Cell Clones:
Manual of Mammalian Cell Techniques

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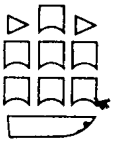

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Mathematical aspects of colony growth, transplantation kinetics and cell survival

COLONY GROWTH

Definitions

The ability of cells to divide repeatedly has long been considered to be the most important parameter to be quantitated in renewing tissues exposed to cytotoxic agents. Cells with this ability are commonly called *viable cells*. (It should be noted that this term is also sometimes used to describe whether a cell is morphologically and often functionally intact.) When radiation is the cytotoxic agent, the major expression of injury is the reduction in viability i.e. the proportion of cells which retains *reproductive integrity* or the *surviving fraction* of cells according to this criterion.

When cells divide repeatedly and form discrete isolated groups of progeny, these groups are called *colonies*. In cases where colonies have been shown to arise from divisions of a single parental cell, for example, by radiation-induced chromosomal markers in the case of spleen colonies (Becker et al, 1963), the originator of a colony should be called a *clonogenic cell* (*clonogen*) or *colony-forming cell* (CFC). The cells in the clone would be genetically identical, although they may have differentiated and be phenotypically different. When colonies contain several cell types of unknown origin, the originators should be called a colony-forming unit (CFU).

Many authors use the term clonogenic cell or a similar connotation, e.g. microcolony-forming cell, in situations where clonality has not been demonstrated but is inferred, for example by agreement with mathematical predictions of single cell responses e.g. exponential survival or transplantation kinetics. However, there are some examples in vitro of a curvilinear relationship between the number of cells plated and the number of colonies produced (see Xu et al, 1983 and Ch. 3), and of an unexpected similar relationship in vivo between the number of cells injected and the number of tumours produced (Porter et al, 1973). In such situations more colonies are produced than would be expected from the higher number of cells seeded, and cell-cooperation, a density-dependent effect of helper cells or factors, or an immune mechanism in vivo acting preferentially on single cells rather than on cells in groups, can be invoked. Furthermore, exponential survival data, although predicted from single-cell considerations, cannot in some instances exclude the necessity of several cells for survival (discussed further below). Hence the term CFU (or micro-CFU), which can embody these effects, is preferable in these cases to the term clonogenic cell.

When colonies arise from single cells, and in the simplest case where all the daughter cells are also capable of division, the number of cells in the colony will increase from 1 to 2, 4, 8, 16, 32 etc. This is exponential growth, and after 'n' divisions, there will be $2^{(T/Tc)}$ cells in the colony. If the time between divisions is constant (the cell cycle time, Tc), then after time T there will have been (T/Tc) divisions. Hence after time T there will be $2^{(T/Tc)}$ cells in the colony. For example, if Tc is 12 hours, then after 7 days (a common assay time in vitro) the colonies should contain 2^{14} or 16 000 cells. If there is an initial lag of one cycle time before growth commences at this rate, the final number of cells would be less by a factor of 2, and if 2 cycles, by a factor of 4. Other combinations can be calculated similarly. Colonies of 1000 cells are often grown and counted in assays in vitro, but in assays in vivo the number of cells per colony can vary markedly, e.g. about 10^6 for spleen colonies (Ch. 2) and about 50 cells for liver follicles in fat pads (Ch. 16). Colonies are defined generally as those containing more than 50 cells. Those containing less than 50 cells are sometimes called small colonies or clusters. The number 50 is relatively arbitrary, but it indicates that cells have undergone more than 5 divisions. It is common experience that most cells capable of undergoing 5 divisions are capable of many more, so that these can be classified as the true survivors.

Self-renewal versus differentiation of clonogenic cells

Most colonies of normal cells differentiate into one or more lineages of maturing cells, and the presence of mature non-dividing cells will lead to a slowing of colony growth in terms of total cell number. This may also occur to some extent if the maturing dividing cells have longer cycle times than their precursors. Small colonies or clusters can arise from maturing cells which still pos-

sess a limited division potential, e.g. BFU-E (Ch. 3). However, these will be temporary, disappearing at the end of the life-span of the mature cells, as the colony will contain no further progenitor cells. Larger colonies can originate from stem cells, defined as those cells which can renew themselves as well as differentiate into all types of maturing progeny characteristic of that cell hierarchy. The content of stem cells at any time can be calculated in some situations, as follows.

When a stem cell divides it could give rise on average to '2p' stem cells and 2(1-p) differentiating cells, where p is the self-renewal probability (Till et al, 1964). If p is constant throughout colony growth, the total number of stem cells after n divisions would be $(2p)^n$. Clearly, if p = 1 there is only self-renewal and no differentiation, and if p = 0.5 the number of stem cells will remain constant. The latter is analogous to the steady-state situation in vivo, where cell production balances cell loss. As the total number of cells after n divisions will be $(2)^n$ if all the differentiating cells are dividing, then the fraction of cells which are stem cells after n divisions will be $(2p)^n/(2)^n = (p)^n$. Also, after time T, the number of stem cells present will be $(2p)^{T/Tc}$, where Tc is the cell cycle time, and the time for the stem cells to double in number, the doubling time (TD), will be $Tc.\frac{\ln(2)}{\ln(2p)}$. Hence, if there is a fixed average probability of differentiation at each cycle, the stem cell population will grow more slowly than the total cell number, which is doubling at a rate given directly by Tc. In the later stages of colony growth the maturing cells may well have longer cycle times, or emigrate from the colony, and hence the fraction of total cells which is stem cells could be higher than predicted. The above considerations are based on a constant value of Tc among stem cells. If there is a distribution of cycles times, which is likely, then the number of stem cells present at any time (T) will be different from the simple relationship above. An example of this effect, which is probably the other extreme but which is amenable to calculation, is where there is an exponential distribution of cycle times. In this case the average number of stem cells (M) present at any time (T) is given by:

$$\ln M = [0.693.(2p-1).\frac{T}{Tc}]$$

(Schofield et al, 1980).

The degree of spread of the stem-cell content between colonies depends on the value of p. This has been discussed with reference to spleen colonies and can be expressed mathematically as:

$$V^2 = \frac{(2-2p)}{(2p-1)} + \frac{1}{M}$$

(Vogel et al, 1968, 1969), where V = coefficient of variation of CFU-S per colony = standard deviation/mean CFU-S per colony; p = probability of self-renewal; M = mean CFU-S per colony. Hence p can be measured when the distribution of stem cells among colonies can be assessed. Values of p for normal CFU-S range between about 0.62 (Vogel et al, 1968) and 0.68 (Schofield et al, 1980). As the doubling time of CFU-S in spleen colonies is about 20 hours, a value for p of 0.65 would give Tc = 7.5 hours.

The number of CFU-S per colony can be changed by various cytotoxic treatments to the original graft, e.g. after IMS (Schofield & Lajtha, 1973). The reduced content of CFU-S in colonies of a given age is commonly used as a measure of an induced qualitative defect in the surviving stem-cell population, e.g. Botnick et al (1981). However, this should not be interpreted automatically in terms of a reduction in the value of p, because if this were so, the doubling time of CFU-S in the colony would change assuming that the cycle time is unchanged. Most examples where this has been measured show a *similar* rate of growth of the CFU-S population, but the growth curve is shifted in time due to a lag or some other mechanism (Schofield & Lajtha, 1983).

A parameter related to p is the extinction probability 'ω' (Vogel et al, 1968, 1969). If p is constant throughout colony growth, then a fraction (1-p) of stem cells will differentiate at the first division and will not form a colony. A similar fraction should differentiate at the second and subsequent divisions, and this effect will reduce the initial number of CFC which finally produce colonies. The probability of 'extinction' reaches an asymptotic value of [(1-p)/p] after about 5 generations (Vogel et al, 1968), and this is about 0.63 for normal CFU-S. Thus, only (1-ω) = 37 per cent of CFC would produce colonies, and hence the expected number of potential CFC would be greater than the measured number of CFC by a factor (1/0.37) = 2.7.

These techniques have not yet been applied to other colony assays, but the advent of grafting techniques for producing colonies derived from cells in other tissues makes their application possible.

In contrast, a cell loss factor (ϕ) was introduced for the growth of tumours (Steel, 1968). This is the rate of cell loss i.e. cells lost per unit time expressed as a fraction of the rate of cell production. If cell 'loss' is taken to denote solely loss of self-renewal ability, then ϕ = [(1-p)/p], which is the same expression as for ω. However, cell 'loss' in tumours refers to the physical removal of cells, not solely their loss of self-renewal ability. A discussion of 'p' in differentiating cell populations in tumours can be found elsewhere (Mackillop et al, 1983).

SAMPLING TECHNIQUES

The Poisson distribution

All the assays described in this book are based on tech-

niques using estimated sample sizes. When the total number of cells (N) plated in vitro or at risk in vivo can be counted accurately, rather than estimated from a count of a small aliquot, binomial statistics should be applied as the surviving number ranges between N and zero. When N is estimated from a random sample, i.e. when aliquots of a suspension are plated out, Poisson statistics can be used as an approximation because N could range between zero and very large (almost infinite) values. When the mean number of cells is small, the Poisson distribution will be skew, as many of the counts will be zero by chance. When the mean is large (above about 10), the distribution becomes more symmetrical and tends towards a normal distribution.

The Poisson distribution is very common in radiobiology, not only because of the sampling techniques employed but also because of the random nature of deposition of energy, and hence the production of biological events, by radiation. The probability of any given count can be calculated from: $f = \frac{e^{-m}.m^n}{n!}$ where m is the mean count, n is the count which occurs with probability f, and $n! = [n \times (n-1) \times (n-2) \text{ x.....x1}]$. Common examples of its applications are given below.

Intracellular events (distribution of hits)

If cells receive on average 1 lethal hit, the fraction of cells escaping i.e. not being hit, will be $e^{-1} = 0.37$ or 37 per cent. Another fraction $(e^{-1} \times 1^1)/1 = e^{-1} = 37$ per cent of cells will receive 1 lethal hit, $(e^{-1} \times 1^2)/(2 \times 1) = (e^{-1})/2 = 18.5$ per cent of cells will receive 2 hits, and $(e^{-1} \times 1^3)/(3 \times 2 \times 1) = (e^{-1})/6 = 6$ per cent will receive 3 hits. With 2 lethal hits on average per cell, $e^{-2} = 13.5$ per cent of cells will escape, 27 per cent will receive 1 hit, and 27 per cent will receive 2 hits, etc.

Clearly, the fraction of cells escaping 'x' lethal hits is e^{-x}, and when x is linear with the dose delivered this forms the basis of a simple exponential survival curve (see below).

Extracellular events (groups of cells)

In many of the assays for survival, the clonogenic cells are grouped into structures containing similar numbers of these cells. After radiation there will be a distribution of the number of surviving cells per structure which approximates to a Poisson distribution when the initial number of clonogenic cells per structure is large and the number of survivors is small, so that the range in numbers about the mean can be from zero to this large initial value. When colonies can grow from 1 or more clonogenic cells, the fraction of ablated structures (F), can be used to calculate the corresponding mean number of surviving clonogenic cells per structure (m). $F = e^{-m}$, and hence $m = -\ln F$. Values of m can be plotted on the logarithmic scale of semi-log graph paper versus dose on the linear scale to give a conventional cell survival curve. However, this method can be used only at relatively high doses where the number of surviving cells per structure has been reduced on average to near unity or below so that some structures are ablated (see Chs 5, 12, 17). If the standard error on F is f, then the standard error on $m = f/F$. Also, it should be recognised that the number of surviving cells per surviving structure $= [m/(1-F)] = [-\ln F/(1-F)]$. The validity of using these calculations has been confirmed in two assays by measuring the distribution of colonies between different areas of epidermis (Withers, 1967; Hendry, 1984) or intestine (Ch. 5), where the distribution was not significantly different from a Poisson distribution.

Two related extensions of these principles are (1) when the possibility is considered that one clonogenic cell cannot form a colony, and co-operation of two or more is required, as noted in Chapter 17, and (2) when a larger number of clonogenic cells is required to rescue a tissue or an animal, where each cell has a small but finite probability of rescue. These effects are described below in the section on cell survival curves and multicellular structures.

Transplantation kinetics

Another similar and common situation is where colonies arise from one or more CFU after injecting serially-diluted inocula of a cell suspension. This applies to assays in fat pads (Chs 15, 16), and also to TD_{50} assays for tumours (Ch. 25). These are sampling techniques which should be describable by Poisson statistics. In these cases, the chance of no colony growing will be e^{-m}, where m is the mean number of CFU injected. Hence, when 50 per cent of the injected sites show no growth, $e^{-m} = 0.5$, $m = 0.693$, and thus 0.693 viable CFU were injected on average. When $m = 1$, $e^{-m} = 0.37$, and hence the inoculum size resulting in 37 per cent of sites with no colony (TD_{63}) gives the plating efficiency directly, i.e. 1 CFU exists within a certain number of cells. In practice, serial dilutions are made and injected into different groups of animals to 'bracket' for example the TD_{63}. Sophisticated statistical methods are now commonly available for estimating the most likely value of TD_{50} or TD_{63} with their associated uncertainties. These methods have been developed largely by Finney (1964) and also by others for use in radiobiology (Porter & Berry, 1963; Porter et al, 1973; Gilbert, 1974; Porter, 1980a,b).

Briefly, the probability (F) of lack of growth in an injected site is given by, $F = e^{-m}$ (as above). m is proportional to the inoculum size (z) so that $m = kz$, and: $\ln m = \ln k + \ln z$. As $m = -\ln F$, $\ln(-\ln F) = \ln k + \ln z$. Hence a plot of $\ln(-\ln F)$ against $\ln z$ should give a line with a slope of 1 and an intercept of $\ln k$ on the ordinate, where k is the plating efficiency, i.e. n/z.

Also, k is given by the value of 1/z when F = 1/e = 0.37, i.e. at the TD_{63}.

Estimates of the parameters with associated error limits can be made using computer programmes with maximum likelihood (Finney, 1964; Porter, 1980a, b) or minimum chi-square techniques (Gilbert, 1969, 1974). We use a modified version of an earlier programme (Gilbert, 1969), as noted elsewhere (Gilbert, 1974), which calculates directly the plating efficiency with the expected standard deviation (sometimes called the standard error) of the mean (Fisher & Hendry, unpublished). Others who are using the above techniques include Clifton & Gould (Ch. 15), Jirtle & Michalopoulos (Ch. 16), Hill (Ch. 25), Rice et al (1980) and Porter (1980a,b). New users are advised to contact one of the current users and either send them the data for fitting if their needs are only occasional or obtain a copy of their computer programme if their respective computers use the same language and format.

Porter et al (1973) also discussed the case where the sigmoid curve is not in accordance with single-cell transplantation kinetics, i.e. when the number of clonogens in an inoculum is not related linearly to inoculum size. In this case other fitting procedures can be used (see Finney, 1964 and Ch. 25) which do not depend on any biological model. However, the effect can be accommodated in the fitting procedure by introducing another parameter 's', where $m = k.z^s$. Values of $s > 1$ could be explained by marked variability between recipients. Values of $s<1$ could result from 2 or more clonogenic cells being required for colony growth, as follows.

If 2 or more clonogenic cells are required, then the probability of there being no growth is the sum of the probabilities of there being (1) no cells, and (2) 1 cell, in the site, i.e. $F = e^{-m} + (m.e^{-m}) = (1 + m)e^{-m}$. Hence $\ln F = [\ln (1 + m)] - m$. An approximate value for 's' can be deduced by approximating $\ln(1 + m)$ by $(m - \frac{m^2}{2})$, when m is much less than 1.

Thus $\ln F = -m' \simeq \frac{-m^2}{2}$,

and $\ln(-\ln F) \simeq \ln 0.5 + 2 \ln m = \ln \frac{k^2}{2} + 2 \ln z$.

Hence, a plot of $\ln(-\ln F)$ against $\ln z$ would give a slope of 2 i.e. $s = 0.5$. However, the approximation is valid only for values of m less than about 0.2, which gives m' less than 0.02, and which corresponds to less than TD_2. For the majority of data, ranging between TD_5 (m' = 0.05) and TD_{95} (m' = 3), s can be shown by graphical means to approximate to about 0.65. Also, if 3 clonogenic cells or more were required for growth, s would be about 0.5 over the range TD_5 to TD_{95}.

The above considerations provide a slope that is steeper than expected from single cell transplantation kinetics, as observed with some of the tumour data, but this invokes the peculiar situation where 1 clonogenic cell has zero probability of growth! Thus, either cooperation between 2 cells (or 3 cells etc.) is essential for growth, or single cells are selectively inactivated compared with cells in pairs or in groups. This contrasts with the conventional situation described above, where *every* viable cell has a finite probability of forming a colony or a tumour, but the colony-forming efficiency of the injected cells is less than 1, due for example to the low concentration of viable cells in the inoculum, to a relative lack of helper cells or factors, or to the random cytotoxic action of enzymatic disaggregation procedures and/or the immune system in the grafted host.

With the normal tissue assays, where structures of mammary epithelium and thyroid (Ch. 15) or liver (Ch. 16) are produced in fat pads, values of $s = 1$ are good evidence for growth from a single cell rather than simply an aggregation of the injected cells. The latter situation would give $s<1$. Aggregation is considered to be a problem when large numbers of cells are injected, but this can be avoided by using multiple injection sites (Ch. 16). The clonal origin of these colonies is discussed in detail in Chapter 16. Values of $s = 1$ are compatible with the results obtained for all the normal tissues tested so far using this method (mammary CFU — Gould & Clifton, 1977; thyroid CFU — De Mott et al, 1979; liver CFU — Jirtle et al, 1981) and some but not all tumours (Porter et al, 1973). Linearity is also observed for many other CFU, which has been tested directly by the relationship between colony number and cells injected or plated, e.g. spleen colonies (Ch. 2) and other haemopoietic colonies (Ch. 3).

The expressions $F = e^{-m}$ and $m = kz$ are similar to those invoked earlier by Lange and Gilbert (1968) to describe the probability (α) of a single neoblast repopulating a planarian. Thus, the probability of a grafted planarian dying $(F) = (1-\alpha)^N$ when N neoblasts had been grafted, and this was approximated to $\exp(-\alpha N)$. Hence, α corresponds to k, and N to z.

Sampling errors

A characteristic of the Poisson distribution is that the mean M equals the variance which is the square of the standard deviation (SD) i.e. $M = (SD)^2$. The standard deviation of the mean is commonly called the standard error (SE) and $SE = (SD/\sqrt{N})$ where N is the number of estimates of the mean.

Two common procedures in colony experiments are (1) to calculate the number of counts required to reduce the SE to a given percentage of the mean, for example 5 per cent or 10 per cent, (2) to test whether two means are significantly different e.g. for a control and a treated sample. With the first procedure, if the standard error on the mean M is required to be 5 per cent, so that the mean equals $M \pm 0.05M$, then:

0.05M × √(N samples) = SD = √M (see above). Hence, N = 1/ (0.0025M), and N can be calculated for any value of M. If the mean is 10, as for example in the spleen colony assay, 40 samples (spleens) should be counted, i.e. a total of 400 colonies. If the mean is 100, as in some assays in vitro and in vivo, e.g. crypts per circumference (Ch. 5), only 4 samples need be counted with a total of 400 colonies. If a standard error of 10 per cent of the mean is acceptable, the calculated number of samples can be reduced by a factor of 4. This could mean that only 1 plate in vitro was necessary, although the majority of investigators would in any event use 2 or 3 to cover the possibility of infection or poor growth in a single sample. It is considered that when few samples are counted, the error quoted should be at least the sampling error and not simply the value calculated from the few samples taken (Boag, 1975). This is because the few samples could easily by chance be selected from a narrow range in the distribution and hence appear to have an associated error smaller than was representative. Also, there may well be an additional variance to the sampling error due to pipetting or injection inaccuracies, or variations between recipients, e.g. in mice or in feeder layers in vitro.

For the second procedure, a t-test is commonly used to test whether two means are significantly different. A worked example is given in Mather (1964), and these tests are usually included in computer software packages. The t-test applies when the data are approximately normally distributed. For more than 2 groups, an analysis of variance is used or the non-parametric equivalents (Siegel, 1956). The Poisson distribution deviates from the normal distribution for low values of the mean (much less than 10), and hence the t-test is more appropriate for distributions with higher mean values. If the means are small then the data will be skewed (i.e. non-normal) and either a chi-squared test should be undertaken to compare the observed and the expected frequencies, or the non-parametric equivalent to the unpaired t-test should be used, i.e. the Mann-Whitney U-test. The application of the t-test to colony counts has been discussed by Blackett (1974) and Hazout & Valleron (1977) with special reference to the 'thymidine suicide' technique where small or large differences in the amount of cell kill are used as indicators of the cycling status of the cell population in question (Ch. 2). Relationships are given in Hazout &Valleron (1977) which give the total number of colonies to be counted to give various levels of significance to observed differences between 2 mean values. For example, they calculate that 2000 colonies should be counted for each mean value to make a 10 per cent difference between mean values significant ($P<0.05$) in 90 per cent of cases. 2000 colonies corresponds to 200 spleens with 10 colonies per spleen, or 200 mice with 10 intestinal crypts per circumference, or 20 plates in vitro with 100 colonies per plate. If the difference between the means is 40 per cent, the total number of colonies can be reduced drastically from 2000 to 100.

The fraction of cells surviving is the ratio of the number of colonies in the treated sample to the number in the control sample. If the ratio is (A/B) and the standard errors on A and B are 'a' and 'b' respectively, then the error on the ratio is given by: $\frac{1}{B^2} \cdot \sqrt{(a^2B^2 + b^2A^2)}$.

CELL SURVIVAL CURVES

Parameters and models

As energy from ionising radiation is deposited at random in discrete volumes, resulting generally in randomly-distributed biological injury, it is conventional to describe radiation dose-response curves using an exponential (Poisson) distribution. Hence, dose is plotted on a linear scale and cell survival is plotted on a logarithmic scale (see Fig. 1.1). A line on such a plot indicates that equal increments of dose produce equal decrements in log cell survival. If the line extrapolates to the origin, as for example with human fibroblasts (Ch. 19), the relationship between dose and survival can be described by one parameter, the mean lethal (or inactivation) dose D_{37} or D_o (Lea, 1946). If the survival curve is truly exponential, this is the dose which reduces the number of viable cells to 37 per cent of the original number. This value was chosen because according to the Poisson distribution, when all cells have received on average 1 lethal hit at random, e^{-1} (=0.37) should receive no hit i.e. 37 per cent should survive. Because of the exponential nature of the survival curve, this will apply at any level of survival fraction i.e. from 1 to 0.37 or from 0.1 to 0.037.

The term D_o is preferred so as to cover the more general case where, for most mammalian cells, there is a shoulder or initial region demonstrating less sensitivity. Hence, D_o is the mean lethal dose describing the *terminal* exponential region (see left panel, Fig. 1.1). If there is a finite initial slope in the shoulder region this is often described by ${}_1D_o$. The size of the shoulder is described most simply by the extrapolation number 'n' which is the point of extrapolation of the terminal exponential slope on the (log) ordinate. Hence, for many of the survival curves presented in this volume as examples of the use of various assay techniques, comparisons can be made of cell sensitivity (D_o) and of shoulder size (n). Alternatively the quasi-threshold dose Dq can be used (Alper et al, 1962), which is the point of extrapolation of the terminal slope on the linear abscissa (Fig. 1.1, left panel), and which equals (D_o.ln n). It is not the true threshold dose, and for cells demonstrating a marked initial slope there may be a significant decrease in survival at a dose Dq. Interestingly, with a multi-

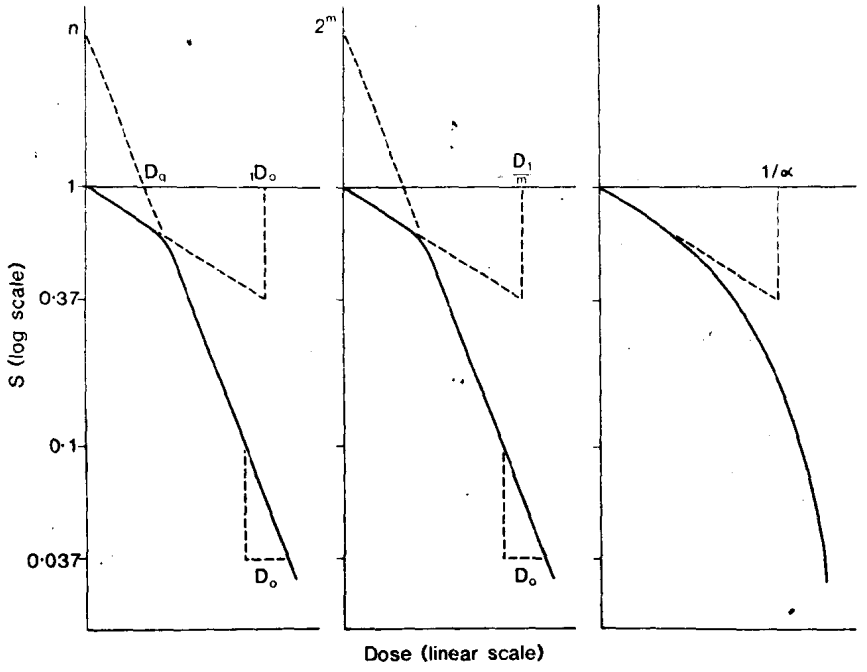

Fig. 1.1 Shapes of survival curves.
Left panel, conventional multi-target survival curve. n = extrapolation number; $_1D_o$ = mean lethal dose at low doses; Dq = quasi-threshold dose = $D_o.\ln n$.
Middle panel, target-pair model. 2^m extrapolation number, with m pairs of targets; $\left[\frac{D_1D_2}{m(D_1+D_2)}\right]$ mean lethal dose at high doses = D_0; $[\frac{D_1}{m}]$ = mean lethal dose at low doses. For D_2 see text.
Right panel, α β curve. Extrapolation number (2^m), very high. $1/\alpha$ = mean lethal dose at low doses. Continuous curvature, terminal exponential slope usually never reached.

target curve, Dq is the dose at which the linear decrease in survival per unit dose is maximal (Okumura et al, 1974).

Although assays for mammalian cell survival have been developing over nearly 30 years, the mechanisms responsible for values of n and D_o have not yet been elucidated. However, in the literature there is an increasing use of alternative mathematical models for cell survival curves which are based on various 'plausible'mechanisms. These are often considered to be 'better' models but because of the amount of scatter in most data, the newer models cannot usually be shown to fit the data better when the number of variables is the same as in the old models. As users of colony techniques for dose-response measurements are becoming increasingly confronted with descriptions of survival curve shapes in terms other than D_o and n, for example α and β (Ch. 18), a brief description is given of the meaning and use of such parameters.

All target-type models invoke the Poisson distribution to give the probability of an event not happening, when the mean number of events is specified. A simple case, described above, is where one event kills the cell and where the mean number of events per cell is linear with dose (D) so that survival (S) of the cell is given by:

$$S = \exp(-D/D_0) \qquad \text{equation (1)}$$

If there are 'n' targets in a cell, all of which have to be inactivated to kill that cell, and if the number of inactivating events is linear with increasing dose, then survival of single *targets* could also be described by [exp $(-D/D_o)$]. The chance of a single target being inactivated is [1 – exp $(-D/D_o)$], and the chance of inactivating all n targets is [1 – exp $(-D/D_o)]^n$. Hence, cell survival (S) is given by:

$$S = 1-[1-\exp(-D/D_o)]^n \qquad \text{equation (2)}$$

This is the conventional multi-target equation, which approximates at high doses to [n. exp $(-D/D_o)$]. A finite slope to the cell survival curve at low doses is often characterised by an additional exponential term, so that:

$$S = \exp(-D/{}_1D_o) \times (1-[1-\exp(-D/D_o)]^n) \qquad \text{equation (3)}$$

(Bender & Gooch, 1962), where $_1D_o$ is the mean lethal dose for the cell from single lethal events, and a different

target is implied from the n targets already described. At high doses S becomes $(n.\ \exp\ [-D\ (\frac{1}{{}_1D_o} + \frac{1}{D_o})])$. Equation (3) is adequate for empirical description and comparative purposes, but its use for interpretation of the parameters in terms of mechanisms is now not favoured by many people. This is because of the conceptual difficulties in the postulate of a multiplicity of essential targets, e.g. Alper (1979).

A more plausible version of target-type models is based on the assumption that *only one* target of many has to be inactivated to sterilise the cell, e.g. one unrepaired break in a double strand of DNA which leads to lethal chromosomal injury. If such a break can be produced by one radiation event with a probability α and by a combination of two events with a probability β, then the number of lethal events (L) can be expressed by a quadratic function: $L = \alpha D + \beta D^2$. Hence, the surviving fraction (S) of cells will be given by:

$$S = \exp(-L) = \exp -(\alpha D + \beta D^2) \qquad \text{equation (4)}$$

e.g. Chadwick and Leenhouts (1973). This equation predicts a continuously-bending survival curve, and although it can also accommodate curves which are simple exponentials, in which case $\beta = 0$, it cannot accommodate conventional multi-target-type survival curves which show quite clearly, the presence of a shoulder and a terminal exponential region, e.g. Puck & Marcus (1956).

However, the multi-target curve and the α, β formulation can be reconciled if the same mathematical principles are applied to derive both. The relationship between them can be demonstrated as follows.

If a potential target-pair is considered, the chance of one strand of the pair not being broken would be given by $\exp(-D/D_2)$, where D_2 is the mean number broken per unit dose. The chance of the pair remaining intact would be $(1-[1-\exp(-D/D_2)]^2)$. If there are 'm' potential site-pairs in a cell, the chance of the cell surviving is: $S = (1-[-\exp(-D/D_2)]^2)^m$. If the pair can also be inactivated by *single* events, another exponential factor is added so that:

$$S = [\exp(-D/D_1)]^m \cdot (1-[1-\exp(-D/D_2)]^2)^m \qquad \text{equation (5)}$$

(Gilbert, 1975; Ehrenberg, 1977; Gilbert et al, 1980). At high doses, S approximates to

$$\{2^m \cdot \exp[-\ mD\ (\frac{1}{D_1} + \frac{1}{D_2})]\}$$

and hence the survival curve (see middle panel, Fig. 1.1) has an extrapolation number of 2^m (Neary, 1965) and a sensitivity $(\frac{1}{D_o})$, of $m(\frac{1}{D_1}+\frac{1}{D_2})$. When $m = 1$, the equation becomes a conventional multi-target equation with $n = 2$. When m is greater than 1, the extrapolation number becomes very large and the terminal sensitivity, which may never be reached in practice because of the enormous shoulder, includes the parameter m. This is because in the original multi-target model, no matter how many targets there were initially in the cell, the sensitivity for lethality is given always by the last surviving target. In the newer version, if there are more potential site-pairs per cell there is a greater chance, in proportion to their number, of hitting one of them. The now widely used expression for survival, $S = \exp-(\alpha D + \beta D^2)$ is an approximation to equation (5), and it applies well either for low doses or for large values of m, but not at high doses when m is small. The approximation applies when D is much less than D_2 so that the chance of 2 coincident events on a single site is negligible. In this case S approximates to:

$$S = \exp-(\frac{m}{D_1}.D + \frac{m}{D_2}.D^2) \qquad \text{(Gilbert, 1975).}$$

Hence $\alpha = \frac{m}{D_1}$, and $\beta = \frac{m}{D_2{}^2}$. Also, $\frac{\alpha}{\beta} = \frac{D_2{}^2}{D_1}$, and this is the dose at which the contributions of single and double-events to lethality are equal. When m is very large, which is plausible for the number of potential sites where a lethal event may occur, D_2 must also be large for values of S to be in the common measurable range. Hence, the approximation will be valid to higher values of dose (D). It is interesting therefore that the (α,β) formulation is in effect describing the shape of the huge 'shoulder' of one type of multi-target survival curve. Also, as a quadratic is a good approximation to other equations for survival, e.g. Burch & Chesters (1981), it can be applied with differing limitations in most situations.

The initial slope $\alpha = (m/D_1)$ (see right panel, Fig. 1.1) corresponds to $(1/{}_1D_o)$ in the original multi-target formula (equation 3). As described above, the (α, β) formulation can be derived directly from an assumed quadratic relationship for the production of lethal events. However, this does not allow possible interpretation of the values of the parameters in terms of target sizes, nor the accommodation of expected deviations from a quadratic due to considerations of target number and target 'overkill', as already described.

There are many other models, e.g. the 'multihit' and the 'pool' models, which have biological significance but which have not yet been used by many investigators. Readers are referred to radiobiological texts for further information (Elkind & Whitmore, 1967; Proceedings of the 6th L. H. Gray Conference, 1975; Alper, 1979; Proceedings of the 11th L. H. Gray Conference, 1984).

Cell survival parameters and groups of cells

If cells are irradiated *in vitro* after they have all divided once, then the extrapolation number per initial seeded cell should be double the value obtained if they had not divided. After 2 divisions, it should be 4x, and after 3

divisions it should be 8x. However, because of asynchrony in most cell populations, these expectations will not be fulfilled exactly, and there will be a distribution of sizes of cell clusters. Further information on these aspects has been discussed by Elkind &Whitmore (1967).

Many structures in vivo contain presumably similar numbers of clonogenic cells, and hence these could be regarded as multi-target-type structures, where one surviving cell per structure is required to regenerate the structure. Hence data expressed as surviving fractions of structures could be analysed directly using computer methods and multitarget equations (see below).

The response of single cells in these structures can be deduced because if one clonogenic cell or more regenerates a structure, then the fraction (F) of structures ablated will be given by $F = \exp(-m)$ where m is the mean number of clonogenic cells surviving per structure. If m decreases exponentially with increasing dose at high doses, so that $m = [n.\exp(-D/D_o)]$, where n is the total extrapolation number per structure, then:

$$\ln(-\ln F) = \ln n - D/D_o.$$

Hence if semi-logarithmic graph paper is used, and $(-\ln F)$ is plotted on the logarithmic ordinate scale versus dose on the linear abscissa, the line produced would have an intercept of $\ln(n)$ on the ordinate and a slope$(-1/D_o)$ (Withers and Elkind, 1970). Alternatively, $\ln(-\ln F)$ can be plotted on a linear ordinate (Gilbert, 1974). The relationship between these two graphical methods can be found in Chapter 5.

On the other hand, if 2 clonogenic cells are required to regenerate a structure, and it will fail to grow with zero cells [probability = $\exp(-m)$] and with 1 cell [probability = $m.\exp(-m)$] then:

$$F = \exp(-m) + m.\exp(-m) = (1 + m)\exp(-m).$$

Hence, $\ln F = \ln(1 + m) - m = -m'$.

At high doses when m is much less than 1, $\ln(1 + m)$ approximates to $(m - \frac{m^2}{2})$ and hence $m' \simeq \frac{m^2}{2}$.

Thus, $m' \simeq \frac{n^2}{2}\exp(-D.\frac{2}{D_o}) = n'.\exp(\frac{-D}{D_o'})$

Hence, if m′ is calculated from F, and n′ and D_o' are deduced as described above, the sensitivity will appear greater by a factor of 2 i.e. $D_o' = D_o$ (as noted in Ch. 17), and the extrapolation number will be much greater, i.e. $n' = (\frac{n^2}{2})$.

The change in the threshold dose for the ablation of structures can be calculated, because when say $F = 0.1$, $m = \ln 0.1 = 2.3$. If 2 cells are required to regenerate the structure, then when $F = 0.1$,
$\ln 0.1 = \ln(1 + m_1) - m_1$, and hence $m_1 = 3.89$.
As $m = n\exp(-D/D_o)$,

$\ln(\frac{m}{m_1}) = (D_1 - D_2)/D_o$ where $(D_1 - D_2)$ is the reduction in dose between the two models when $F = 0.1$. In the above example, $(D_1 - D_2)/D_o = 0.59$, and hence if $D_o \simeq 1$ Gy, $(D_1 - D_2) \simeq 0.6$ Gy, so that the threshold dose would not change by much.

If the above approximation is used directly, then

$$D_q' = D_o'.\ln n' = (\frac{D_o}{2}).\ln(\frac{n^2}{2}) = Dq - 0.35\ D_o.$$

However, this method is less accurate because the approximation is valid only for values of n less than about 0.2.

This situation, where the survival curve almost 'pivots' about the threshold dose and has a lower D_o and higher n, has indeed been observed in several situations and no satisfactory explanation has yet been found, e.g. the diurnal changes in sensitivity of intestinal crypts (Hendry, 1975).

As already noted, the approximation of $\ln(1 + m)$ by $(\frac{m - m^2}{2})$ is valid only when m is less than about 0.2, i.e. when $F > 0.98$ or when less than 2 per cent of structures survive. In the common measurable range of 95 per cent down to 1 per cent survival of structures, the deduced cell survival curve would appear gently bending with an average sensitivity characterised by $D_o' \simeq \frac{D_o}{1.6}$. Similar reasoning can be applied to the cases where >3, or >4 etc. clonogenic cells are required for regenerating a structure. For x cells, the terminal D_0' would be $(\frac{D_o}{x})$, and the extrapolation number n′ would be $(\frac{n^x}{x!})$. However, with increasing values of x, the terminal D_o' would never be reached, and hence the apparent values of D_o' and n′ over the range considered would be greater and lesser respectively. This reasoning is very similar to that applied above in the section on Transplantation Kinetics.

An extension to these ideas is where *many* cells are required to regenerate a larger structure such as a tissue or an animal. The above approach would give a *much* smaller value for D_o', but in this situation a number of surviving cells below a critical level has *zero* probability of rescue. This is clearly unrealistic when the difference between two numbers of surviving cells is very small, and a more logical approach is that proposed by Lange & Gilbert (1968), where *each* cell has a small probability (α) of rescuing the tissue. Hence, the probability of failure is $(1-\alpha)$, and with N surviving cells, the probability of failure (F) would be given by:

$F = (1-\alpha)^N \simeq \exp(-N\alpha)$ when α is small.
Hence $\ln F \simeq (-N\alpha)$.

If N is related exponentially to dose by $N = No.\exp(-D/D_o)$ where No is the total extrapolation number per tissue, then:

$\ln(-\ln F) = \ln(\alpha No) - D/D_o$.

Hence, a plot of $\ln(-\ln F)$ against dose D would give a line with slope $(-1/D_o)$ and an extrapolation number of $\ln(\alpha No)$ on the ordinate. Alternatively, $(-\ln F)$ can be plotted on the logarithmic ordinate of semi-logarithmic graph paper. This approach was developed from ideas concerning tumours presented by Munro & Gilbert (1961), and it has been further applied to the sensitivity of 'target' cells in tumours (Andrews &Mossman, 1976; Wheldon et al, 1977), haemopoietic tissue (Robinson, 1968), intestine (Hendry et al, 1983) and epidermis (Hendry, 1984). The D_o values which can be deduced for the target cells responsible for failure of haemopoietic tissue, intestine and epidermis are very similar to the D_o values measured directly for their respective colony-forming cells. Thus this validates the use of this approach.

As discussed in detail elsewhere (Potten & Hendry, 1983), the necessary killing of all clonogenic cells in a structure in order to ablate it, is analogous to the multi-target theory for *cell* survival where all targets in a cell have to be inactivated to kill it. Hence, the survival (S) of structures will correspond to the survival of (nA) targets, where A target cells per structure each have n subcellular targets, and $S = 1 - [1 - \exp(-D/D_o)]^{nA}$ (see equation 2, p. 6). Thus, computer programmes which fit data to multi-target equations (Gilbert, 1969), can be used directly to fit data for the survival of structure versus dose, and values for the cell D_o and the total number of targets per structure can be calculated. Also, the double-logarithmic transformation described above is a good approximation to the multi-target formula (Watson, 1978; Potten & Hendry, 1983). When (nA) is large and $\exp(-D/D_o)$ is small, the multi-target equation for S approximates to:

$S \simeq 1 - \exp[-nA.\ \exp(-D/D_o)]$
Hence, $F = 1-S \simeq \exp[-nA.\ \exp(-D/D_o)]$
and $\ln(-\ln F) \simeq \ln(nA) - D/D_o$.

nA per structure corresponds to No per tissue (see above). α, which is applied above to the ability of a cell to regenerate a structure, is 1 according to the definition of a colony-forming cell.

Thus, the double-logarithmic transformation of F is a good approximation to the multi-target formula not only in the region where cell survival is related exponentially with dose, but also at lower doses in the shoulder region of the cell survival curve, if this is described adequately by a conventional multi-target equation. Specific examples are calculated in Potten & Hendry (1983). The practical use of a linear transform of the multi-target equation has been discussed by Watson (1978).

Finally, the exponential function for cell survival can be replaced by more complex functions and similar analyses of cell survival in multicellular structures can be undertaken. Readers are referred elsewhere for further information on this subject (Yau & Cairnie, 1979; Thames et al, 1981; Potten & Hendry, 1983).

Curve fitting

Cell survival data are plotted conventionally on a semi-logarithmic plot of dose versus survival. A line or curve can be drawn by eye through the data and values, for example of D_o and n, can be estimated. This is satisfactory for many purposes but many people employ computer methods so that (1) the statistical weighting of individual datum points can be automatically taken into account, (2) error limits on the fitted parameters can be more easily calculated, (3) curves of specified shape can be fitted to the data.

Mean survival values derived from widely-spread results are clearly not very accurate, and data points are weighted by their inverse variance. If N cells survive out of No at risk, the surviving fraction (P) will be N/No and this fraction could vary between 1 and zero. If No is actually counted (and not estimated from a sample count), binomial statistics apply, and the variance of P is $[\frac{P(1-P)}{No}]$. If No is estimated from a sample count, and $No \gg 1$, $P \ll 1$, Poisson statistics can be applied, and the variance of P is $[\frac{P(1+P)}{No}]$ when *survivors* are counted, and $[\frac{1}{No}.(1-P).(2-P)]$ when *non-survivors* are counted. Clearly the variance of P is reduced by using (1) large samples, (2) counted numbers of cells at risk rather than numbers estimated from an aliquot, and (3) at low doses (values of P near 1) when non-survivors are counted rather than survivors.

There are several methods of curve fitting. Linear regression can be used for simpler models where a linear transformation of the data can easily be made, for example with an exponential survival curve (Pike & Alper, 1964). In this case, the sums of squares of the differences between the observations and the fitted line are minimised to provide the line of best fit, and sampling errors on the regression constants can be calculated. A worked example can be found in Mather (1964), but computer methods using iterative procedures are now widely available. The main difficulty with the basic technique is in cases where a decision has to be made as to which points to include in the fitting procedure, for example if there is a marked curving shoulder to the survival curve.

Maximum likelihood or minimum chi-square techniques are often preferred. No initial transformation of the data is necessary except if required for subsequent plotting purposes, and the data can be fitted to any equation by iterative procedures. These techniques have

been developed notably by Finney since 1947 (updated in Finney, 1978), but also by others specifically for radiobiological applications (Gilbert, 1969; Porter, 1980a,b). Best-fit curves can be obtained, with values for the fitted parameters and their associated error limits.

Several investigators who have used one or other of these techniques can be found in the Proceedings of the 6th L. H. Gray Conference (1975), e.g. Gillespie et al, Bryant & Lansley, Phillips et al, including this author. New users are advised to contact someone who has experience with the use of these techniques and either send them the data for fitting or obtain a copy of their programme. The latter approach nearly always requires the assistance of a computer programmer because of the numerous variations in format between similar computers. Some programmes include options for fitting one or more common parameters to separate survival curves, e.g. fitting a common extrapolation number to extract dose-modifying factors (Pike & Alper, 1964; Gilbert, 1969).

Effect of colony size

Many investigators have observed an increase in the number of small colonies after increasing radiation doses. This effect implies that when colonies containing fewer cells are counted the CFC will appear less sensitive. Two examples of this effect are shown in Figure 1.2. Nias & Fox (1968) demonstrated that when progressively smaller colonies were included in the measurement of surviving fraction, the latter increased gradually (left panel, Fig. 1.2). The effect was slightly greater at the higher doses, suggesting that both n and D_o were affected. Also, when compared with the strictest test for survival — back extrapolation of growth curves where numerous cell divisions are needed

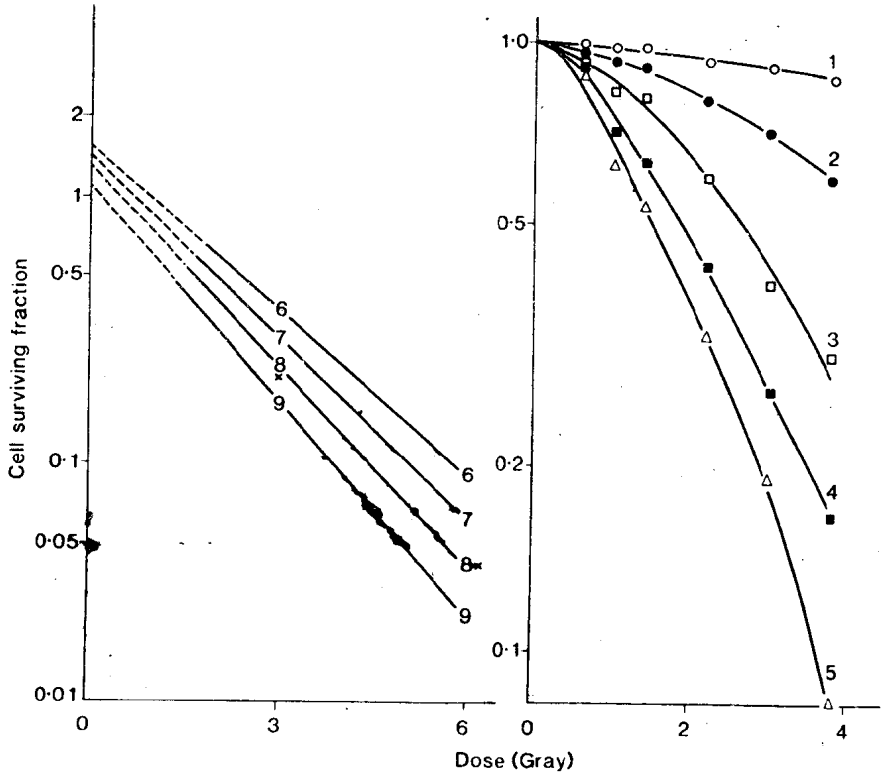

Fig. 1.2 Effect on a survival curve of different criteria for counting colonies.
Left panel, data obtained using HeLa cells by Nias & Fox (1968). Numbers on lines correspond to minimum number of divisions required for a colony to be counted, i.e. 6 divisions, all colonies with 33 cells or more; 7 divisions, 65 cells or more; 8, 129; 9, 157. Crosses, survival level deduced from a back-extrapolation of growth curves, and corrected for mitotic delay. Right panel, data obtained using BHK cells, counting maximum number of cells reached per colony up to day 5½ after plating (data courtesy of Dr S. Revell). Numbers on lines correspond to minimum number of divisions required for a colony to be counted. 1 division = 2 cells; 2 divisions = 3–4 cells; 3 divisions = 5–8 cells; 4 divisions = 9–16 cells; 5 divisions = 17–31 cells. Lower limit on number of cells due partly to cells 'floating off' in the culture.