Focus Formation by a Murine Sarcoma-Leukemia Virus Complex

I. Theoretical Analysis

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The transformation of cells in culture by a murine sarcoma-leukemia virus complex leads to the formation of identifiable foci. Titration patterns of focus counts vs. virus dilutions have been explained as being due to the interactions of three virions: leukemia, defective sarcoma, and competent hybrid. This interaction is analyzed quantitatively and equations are derived which permit determination of the composition of a sarcoma virus pseudotype as well as use of the technique for the assay of exogenous leukemia virus. The analysis is based on the statistical distribution of virions in the culture cells and assumes that all virions have equal probability of infecting a cell and that transformation of any one cell will lead to focus formation. Limitations of the assumptions and expected measurement accuracy are discussed.

Viruses of the murine sarcoma-leukemia complex can cause the formation of recognizable foci of transformed cells in tissue culture (3). In this respect, the murine viruses closely resemble the avian Rous viruses, studied by Rubin and associates (5, 6). Titration patterns of focus formation have been discussed by O'Connor and Fischinger (4), who postulate that the sarcoma-leukemia virus complex encompasses three types of virions: leukemia virus, defective sarcoma virus, and a hybrid "competent" virus. Focus formation can be initiated by the hybrid alone (hence the name "competent") but not by the defective sarcoma virus, which needs the helper activity of leukemia virus.

One of the interesting features of this system is that it can be used to assay leukemia virus preparations in vitro. For this purpose, a theoretical formulation of the virus kinetics leading to focus formation is essential. Fischinger and O'Connor (2) have developed approximate equations permitting some quantitation under restricted circumstances. The assay apparently has wide applicability, and this consideration has led us to derive expressions governing the probabilities of focus formation. The resulting equations can be applied directly, or, alternately,

graphic techniques can be used which yield the compositions of the virus mixtures involved in the assay. The usefulness of the equations is demonstrated in a companion paper (1).

FOCUS FORMATION

For the purposes of this analysis, focus formation may be considered to result from infection and transformation of a single cell. How the process continues from there (i.e., via production of additional virus and subsequent infection of adjacent cells, or via proliferation of transformed cells) does not affect the arithmetic presented here. We followed the experimental procedure outlined in the literature: a fixed volume of well-dispersed virus-containing material was added to a more or less confluent monolayer of cells in culture, and several days later the number of foci were counted. We then derived the equations which allow prediction of the number of foci expected for arbitrary combinations of the three types of virions.

We assume that infection of an individual cell, leading to focus formation, can occur in the following ways: (i) infection by one or more "competent" virus particles (C particles), (ii) joint infection by one or more "defective" virus particles (D particles) and one or more leukemia particles (L particles), and (iii) by a combination of the two methods just listed.

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We first derive the probability of infection of any one cell. Let the number of cells be denoted by N , the concentrations of C, D, and L particles (in particles per milliliter) by c , d , and l , respectively, and assume that a volume V of fluid containing the particles is added to the cell culture. The average number of particles available per cell is then cV/N , dV/N , and lV/N , respectively. Since the particles will distribute themselves according to Poisson statistics, the probability that a given cell is not infected by, say, a C particle is $e^{-cV/N}$ and the probability that it is infected by one or more is

$$P_C = 1 - e^{-cV/N} \quad (1)$$

For any Poisson process involving distributions of discrete elements the following is true: if, on the average, there are λ elements per unit volume, then the probability of finding exactly K elements in one unit volume is $P(\lambda, K) = e^{-\lambda} \lambda^K / K!$. Therefore, the probability of finding 0 elements is $P(\lambda, 0) = e^{-\lambda}$. Furthermore, since $\sum_{K=0}^{\infty} P(\lambda, K) = 1$, the probability for finding one or more particles in a given unit volume is $1 - P(\lambda, 0)$, which, after substituting for $P(\lambda, 0)$ leads to equation 1. Similar equations hold for P_D and P_L , the probabilities of infection with one or more D or L particles, respectively. The desired probability of transformation, P_T , is then, using basic laws of probability addition,

$$P_T = P_C + P_L P_D - P_C P_L P_D \quad (2)$$

If we now make the additional assumption that every cell transformed by virus produces one focus, then the expected number of foci is simply NP_T ; furthermore, Poisson statistics determine that the variance of the probability distribution, $\sigma_{P_T}^2$, is also NP_T .

Figures 1 and 2 are computer-calculated results of equation 2 for various combinations of parameters. The concentrations of leukemia and defective sarcoma virus assumed in the calculations are chosen to overlap some measured concentrations (1). The data are plotted in two ways: as expected number of foci per dish versus progressive virus dilution and as focus-forming units per ml versus dilution. Focus-forming units are obtained from focus counts by multiplying these by the dilution factor and then normalizing for the volume of test material used. This latter way of plotting the data is convenient for some of the extrapolations to be discussed in the following sections.

APPLICATION TO QUANTITATIVE LEUKEMIA VIRUS ASSAY

The assay is performed utilizing two virus preparations: the "indicator," a virus suspension containing unknown amounts of C, D, and L particles, and the assay material, containing only L virions. In principle, equation 2 can be used directly to estimate the concentrations of the various virus types. By carrying out serial dilutions of indicator material and determining the number of foci for each dilution, one obtains experimental curves analogous to the theoretical ones of Fig. 1 and 2. The computer can then be used to provide a best estimate (say, by a non-linear least-square procedure) of the values of c , d , and l_{ind} . Then, after adding a known amount of assay material, the titration is repeated and an estimate of c , d and $l(\text{total})$ is obtained, from which the concentration of leukemia virus in the assay material is obtained by subtraction. Although such a procedure is quite straightforward, it does require access to a digital computer (hardly an insuperable obstacle these days!) and availability of an appropriate program. In the absence of one or the other of these, a technique like the one of Fischinger and O'Connor (2) yields approximate results, as will now be shown.

FIG. 1. Titration patterns for indicators of different compositions (excluding effects of competent virions). Panel A, expected number of foci per dish. Panel B, expected number of focus-forming units per milliliter. Cell number was 3.5×10^5 ; volume of material per dish, 0.2 ml. Composition of indicator: curve f, $l = 10^{10}$, $d = 10^8$; curve g, $l = 10^8$, $d = 10^4$; curve h, $l = 10^8$, $d = 10^8$; curve i, $l = 10^7$, $d = 10^8$; curve j, $l = 3.3 \times 10^8$, $d = 3.3 \times 10^6$ (all in virions per milliliter). The indicator represented by f is not suitable for the assay; that of g only marginally so. Indicators represented by h, i, and j are all suitable. Their titers in focus-forming units are experimentally indistinguishable. However, obviously they differ substantially in concentration of total virions as well as in the ratio of L to D particles.

FIG. 2. Titration patterns for indicators of different compositions including competent virions. Panels A, C, E, expected number of foci per dish. Panels B, D, F, expected number of focus-forming units per milliliter. Panels A, B, $l = 10^6$, $d = 10^6$. Panels C, D, $l = 3.3 \times 10^7$, $d = 3.3 \times 10^7$. Panels E, F, $l = 10^8$, $d = 10^8$ (all in virions per milliliter). Other data same as in Fig. 1. Concentrations of competent virions are expressed as percentage of defective virions.

The first step in the procedure is to evaluate the number of competent particles contained in the indicator. Assume that all dilutions are carried out in uniform steps, so that the concentration of virus at the i^{th} dilution is related to that of the $i - 1^{\text{st}}$ by $c(i - 1)/[c(i)] = K$, where K is the dilution constant. Similar equations hold for D and L particles. For large i , $(cV/N)K^{-i}$, $(dV/N)K^{-i}$, and $(l_{\text{ind}}V/N)K^{-i}$ all become small

compared to one. Expanding the exponentials in equation 2 and retaining only terms in K^{-i} yields for the expected number of foci at the i^{th} dilution:

$$NP_i(i) \cong cVK^{-i} \quad (3)$$

The right side of equation 3, if plotted on log-log paper against i , has a 45° slope; the 45° part of the curve, when extrapolated to the 0^{th}

dilution, yields $c = NP_t(0)/V$. The value of c so obtained is now used to calculate the number of foci to be expected for various dilutions on the basis of competent virus alone. These values are subtracted from the experimentally obtained focus counts and a new, approximate graph is made of "competent corrected" foci. Then for most indicators there will be a range of values of i for which the dominant term in equation 2 is of the order of K^{-2i} :

$$NP_t(i) \cong \frac{l_{\text{ind}} d}{N} V^2 K^{-2i} \quad (4)$$

Equation 4, plotted on log-log paper has a 63.5° slope and extrapolation to zero dilution allows determination of $l_{\text{ind}} d$:

$$l_{\text{ind}} d \cong \frac{N[NP_T(0)]}{V^2} \quad (5)$$

Earlier authors do not specify the indicator beyond this point, referring to indicators as having a "titer of FFU/ml." Examination of Fig. 1 shows that the indicators whose titration patterns are represented by the curves h , i , and j all extrapolate to values of focus-forming units per milliliter which experimentally are probably indistinguishable (c.f., discussion of assay accuracy). Yet these indicators vary in total virion content by a factor of about 10^3 and also vary greatly in the relative composition of defective and leukemia virions. Examination of Fig. 1 shows that not all hypothetical indicators are suitable for this procedure; some indicators (e.g., that of curve f) do not have a range of dilutions for which equation 4 applies, as evidenced by the absence of a 63.5° slope.

The next step in the assay procedure is to determine a suitable indicator dilution (say the j^{th}) and to add to it progressive dilutions of the preparation containing only leukemia virus [$L(\text{assay})$].

We use volumes V_{ind} and $V(\text{assay})$, respectively. The expected number of foci at various dilutions will still be given by equation 2, except that lV must be replaced by $l_{\text{ind}}K^{-i}V_{\text{ind}} + l(\text{assay})K^{-i}V(\text{assay})$ and dV by $(dK^{-i})V_{\text{ind}}$. If $l(\text{assay})$ is sufficiently large, then the system will be saturated with leukemia virus and, at low dilutions (small i), the linear approximation to equation 2 becomes:

$$NP_T(i) = (dK^{-i})V_{\text{ind}} \quad (6)$$

Since the right side of equation 6 is independent of i , it represents a plateau (with zero slope) and d can be obtained immediately:

$$d = \frac{(NP_T)}{V_{\text{ind}}} K^i. \quad (7)$$

For higher dilutions, beyond saturation, equation

4 will again become applicable and then, remembering the indicated substitutions,

$$NP_T(i) = \frac{[l_{\text{ind}}K^{-i}V_{\text{ind}} + l(\text{assay})K^{-i}V(\text{assay})] dK^{-i}V_{\text{ind}}}{N}$$

which can be rewritten as

$$NP_T(i) = \frac{l_{\text{ind}} V_{\text{ind}}^2 d K^{-2i}}{N} = \frac{[l(\text{assay}) d] V(\text{assay}) V_{\text{ind}} K^{-i} K^{-i}}{N} \quad (8)$$

FIG. 3. Titration of exogenous RadLV (with constant amount of indicator). Dilution factor (K^{-i}) = $1/160$; volume = 0.2 ml. Assay virus: $l(\text{assay}) = 6.3 \times 10^7$; volume = 0.1 ml. Cell number: 2.5×10^5 . These values were chosen to approximate the numbers of the experiment of Fig. 6 in ref. 1. The close resemblance to the experimental curve of that figure indicates internal consistency of the model.

FIG. 4. Expected error of the mean (per cent) for different numbers of dishes per measurement point at different numbers of foci. The errors calculated are those due only to Poisson statistics. Experimental errors (dilution, counting, etc.) add to these to make true errors even larger. Errors were calculated according to per cent error = $\sim (1/\sqrt{m}) (1/\sqrt{n-1})$ where m is the expected mean and n the number of dishes used. Curve a, three dishes per measurement point; curve b, 5 dishes per measurement point; curve c, 10 dishes per measurement point.

But the second term on the left in equation 8 is the expected number of foci for the j^{th} dilution of the indicator in the absence of assay material. Hence, the left side of equation 8 represents the additional number of foci expected as a result of adding the i^{th} dilution of assay material to the indicator. Call this difference $\Delta F(i)$. On log-log paper, a plot of $\Delta F(i)$ versus i takes on a 45° slope. Extrapolation to $i = 0$ then yields $l(\text{assay})$:

$$l(\text{assay}) = \frac{N\Delta F(0)}{V(\text{assay}) V_{\text{ind}} d} K^i \quad (9)$$

A computer calculation, based on equation 2, of $\Delta F(i)$ versus i is presented in Fig. 3. As can be seen from that curve, the linear approximation (equation 8) is appropriate only for dilutions in excess of 1:100. Equation 9, after some manipulation, can be shown to be essentially equivalent to the equation for ΔF of Fischinger and O'Connor. However, in the procedure of Fischinger and O'Connor, only the assay material is completely specified. The treatment presented here allows graphic determination of all particle concentrations, c , l_{ind} , d , and $l(\text{assay})$, using equations 3, 5, 7, and 9, provided that the indicator used has regions over which the approximate equations are valid.

ACCURACY OF THE ASSAY

There are really two independent aspects to the question of how accurately the assay measures what one hopes it measures (i.e., concentrations of virions). The first aspect is: how accurately do the mathematics represent biological reality? Consider for instance the assumption that the efficiency of viral infection is very high and that it is the same for the several virions of the leukemia-sarcoma complex. If the first half of the assumption is in error but all efficiencies (though not high) are still identical, then the assay measures quantities linearly related to concentrations. This would not be a serious defect. However, if the efficiencies differ appreciably, then the quantities measured are not necessarily linearly related to concentrations and the usefulness of the method would be seriously in doubt. We have expressed l_{ind} and $l(\text{assay})$ in particles (or virions) per ml; Fischinger and O'Connor prefer the term leukemia virus helper units (LVHU). Their definition of this quantity is in terms of an equation similar to equation 9 of the text. Thus LVHU applies only to exogeneous (assay) material; furthermore, because of the restricted applicability of equation 9, this definition requires a suitable indicator. We prefer to state

explicitly the assumptions under which our mathematical results are applicable and thus avoid the problems inherent in the operational definition of the LVHU.

The other aspect of assay accuracy relates to measurement uncertainties. The focus formation assay is governed by Poisson statistics. Confluence limits the number of countable foci to 150 or less. Frequently, one is forced to work at low expected focus counts (say 3 to 20). At such low numbers the standard deviation ($\sqrt{3}$ to $\sqrt{20}$) is a high percentage of the expected mean and it becomes necessary to use a large number of dishes if the estimate of the mean is to have much precision. Some of these considerations are demonstrated in Fig. 4, which illustrates the expected percentage error at low focus counts for various numbers of dishes per measurement point. The situation is even worse when plotting Δ foci; the values there result from taking the difference between two quantities, each of which may have a large percentage of error associated with it. Hence, values of Δ foci are in considerable doubt. Careful experimental design may yield measurement values of virion concentrations with a minimum standard error of perhaps $\pm 10\%$ of the estimate values. Some of the published data in the literature are quoted to two significant figures. The implied accuracy must be regarded with considerable skepticism.

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