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Inherited Resistance to N- and B-Tropic Murine Leukemia Viruses In Vitro: Titration Patterns in Strains SIM and SIM.R Congenic at the Fv-1 Locus

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We have investigated the titration patterns of murine leukemia viruses on mouse embryo cultures derived from a pair of congenic strains differing at the Fv-1 locus. XC plaque and infectious center assays carried out with N- and B-tropic viruses on both SIM(Fv-1^{nn}) and SIM.R(Fv-1^{bb}) host cells yielded results that were best approximated by Poisson one-hit curves. Titration curves of N-tropic virus by direct XC plaque assay were linear and parallel on the different hosts, with titers 1.8 to 2.7 $\log_{10}$ lower on SIM.R and on (SIM × SIM.R)F_1 than on SIM cells; similar linear and parallel curves were found for B-tropic virus, with titers 1.4 to 2.0 $\log_{10}$ lower on SIM and (SIM × SIM.R)F_1 than on SIM.R cells. In the infectious center assays, the proportion of infected cells was linearly related to multiplicity of infection on both permissive (N- on SIM and B- on SIM.R) and restrictive (B- on SIM and N- on SIM.R) genotypes at multiplicities of infection below 0.5; the line relating the variables was about 1 $\log_{10}$ lower in the restrictive than in the permissive situations. At multiplicities of infection where the proportion of infected cells reached a plateau, differences between the results on permissive and restrictive genotypes were considerably reduced. This appeared to be due to the action of non-Fv-1 factors in permissive hosts. We conclude that the major action of the restrictive allele at the Fv-1 locus in this system is to reduce the probability of successful murine leukemia virus infection without a change in hitness.

Cellular resistance to infection by N- and B-tropic murine leukemia viruses (MuLV) is known to be controlled at the Fv-1 locus (10, 11), linkage group VIII, chromosome no. 4 (14). Titrations of murine leukemia viruses on Fv-1 permissive cells exhibit a single-hit pattern, whereas in Fv-1 restrictive cells the titration pattern has been described as at least two-hit (4, 5, 9, 12, 15, 16). We wished to explore further (2, 15) the titration pattern of murine leukemia viruses of different tropism on cells derived from mouse strains congenic at the Fv-1 locus, strains SIM (Fv-1^{nn}) and SIM.R (Fv-1^{bb}), and their F_1 hybrids (Fv-1^{nb}).

Evidence that these mouse strains differ at Fv-1 is provided by the fact that SIM cells are relatively restrictive for B-tropic virus and permissive for N-tropic virus, SIM.R cells show the reciprocal behavior, and (SIM × SIM.R)F_1 hybrid cells are restrictive to viruses of both tropisms (15). Furthermore, SIM and SIM.R mice differ with respect to their isozymes of glucose phosphate dehydrogenase, controlled at the Gpd-1 locus (14) which is known to be linked to Fv-1.

We now report that titrations of N- and B-tropic MuLV by XC plaque and infectious-center assays have yielded single-hit patterns on restrictive as well as on permissive host cells.

MATERIALS AND METHODS

MuLV's. The N-tropic virus used was originally Lilly's F-S virus (S-2597) obtained as a spleen extract from J. Hartley and W. P. Rowe in August, 1970 and passaged initially three times on noninbred Swiss, SIM secondary mouse embryo or NIH Swiss 3T3 (15) and then four to six times exclusively on SIM secondary mouse embryo cells before being used in the present experiments.

The B-tropic virus used was originally C57MC pool 1636 obtained from a methylcholanthrene-induced tumor in a C57BL mouse. It was received from Hartley and Rowe at the seventh passage on BALB/c secondary mouse embryo cells in August 1970. In our laboratory it was first propagated on BALB/c secondary mouse embryo or 3T3 cells (15) and since then exclusively on SIM.R mouse embryo cells. At the time of the present use it had been passaged six to nine times on SIM.R cells.

The NB-tropic virus used was originally Lilly's F-B (S-2598) received as a spleen extract from Hartley

and Rowe in August, 1970, initially propagated on any of several mouse strains (15) and then exclusively on SIM secondary mouse embryo cells for five passages before use.

The viruses were passaged on mouse embryo cultures at multiplicities of 0.1 to 0.2 PFU/cell. Tests to be detailed elsewhere gave no evidence that the Fv-1 status of the cells on which the viruses were passaged had any effect on the subsequent behavior of these viruses. Virus pools were prepared either in stationary cultures or in roller bottle cultures at 37 C for 2 days, 33 C for 4 days, and harvested on day 6. They were clarified by low-speed (2,000 × *g*-min) or high-speed (25,000 to 50,000 × *g*-min) centrifugation. Some clarified preparations were concentrated at 200,000 × *g*-h.

Cell culture. Primary cultures were prepared from pools of 15- to 17-day-old eviscerated SIM, SIM.R, and (SIM × SIM.R)F_1 hybrid embryos. Only the first passage of such cultures were used throughout. The medium was (Flow) supplemented with 10% fetal calf serum (Flow). Falcon plastic petri dishes (60 mm) were seeded at 2.5 × 10^5 cells/dish in a volume of 4 ml. The cultures were used 16 to 20 h later.

Virus assays. Two procedures were used to infect the cells. (i) The cells were treated for 1 h at 37 C with Polybrene (Pharmacia, Montreal) (8 μg/ml) (2 ml/dish) in serum-free medium. After removal of Polybrene, the cells were infected with 0.5 ml/dish of each virus dilution using medium without serum containing 4 μg of Polybrene per ml as diluent. Adsorption of viruses was done for 45 min at 36 C and then the medium with 10% FCS was added (4 ml/dish). (ii) The cells were treated for 45 min at 37 C with DEAE dextran (Pharmacia) (50 μg/ml) (2 ml/dish) in serum-free medium. The DEAE-dextran medium was removed and the cells were infected with 0.1 ml of virus dilution in complete medium per dish. Adsorption was carried out for 45 min at 37 C. The plates were washed with phosphate-buffered saline (PBS) and medium was added.

The XC plaque assay method was used (8, 13). Four days after infection the infected and control cultures were decanted, washed with PBS, irradiated for 30 s with UV (approximately 67 ergs/mm^2 per s), and 10^6 XC cells/dish in 4 ml of medium with 10% FCS added. Twenty-four hours later the medium was exchanged and the cultures were allowed to continue for a further 24 h, after which they were washed with PBS and fixed with methanol for 10 min. The cultures were stained for 45 min with Harris hematoxylin. XC plaques were scored by naked eye and the presence of giant cells was confirmed microscopically. In some experiments, microscopic plaques were scored separately under high power in gridded plates.

Infectious center assays were carried out as follows. Five hours after completion of the last step of infection (as described in ii), the medium was removed and the cells were dissociated with 0.25% trypsin, pelleted by low-speed centrifugation, resuspended, and an aliquot was counted in a Coulter electronic cell counter. Cells at five dilutions were seeded in quadruplicate onto monolayers prepared on the previous day (10^5 cells/well). Plating efficiencies under similar conditions have ranged from 45 to 65%. UV irradiation, addition of XC cells etc. were then carried out as described above in the XC plaque assay.

RESULTS

To examine the titration patterns of MuLV of different tropism on cells differing at the Fv-1 locus, XC plaque titrations of low-speed clarified N-tropic and B-tropic MuLV were performed on secondary MEC derived from strains SIM, SIM.R, and (SIM × SIM.R)F_1 hybrids. The results are shown in Fig. 1 for N-tropic and in Fig. 2 for B-topic MuLV. Each point represents the mean of four plates. It can be seen that in each titration all the experimental points lie within ± 1 standard deviation from the regression line (with the exception of the highest dilutions of the viruses). The slopes of all the regression lines are approximately one. The results thus indicate a single-hit titration pattern for both N- and B-tropic viruses on the permissive and on both of the restrictive genotypes.

The ratio of N-tropic virus titer on SIM (Fv-1^{nn}) cells/SIM.R (Fv-1^{bb}) cells was 2.7 $\log_{10}$ units. The (SIM × SIM.R)F_1 hybrid cells (Fv-1^{nb}) showed the same virus titer as SIM.R (Fv-1^{bb}) cells. For the N-tropic virus, more than half of all PFU registered as macroscopically visible plaques on all three cell genotypes. The ratio of B-tropic virus titer on SIM.R/SIM was 2.0 $\log_{10}$ units. The titer in (SIM × SIM.R)F_1 hybrid cells was close (3.2 × 10^2 PFU/ml) to that on SIM cells (2.9 × 10^2 PFU/ml). For B-tropic virus, only about 17% (14 to 22%) of all plaques registered as macroscopic plaques on the restrictive SIM and about 23% (22 to 27%) on permissive SIM.R cells.

Similar linear titrations were observed when untreated supernatants, high-speed clarified supernatants, and concentrates of N- and B-tropic MuLV were used to infect SIM and SIM.R cells. No differences in titration patterns were found when the infections were carried out in the presence of polybrene or DEAE-dextran.

In an attempt to eliminate virus aggregates, a concentrate of N-tropic virus was filtered through a 0.22-μm membrane filter (Millipore Corp.) and SIM and SIM.R cells were infected with the filtrate. Table 1 presents results of XC plaque assays on the concentrated, unfiltered virus and on the concentrated, filtered virus. The titrations were linear with both filtered and unfiltered virus on both genotypes. There was a 2.2 $\log_{10}$ unit ratio of the titer on SIM/

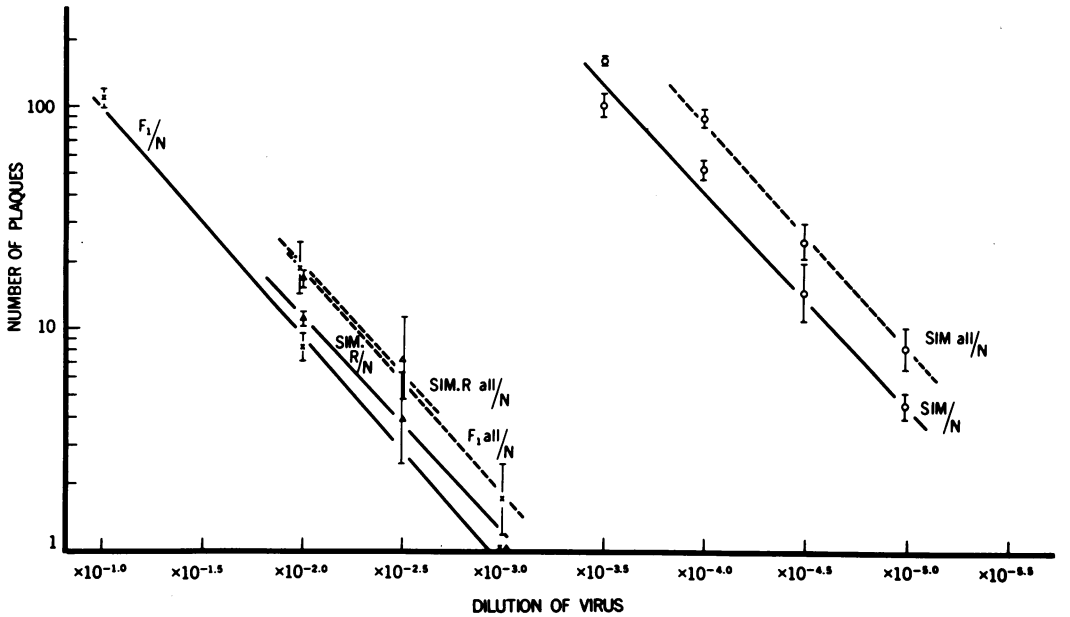

FIG. 1. *XC-plaque assays of N-tropic MuLV on secondary MEC from: SIM (Fv-1^{nn}), macro-plaques--○--, all plaques --○--; SIM.R (Fv-1^{bb}), macro-plaques -△-, all plaques --△--; (SIM × SIM.R)F$_1$(Fv-1^{nb}), macro-plaques -×-, all plaques --×--.*

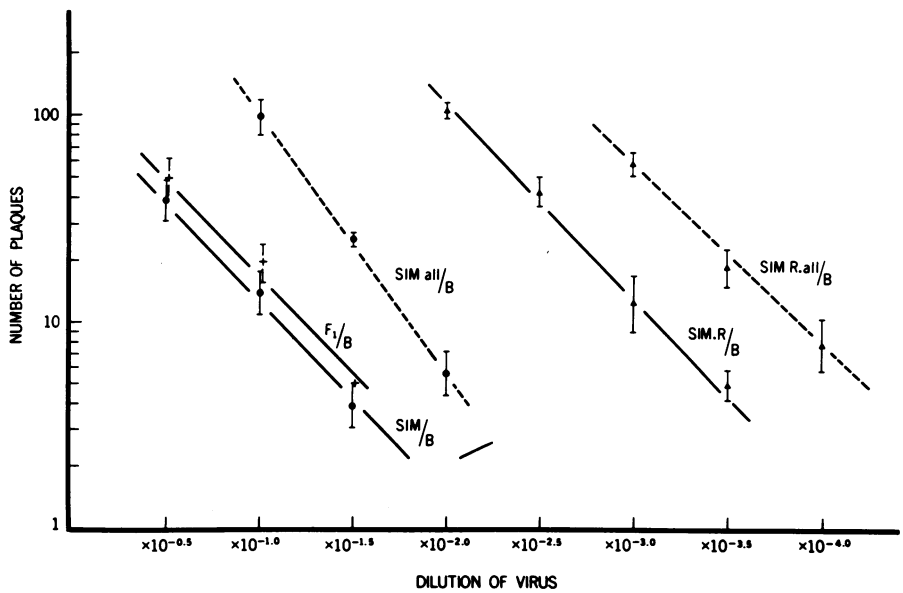

FIG. 2. *XC-plaque assay of B-tropic MuLV on secondary MEC from: SIM(Fv-1^{nn}), macro-plaques -●-, all plaques ---●---; SIM.R(Fv-1^{bb}), macro-plaques -▲-, all plaques ---▲---; (SIM × SIM.R)F$_1$(Fv-1^{nb}), macro-plaques, —+—.*

SIM.R after filtration. In a separate experiment the same N-tropic concentrate was filtered and titrated on (SIM × SIM.R)F$_1$ hybrid cells. The results (Table 1) again show that the titration was linear on these cells of Fv-1 restrictive genotype.

The direct XC plaque assay is restricted to a limited range of titration (1.5 to 2.0 log$_{10}$ units),

TABLE 1. *Effect of filtration of N-tropic MuLV on titration pattern*

	Dilution	No. of plaques/culture	Mean	Titer (PFU/ml)
Virus prepn unfiltered				
N-tropic on SIM	$10^{-5.5}$	T.N.T.C.[a]		
	10^{-6}	97, 77, 78, 84	84.0	8.4×10^7
	$10^{-6.5}$	26, 31, 20, 21	24.5	7.7×10^7
	10^{-7}	12, 5, 6, 8	7.8	7.8×10^7
	$10^{-7.5}$	2, 3, 2, 3	2.5	7.9×10^7
	10^{-8}	0, 0, 0, 0		
N-tropic on SIM.R	$10^{-3.5}$	117, 144, 136, 131	132	4.2×10^5
	10^{-4}	60, 62, 57, 50	57.3	5.7×10^5
	$10^{-4.5}$	18, 19, 12, 13	15.5	4.9×10^5
	10^{-5}	9, 5, 4, 3	5.25	5.2×10^5
	$10^{-5.5}$	0, 0, 0, 0		
Virus prepn filtered				
N-tropic on SIM	10^{-5}	109, 127, 126, 117	119.75	1.2×10^7
	$10^{-5.5}$	61, 56, 62, 64	60.75	1.9×10^7
	10^{-6}	12, 17, 14, 15	14.5	1.5×10^7
	$10^{-6.5}$	4, 6, 2, 6	4.5	1.4×10^7
	10^{-7}	0, 1, 0, 1		
N-tropic on SIM.R	10^{-3}	T.N.T.C.		
	$10^{-3.5}$	70, 61, 59, 63	63.25	2.0×10^5
	10^{-4}	27, 24, 32, 30	28.25	2.8×10^5
	$10^{-4.5}$	7, 9, 5, 8	7.25	2.3×10^5
	10^{-5}	1, 4, 1, 2	2.0	2.0×10^5
	$10^{-5.5}$	1, 0, 0, 0		
Virus prepn filtered				
N-tropic on (SIM × SIM.R)F_1 hybrid	10^{-3}	50, 49, 47, 45, 57, 59, 50, 51	51	5.1×10^4
	$10^{-3.5}$	17, 18, 11, 13, 25, 20, 10, 19	16.75	5.3×10^4
	10^{-4}	9, 7, 6, 2, 8, 6, 2, 1	5.125	5.1×10^4
	$10^{-4.5}$	1, 0, 2, 2, 1, 5, 0, 1	1.5	4.7×10^4
	10^{-5}	0, 0, 0, 0, 1, 0, 0, 1		

[a] T.N.T.C., Too numerous to count.

and requires multiplicities of infection less than 10^{-1} PFU/cell for restrictive cells and less than 10^{-3} PFU/cell for permissive cells. In order to extend this range we utilized the infectious center assay.

B-tropic virus was used to infect 10^5 SIM and SIM.R cells over the range of 189 PFU/cell to 8.0×10^{-2} PFU/cell. The cells were then dissociated and different numbers of cells (from 50 to 12,800) were plated on monolayers of either SIM or SIM.R cells. Four days later the XC procedure was carried out and macroscopic plaques were counted after 2 days.

The number of plaques was found to be directly proportional to the number of infected cells seeded. We found no difference in the number of plaques obtained when the monolayers on which the infected cells were seeded were SIM or SIM.R. It can be seen from Fig. 3 that the shapes of the titration curves of B-tropic MuLV on SIM.R and SIM cells both conformed closely to that of the theoretical Poisson one-hit curve but were significantly different from that of the two-hit curve. The slopes of the linear descending portion of the curves were 0.86 for SIM.R and 0.94 for SIM cells, neither being significantly different from unit slopes. At multiplicities of 1 PFU/cell or less, the proportion of B-tropic virus infected cells was sevenfold lower on SIM than on SIM.R cells. The curve of B-tropic virus on SIM.R cells appeared to be shifted horizontally to the left of the theoretical curve by about 1 $\log_{10}$ unit of multiplicity of infection, and to deviate slightly downwards from the theoretical curve with increasing multiplicity. At multiplicities of 10 PFU/cell or greater, the proportion of infected cells on both SIM.R and SIM reached a plateau with maximum values of 35% and 25%, respectively.

The above preparations of cells were used at the same time in the direct XC assay with the same B-tropic virus and with a preparation of filtered N-tropic virus. The titration patterns (data not shown) were linear over a range of 1.5 to 2.0 $\log_{10}$ units and yielded the following titers: SIM, B-tropic virus 7.9×10^6 PFU/ml, N-tropic virus 1.78×10^8 PFU/ml; SIM.R, B-tropic virus 1.24×10^8 PFU/ml, N-tropic virus 1.42×10^6 PFU/ml.

Figure 4 shows the results of titration of N-tropic virus on SIM and SIM.R cells by the infectious center assay, with a monolayer of SIM cells. Again it can be noted that the shapes of the curves for both conformed closely to that

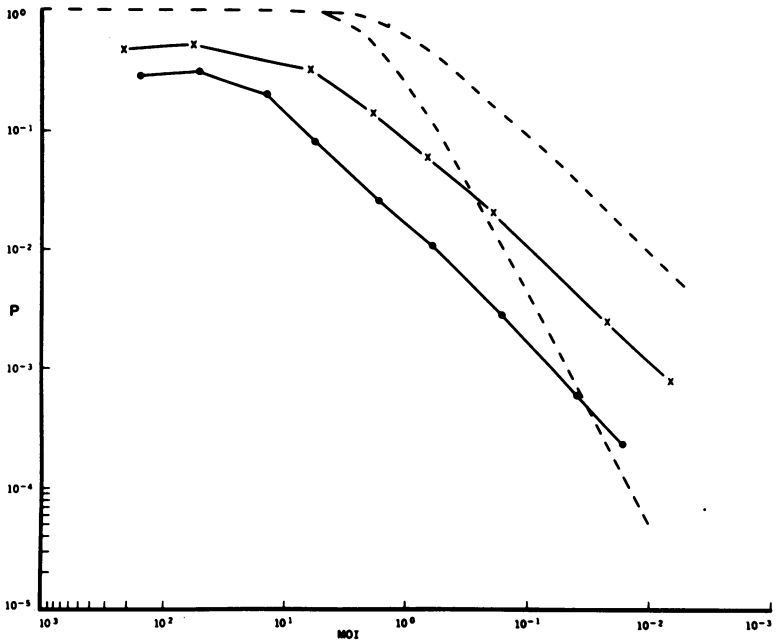

FIG. 3. *Infectious center assay of B-tropic MuLV. Symbols: ×, SIM.R cells and ·, SIM cells both seeded onto a SIM.R monolayer. In Fig. 3 and 4 the fraction of infected cells P (number of plaques/number of cells plated) is plotted against the multiplicity of infection (MOI) (no. of PFU added/no. of cells exposed to virus). The broken lines represent the theroretical Poisson one-hit and two-hit (rapid descent slope) curves plotted at an efficiency of one.*

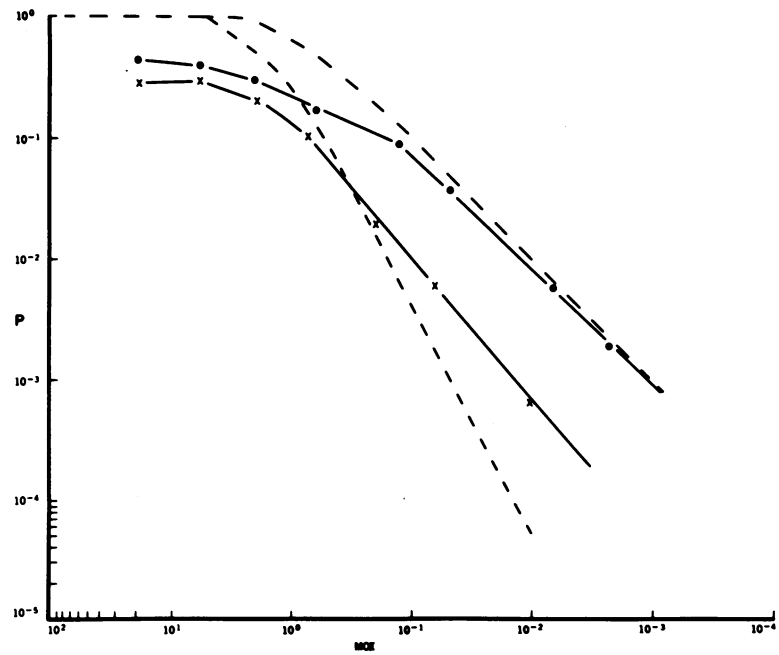

FIG. 4. *Infectious center assay of N-tropic MuLV in (×) SIM.R cells, and (·) SIM cells, seeded on a monolayer of SIM cells.*

of the theoretical Poisson one-hit curve. The slope of the linear descending portion of the curve of N-tropic virus on SIM cells was 0.88 and that on SIM.R cells, 0.96, neither being significantly different from unit slope. At multiplicities of 0.1 PFU/cell or less, the proportion of infected cells was approximately 10-fold greater on SIM than on SIM.R cells. The curve of N-tropic virus on SIM cells also coincided in position with the theoretical curve at multiplicities of infection below 0.1 PFU/cell, i.e., there was no horizontal shift. The curve deviated increasingly downward from the theoretical curve with increasing multiplicity. At multiplicities between 1 and 10 PFU/cell or greater, the proportion of infected cells on both SIM and SIM.R cells reached a plateau, with maximum values of 39% and 30%, respectively.

DISCUSSION

In the results presented here, as well as in others obtained in our laboratory over the last 2 years, we have found one-hit titration patterns with both N- and B-tropic MuLV on Fv-1 restrictive as well as on Fv-1 permissive host cells, using both direct XC plaque and infectious center assay methods. These observations are in accord with those of Jolicoeur and Baltimore (7).

Direct XC plaque assays on permissive and on restrictive genotypes gave rise to a series of parallel lines of approximately unit slope. Titers of virus were 1.4 to 2.7 $\log_{10}$ lower on Fv-1 restrictive than on Fv-1 permissive hosts. The magnitude of the restriction was approximately the same in cells of F_1 hybrids as in those of parent strains carrying restrictive alleles at Fv-1; thus Fv-1 restriction is completely dominant, showing no significant gene dosage effect.

Infectious center assays permitted a comparison of titration patterns over a much wider range of virus concentrations than direct XC plaque assays. They yielded curves which were best approximated by Poisson one-hit curves, parallel at multiplicities below 0.5. The proportion of infected cells was 0.5 to 1.0 $\log_{10}$ lower on Fv-1 restrictive than on Fv-1 permissive hosts.

Our results and those of Jolicoeur and Baltimore are at variance with those of several other workers (4, 5, 9, 12, 16) who have found two- or more-hit titration patterns on Fv-1 restrictive host cells. One-hit titration patterns might be spuriously produced by clumps of two or more virus particles. This is highly unlikely in the present instance since similar results were obtained with clarified virus, concentrated virus, and virus filtered through a 0.22-μm filter. It is possible that the shapes of the curves might be affected by differences in the ability of plaques to reach macroscopic dimensions. This possibility appears to be ruled out by the findings that our MuLV titration curves were of the single-hit type whether plaques were scored macroscopically or microscopically (Fig. 1 and 2). The presence in the host cells of endogenous MuLV acting as helper for a defective virion could theoretically make an infection that requires two particles appear as a one-hit titration curve. This possibility cannot be entirely excluded. However, all our assays were carried out on fresh explants of SIM and SIM.R embryos always at the first passage. In such cultures we have not been able to detect any endogenous virus after IdU treatment either by [^{3}H]uridine labeling of particles banding at type C virus density in sucrose gradients or by co-cultivation with SIRC cells (3) (M. E. Blackstein, unpublished observations).

In the present study, the host cells differed essentially only by the chromosomal segment bearing the Fv-1 locus (14, 15). Since other authors have used mouse strains differing at Fv-1 but not congenic, the multiple-hit titration patterns seen by them in restrictive situations may have been due to effects of gene loci other than Fv-1. Such factors as differing cell growth rates (4), times required for virus expression (4), virus passage histories, or possibly interference by endogenous viruses may also have been responsible.

We had earlier reported a multihit titration pattern of N-tropic virus on SIM.R cells based on direct XC plaque assays (15), whereas the present results are clearly of single-hit type. In the present experiments, which are much more extensive than the previous ones, culture conditions were better, and the virus was passaged on cells of the same genotype as those in which the experimental comparisons were made, and in identical media, all strongly indicating that our present titration patterns are correct. Moreover, reexamination of our previous data from three experiments indicates that even in those cases, though the titration patterns were apparently of multihit type, statistically single-hit patterns could not be ruled out. We conclude that the titration patterns of MuLV on both Fv-1 permissive and Fv-1 restrictive host cells are of single-hit type.

Although this was true in both Jolicoeur and Baltimore's (7) study and in our own, the results in the two studies were not identical at high multiplicities of infection. Jolicoeur and Baltimore found great differences between the proportion of infected cells on Fv-1 restrictive

versus permissive hosts at multiplicities of infection where the values plateaued (7). We observed only minor differences between the restrictive and permissive situations at these multiplicities.

This raises the question as to whether Fv-1 restriction is overcome at high multiplicities of infection in our system. This is obviously an important question since its answer could determine whether or not it will be possible to do meaningful biochemical experiments with this system in which high mutiplicities are necessary for practical reasons. Jolicoeur and Baltimore's data indicated that Fv-1 restriction was not overcome at high multiplicities (7). From our own data, the question cannot be answered with certainty.

It can be seen both in Fig. 3 and 4 that the descending portions of the curves are not exactly parallel but tend to converge upwards to some extent, i.e., the magnitude of the Fv-1 restriction seems to diminish with increasing multiplicity even at low multiplicities of infection. Although this would be compatible with the notion that Fv-1 restriction can be overcome, it is obvious that this convergence represents only a minor effect if significant at all. The curves would have to be extrapolated to 2×10^4 PFU/cell in Fig. 4 and 5×10^6 PFU/cell in Fig. 3 for them to meet. Thus the small differences between the percentage of infected cells in the restrictive versus the permissive situations at the plateau regions of the curves must have some other explanation. One possibility is that the titration curves for N-tropic virus on SIM and B-tropic virus on SIM.R cells may have, at plateau, reached the highest percentage of infected cells possible for this system, irrespective of multiplicity. Such factors as inherent limitation in the proportion of cells in the population available for infection, homologous viral interference, or factors limiting expression of the infected state of these cells in terms of plaque formation (refractoriness [12]) could be responsible. These would be limiting effects in the permissive situation and so could not be attributed to any action of the Fv-1 gene. The same effects may not have been seen in the Jolicoeur and Baltimore study (7) because their maximum percentage of infected permissive cells tended to be lower and the magnitude of their differences between Fv-1 permissive and restrictive hosts even at low multiplicities tended to be greater than ours.

We incline to the view that it is the visibility of the Fv-1 restriction rather than the restriction phenomenon itself that is impaired at high multiplicities of infection. Whichever way this matter is ultimately resolved, the present results provide clear evidence in favor of the concept that the essential effect of the restrictive allele at Fv-1 in this system is specifically to reduce the probability of successful infection by MuLV of opposite tropism, while maintaining the single-hit pattern of the virus infection.

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