^5	1.1×10^6

[a] Total of 2.5×10^5 to 3.0×10^5 cells per dish were counted at the time of infection. Rat-derived RadLV, 0.1 ml of a $1:10^5$ dilution of a stock yielding 9×10^{11} L/ml. P_6 indicator, 0.2 ml of various dilutions of a stock containing 5.5×10^8 L/ml and 5×10^4 D/ml.

[b] Note that although the data are listed to two significant figures, these are merely the result of calculation and do not imply such accuracy. However, the small dilution steps used (1:1.5) do result in relatively good accuracy.

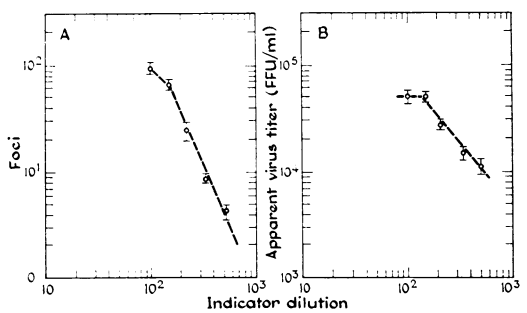

FIG. 7. *Focus titration of P_6 indicator in the presence of a constant amount of helper virus (see Table 1).*

sensitivity. An important consideration in the helper assay is the determination of the indicator dilution which will yield a large value of Δ foci, thereby increasing the sensitivity of the test. For this purpose, focus titrations were performed with P_6 indicator, either alone or in combination with a constant, saturating dilution of RadLV. The T + 1 procedure was followed, and the results are shown in Fig. 8, expressed as foci versus dilution and as focus-forming units per milliliter versus dilution.

At low dilutions of indicator, there is sufficient endogenous leukemia virus present to saturate the system; hence, the same results are obtained with or without added RadLV. As would be expected, the data points thereafter describe a straight line with a 45° slope when the system is saturated with RadLV and a 63.5° slope with indicator alone (Fig. 8A). In Fig. 8B, conversion of number of foci to focus-forming units per milliliter brings these slopes to 0° and 45°, respectively.

The values of Δ foci can be obtained from Fig. 8A by subtracting the numbers of foci in line b from those of line a. These Δ foci values are plotted in Fig. 9, which also shows estimates of standard errors calculated according to: $(SE_{\Delta\ foci})^2 = (SE_{ind})^2 + (SE_{Sat.\ RadLV})^2$.

It is evident that the largest values of Δ foci are obtained at indicator dilutions between 150 and 200, but it is also apparent that, at these dilutions, large standard errors are to be expected. Optimal dilutions appear to lie between 200 and 400.

The failure to reach a value of Δ foci higher than 30 to 35 stems from the high proportion of L_{ind} in the P_6 indicator. It points out the importance of determining the concentration of L_{ind} in an indicator stock, since this concentration plays a dominant role in the sensitivity of the helper assay.

DISCUSSION

In the foregoing experiments, we have described the interactions within a system consisting of RadLV, its MSV pseudotype, and C57BL MEF cells. RadLV was previously described (3) as essentially nonfunctional as a helper in C57BL cells. It should be noted, however, that the indicator, MSV(MLV), used in that study contained an unrelated murine leukemia virus, MLV. In the homologous system described here, the only leukemia virus known to be present in the assay was RadLV, and it behaved as a competent helper.

Our attempts to quantitate the viral components involved were hampered by the interference exerted by exogenous RadLV against MSV(RadLV) in the helper assay. When interference occurred, it was impossible to achieve expression of all the defective sarcoma particles (by saturation with RadLV), and hence to determine their number. Interference was over-

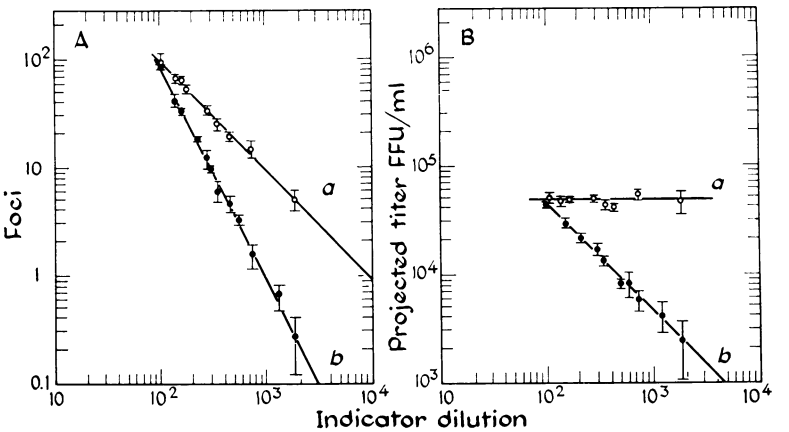

FIG. 8. *Assay of P_6 indicator on C57BL cells, expressed as: (A) foci, (B) focus-forming units per milliliter versus indicator dilution. Curves a (○), titration of indicator in the presence of a saturating concentration of RadLV helper and by using procedure T + 1. Curves b, (●), titration of indicator alone. The predicted slope, the measures slope (least squares estimates), and residual variance are, for the indicator alone, −1 (45°), −1.02, and 0.001, respectively. For the indicator plus helper, they are −2 (63.5°), −1.99, and 0.001.*

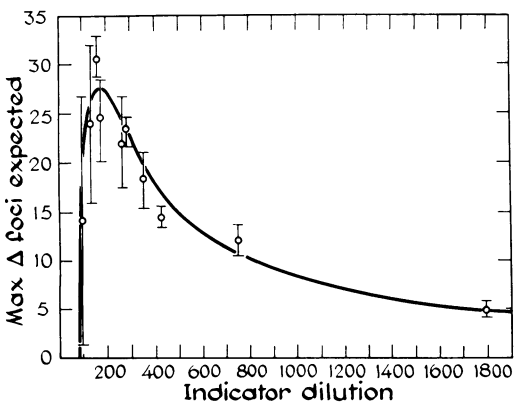

FIG. 9. *Expected values of Δ foci for various dilutions of P_6 indicator when saturating amounts of helper virus are added. Data are obtained. by subtracting curve b from curve a in Fig. 8A. Symbols: solid lines, calculated curve; ○, experimental values.*

come by a modification of the usual assay technique: a 1-hr interval was introduced between inoculation of cells with indicator and the addition of helper. This allowed maximal expression of the focus-forming capacity of MSV-(RadLV) and made possible the quantitative determination of this indicator in terms of its viral components.

It was found that MSV(RadLV) consists, as do other MSV pseudotypes, of defective sarcoma particles, endogenous RadLV, and competent units which probably are aggregates of the two. The 10^4:1 ratio of leukemia to defective particles which obtains in the indicator stock studied here is higher than that observed for other pseudotypes. It should be pointed out, however, that this ratio is based on the assumption (4) that the two types of particles are equally efficient in infecting host cells. A further point which should be noted is that there is no evidence as yet to indicate whether the leukemogenic and the helper activities of RadLV are located in the same particle. Therefore, when mention is made in this article of leukemia virus particles, endogenous or exogenous, no direct reference to their leukemogenic activity is intended.

The data presented are consistent with the theoretical formulation and the computer simulation described in a companion paper (4). The internal consistency of the theory and the data suggests that the assumptions upon which the theory is based are probably not far removed from biological reality.

ACKNOWLEDGMENTS

The competent technical performance of Gayle Hershberger and Laure Paul is gratefully acknowledged.

We are indebted to Florence Wong for programming the statistical analysis on the Stanford Advanced Computer for Medical Education (ACME) system.

This investigation was undertaken during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer to Alain Declève, and was supported by U.S. Public Health Service research grants CA-03352 and CA-10372 from the National Cancer Institute.

LITERATURE CITED

1. Ferrer, J. F., and H. S. Kaplan. 1968. Antigenic characteristics of lymphomas induced by radiation leukemia virus (RadLV) in mice and rats. Cancer Res. 28:2522–2528.
2. Fischinger, P. J., and T. E. O'Connor. 1968. Tissue culture assay of helper activity of murine leukemia virus for murine sarcoma virus. J. Nat. Cancer Inst. 20:1199–1212.

3. Fischinger, P. J., and T. E. O'Connor. 1969. Radiation leukemia virus: quantitative tissue culture assay. Science **165**: 306–309.
4. Hahn, G. M., A. Declève, M. Lieberman, and H. S. Kaplan. 1970. Focus formation by a murine sarcoma-leukemia virus complex. I. Theoretical analysis. J. Virol. **5**:432–436.
5. Hartley, J. W., and W. P. Rowe. 1966. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proc. Nat. Acad. Sci. U.S.A. **55**:780–786.
6. Harvey, J. J. 1964. An unidentified virus which causes the rapid production of tumours in mice. Nature (London) **204**:1104–1105.
7. Huebner, R. J., J. W. Hartley, W. P. Rowe, W. T. Land, and W. J. Capps. 1966. Rescue of the defective genome of Moloney sarcoma virus from a non-infectious hamster tumor and the production of pseudotype sarcoma viruses with various murine leukemia viruses. Proc. Nat. Acad. Sci. U.S.A. **56**:1164–1169.
8. Igel, H., R. J. Huebner, B. Deppa, and S. Bumgarner. 1967. Rescue of the defective murine sarcoma virus genome by radiation induced leukemia virus from C57BL mice. Proc. Nat. Acad. Sci. U.S.A. **58**:1870–1877.
9. Lieberman, M., and H. S. Kaplan. 1959. Leukemogenic activity of filtrates from radiation-induced lymphoid tumors of mice. Science **130**:387–388.
10. Moloney, J. B. 1966. A virus-induced rhabdomyosarcoma of mice. Nat. Cancer Inst. Monogr. **22**:139–142.
11. O'Connor, T. E. 1968. Quantitative *in vitro* studies on a murine sarcoma-leukemia virus complex, p. 64–80. *In* W. Dameshek and R. M. Dutcher, Perspectives in leukemia. Grune and Stratton, Inc., New York.
12. O'Connor, T. E., and P. J. Fischinger. 1968. Titration patterns of a murine sarcoma-leukemia virus complex: evidence for existence of competent sarcoma virions. Science **159**: 325–329.
13. O'Connor, T. E., and P. J. Fischinger. 1969. Physical properties of competent and defective states of a murine sarcoma (Moloney) virus. J. Nat. Cancer Inst. **43**:487–497.