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KINETIC ANALYSIS OF CELL SPREADING

I. THEORY AND MODELLING OF CURVES

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SUMMARY

The spreading of cells on suitably treated surfaces is briefly discussed and the need to analyse this phenomenon numerically is emphasized. Possible mathematical models for fitting experimental data are classified as statistical, kinetic or empirical and examples of each of these types are given. A possible protocol for analysing cell spreading kinetics and determining goodness of fit and parameter redundancy is presented.

INTRODUCTION

Studies of cell behaviour *in vitro* have demonstrated that in normal cell types the supporting substratum plays an active part in the determination of morphology (Aplin & Hughes, 1981; Aplin & Foden, 1982), growth and division (Stoker, O'Neill, Berryman & Waxman, 1968; Folkman & Moscona, 1978; Ben-Ze'ev, Farmer & Penman, 1981; Ben-Ze'ev & Raz, 1981), and the pathway of differentiation (Grinnell, 1978; Grinnell & Feld, 1979). Cells settling on a prepared surface may remain rounded for an indefinite period or flatten and spread, and spreading appears to be a prerequisite for DNA synthesis and cell division (Stoker *et al.* 1968; Folkman & Moscona, 1978; Ben-Ze'ev *et al.* 1981; Ben-Ze'ev & Raz, 1981). Studies with permanent fibroblastic and epithelioid cell lines have in general demonstrated a requirement for exogenous 'promoters' of spreading added either as a supplement to the medium or as a coating on the growth surface (Grinnell, 1978); primary cells in culture may adhere to a prepared substratum or secrete a 'carpet' of adhesive components such as fibronectin, which subsequently allows cell spreading (Grinnell & Feld, 1979). Serum, which is often present as a medium supplement, contains fibronectin (Edsall, Gilbert & Scheraga, 1955), and other adhesion-promoting components (Knox & Griffith, 1980). Cell spreading, which consumes energy (Grinnell, 1978), can occur on a variety of substratum-associated proteins (Aplin & Hughes, 1981). The foregoing attachment process appears to be passive and less specific (Grinnell, 1978).

A variable in adhesion experiments that has been somewhat neglected is that of time. It is evident from the above discussion that the kinetics of cell spreading may depend on the nature of the substratum and cell type in addition to the fundamental molecular processes of membrane and cytoskeletal reorganization. These in turn may be influenced by the position of the cell in the cell cycle, the growth phase of the culture and the method used to obtain a cell suspension (Schor, 1979).

In the present paper we discuss ways of analysing the kinetic behaviour of a population of settled rounded cells, which transforms over a period of time to the spread-out morphology, using the simplifying assumption that each cell can exist in two states: rounded or spread. In the accompanying paper, data collected from fields containing cells that have been defined by an observer as 'rounded' or 'spread' are analysed as a function of time. In this way it is intended to place the phenomenon of spreading on substratum on a quantitative footing, thus allowing detailed study of the variables that influence cellular morphology.

MATERIALS AND METHODS

HeLa cells were grown in Minimum Essential Medium with Earle's salts, non-essential amino acids (1 %), Hepes (20 mM) and 10 % foetal calf serum. They were brought into suspension using 0·01 % trypsin, 0·004 % EDTA in phosphate-buffered saline without divalent cations. Amniotic fluid fibronectin was prepared, and adhesion assays conducted, as described in the accompanying paper.

RESULTS AND DISCUSSION

The course of cell spreading

Data obtained using cultured cell lines spreading as a function of time on substrata containing coatings of adhesion-promoting proteins generally take a sigmoid form (Fig. 1 and accompanying paper). Cell types differ, but light and electron microscopic examination of cultured cells (Wang & Goldman, 1978; Heath & Dunn, 1978) suggest that the process may be divided into stages (Grinnell, 1978; Wang & Goldman, 1978). A tentative series of stages is listed in Fig. 2. The cell settles in a rounded conformation (Fig. 2A) and proceeds to explore the surrounding substratum by extending numerous microspikes or small filipodia (Fig. 2B). Some cell types may extend

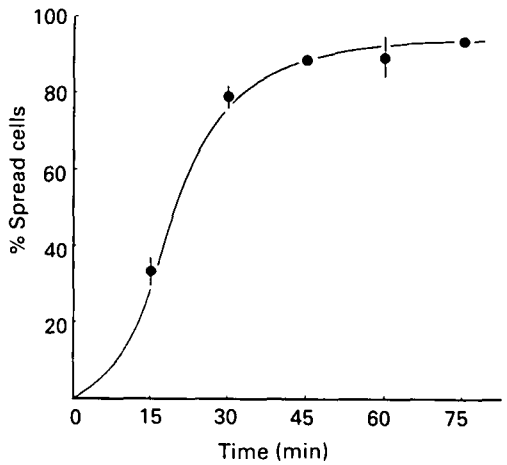

Fig. 1. Kinetics of spreading of HeLa cells on a substratum coated with amniotic fluid fibronectin. Experimental points are shown (± standard deviation) and the curve corresponds to the log normal distribution.

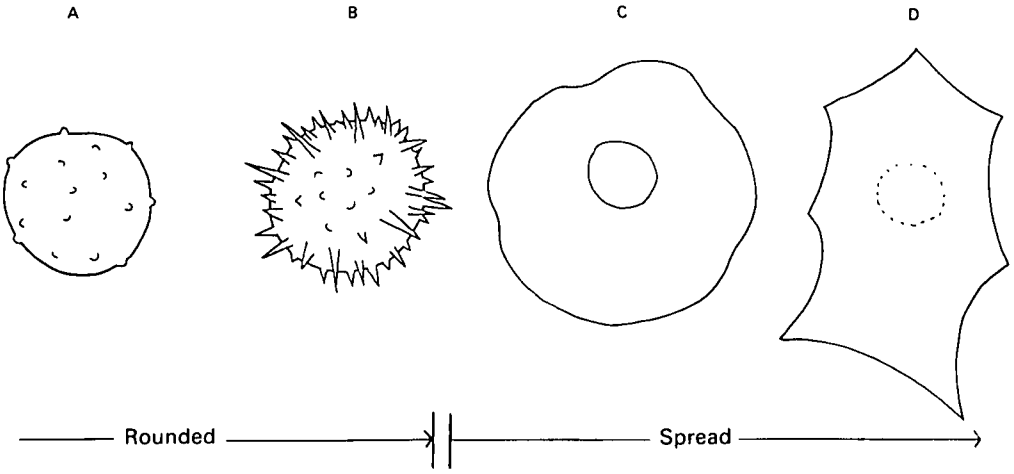

Fig. 2. Hypothetical stages of cell spreading.

lamellipodia. Once stable attachments have been formed from microspike tips, areas between these are filled in, forming areas of lamellar cytoplasm (Fig. 2C). At this stage the observer using a light microscope recognizes the cell as being spread. Further flattening occurs, however, by advancement of lamellar cytoplasm with ruffling activity and, in some cases, formation of tapering cell processes and focal contacts between cell and substratum (Fig. 2D). It is clear that these different steps in the transformation of morphology may show quite different kinetic behaviour; the rate-limiting step may involve cytoskeletal reorganization (Wang & Goldman, 1978; Heath & Dunn, 1978; Badley, Woods, Carruthers & Rees, 1980).

An outline of the possible ways to evaluate cell spreading kinetics

Thus the spreading of cells is a complex process involving many factors. Also, any attempt to estimate the rate at which cells spread will be subject to uncertainty as there is no completely objective way to measure the extent to which any individual cell has begun to spread. Nevertheless, certain observations are suggested by our own experiments.

(1) When cells are observed as quickly as possible after settling no cells are seen to be spread.
(2) When cells are applied to suitably coated surfaces and incubated at 37 °C, then eventually all observers will agree that a proportion of the cells show an altered morphology that can justify the term 'spread'.
(3) Under such conditions as are favourable for cell spreading, all observers agree that a very high proportion of cells, say $> 90\,\%$, become spread eventually after a sufficient time interval.
(4) The rate at which cells spread is dependent on the nature of the surface to which the cells are applied.

Owing to the subjective element in assessing the extent of cell spreading, there will always be some uncertainty in assessing the proportion of cells spread as a function

of time and nature of surface coating. Nevertheless, some quantitative measure of the spreading process is desirable in order that the numerical values of parameters can be estimated, allowing a more rigorous approach to comparing cell spreading kinetics than is possible at the moment.

Let us start by defining our terms of reference. We shall suppose that an experimentalist views a microscope field or photograph of cells and makes a subjective decision that a proportion of cells, say F, is actually spread. Evidently, as a function of time, the value of F is limited by the inequalities:

$$0 \leqslant F \leqslant 1{\cdot}0\,.$$

The percentage of cells spread is simply $100F$ and this proportion, F, is necessarily a function of time and the nature of the surface. For the rest of this paper we shall concentrate on attempts to formulate F as a function of time and in the following paper we shall compare the usefulness of certain mathematical models that can be formulated as applied to data obtained under a variety of experimental conditions. We shall investigate three distinct types of model, i.e. statistical models, kinetic models and empirical models.

Statistical models

When cells are first applied to a suitably coated surface, there is a distribution of the cells throughout various stages of the cell cycle. Also there will be local heterogeneity in the nature of the coated surface and, because of these factors, individual cells will take different times to achieve satisfactory attachment, a necessary preliminary to commencement of the spreading process. Nevertheless as time progresses we should observe a steady increase in the value of F from $F = 0$ at time $t = 0$ to $F \approx 1{\cdot}0$ at large time values. From this point of view, the function $F(t)$ is simply the probability that a cell will have achieved the spread state in time t. So we can regard $F(t)$ as a cumulative probability distribution function defined by:

$$F(t) = \int_0^t f(x)\,\mathrm{d}x,$$

where $f(t)$ is the probability density function. In other words, if we were able to measure the time to achieve the spread state in a large number of cells and plot a histogram of fraction of cells taking time t to spread as a function of t, then in the limit as the number of cells became infinitely large and the subdivision of t became smaller and smaller, the histogram would become a continuous curve, namely $f(t)$. We might expect $f(t)$ to be unimodal, the root of $f'(t) = 0$ representing the average time to spread for the individual cell and we might also expect $f(t)$ to be roughly symmetrical when the variance would give some idea of the dispersion of spreading times about a mean position. However, there is no reason why the distribution could not be skewed and such possibilities could only be settled by experiment. Let us now turn attention to some possible formulae for $f(t)$ and, in doing so, we must not lose sight of the fact that, since $F(t)$ values obtained experimentally are subject to error and since $F(t)$ profiles are usually simple sigmoid curves there is not likely to be any need for models with more than two or three parameters.

The truncated normal distribution

An obvious first choice for a simple distribution would be to assume a normal distribution with parameters μ and σ. However, there is one objection to this; the normal distribution is symmetrical about $t = \mu$ and allows negative t values. Since this is physically impossible we suggest a special form of the normal distribution, truncated so as to exclude negative t values. According to this model the probability density, $f_T(t)$, function would then take the form:

$$f_T(t) = \frac{(1/\sigma_T)\exp[-\frac{1}{2}((t-\mu_T)/\sigma_T)^2]}{(1/\sigma_T)\int_0^\infty \exp[-\frac{1}{2}((x-\mu_T)/\sigma_T)^2]\mathrm{d}x}, \quad 0 \leqslant t < \infty \qquad (1)$$

$$= 0 \text{ otherwise,}$$

where the definite integral in the denominator replaces the usual $\sqrt{(2\pi)}$. The interpretation of μ_T and σ_T is then simply that the time to spread is normally distributed with mean time μ_T and standard deviation σ_T but t values are confined to $t \geqslant 0$ making the distribution unsymmetrical.

The symmetrical normal distribution

A possible objection to the previous distribution is that it is unsymmetrical and an experimentalist could argue that a time of $t = \mu - t_0$ is just as likely as a time $t = \mu + t_0$. A way round this is to define a distribution that declares $t > 2\mu$ as representing all cells spread and takes $f(0) = f(2\mu) = 0$. The resulting symmetrical distribution is derived from the normal one and has the probability density function, $f_s(t)$, defined by:

$$f_s(t) = \frac{(1/\sigma_s)\{\exp[-\frac{1}{2}((t-\mu_s)/\sigma_s)^2] - \exp[-\frac{1}{2}(\mu_s/\sigma_s)^2]\}}{(1/\sigma_s)\int_0^{2\mu_s}\{\exp[-\frac{1}{2}((x-\mu_s)/\sigma_s)^2] - \exp[-\frac{1}{2}(\mu_s/\sigma_s)^2]\}\mathrm{d}x}, \quad 0 \leqslant t \leqslant 2\mu_s \qquad (2)$$

$$= 0 \text{ otherwise.}$$

Now μ_s has the interpretation of the mean time to spread and σ_s is a measure of the dispersion of values around the mean. This distribution can be thought of as arising from the normal one by raising the horizontal axis and redefining the time scale to run from $t = 0$ to $t = 2\mu$.

The log normal distribution

In the usual way we can define a log normal distribution with probability density function $f_L(t)$ given by:

$$f_L(t) = \frac{\exp[-\frac{1}{2}((\ln t - \mu_L)/\sigma_L)^2]}{(\sqrt{2\pi})\sigma_L\, t}, \quad 0 \leqslant t < \infty \qquad (3)$$

$$= 0 \text{ otherwise}$$

and the interpretation of μ_L and σ_L is straightforward. We actually perform the experiment in real time t but if we think of the 'biological time scale' as being $\ln t$, then in

the ln$t$ space μ_L and σ_L are the mean and standard deviation of ln$t$. There is no problem with $t < 0$ but the distribution is, of course, skew.

Kinetic models

It is tempting to see if differential equations could be written to describe the rate of transformation of cells, for then the integrals would be possible functions for $F(t)$ with no probabilistic interpretation. Now there are clearly several ways in which this could be done. We know that there is, first of all, a phase during which the cells settle under gravity. However, this phase is very short in experiments in shallow dishes and can safely be neglected (unpublished data; accompanying paper). Morphological changes then begin (Figs 1, 2). A possible analogy with chemical kinetics could presume that there is a stochastic transfer constant, k say, and that the rate of transformation from rounded to spread could be taken as proportional to the number of rounded cells remaining. Assuming (Fig. 2) that the observer differentiates only two states as in:

$$\text{Not spread} \xrightarrow{k} \text{spread},$$

then $F(t)$ would be defined by:

$$\begin{aligned} F(t) &= \text{spread}/(\text{not spread} + \text{spread}) \\ &= 1 - \exp[-kt] \end{aligned} \tag{4}$$

and it only remains to enquire more closely into the meaning of k. It is proposed (Fig. 2) that at the contact surface cells send out processes to explore the nature of the surface. Occasionally a stable contact forms and then the cell transforms via a rate process into the altered morphology. Obviously the rate at which this happens is roughly proportional to the number of cells remaining in the unspread state and so k constitutes an overall measure of the proportion finding satisfactory contact and the rate of transformation.

A more satisfactory model could involve an intermediate state, say a primed state, that would not be morphologically distinct from the unspread state at the resolution of the light microscope (Fig. 2). This would be formulated using two transfer constants k_1 and k_2 as in:

$$\text{Not spread} \xrightarrow{k_1} \text{primed state} \xrightarrow{k_2} \text{spread},$$

where now k_1 would measure the rate of forming contact sites and k_2 would estimate the rate of conversion into the spread state. The equation for $F(t)$ would be:

$$\begin{aligned} F(t) &= \text{Spread}/(\text{not spread} + \text{primed state} + \text{spread}) \\ &= 1 + \frac{1}{(k_2 - k_1)} \{k_1 \exp[-k_2 t] - k_2 \exp[-k_1 t]\}, \; k_2 \neq k_1 \\ &= 1 - (1 + kt) \exp[-kt], \; k_2 = k_1 = k \end{aligned} \tag{5}$$

and this allows a sigmoid $F(t)$ profile unlike equation (4).

In defence of using such kinetic models with irreversible steps, we simply indicate that in the time course and experimental situation envisaged the reverse transformation from spread to rounded morphology is not appreciable.

Empirical equations

In a sense, all of the equations proposed so far are empirical equations for $F(t)$, since very sweeping assumptions have been made in developing them. However, the statistical models can be strongly defended on the grounds that the μ and σ values obtained certainly do have the significance described whatever underlying molecular interpretation there is. It is perhaps not so easy to defend the interpretation of the k_i values obtained using the kinetic equations, since exponential models are quite versatile and the sort of $F(t)$ profiles obtained experimentally would be well fitted even if the interpretation of the meaning of the k_i was in error. The only way to settle this would be to measure the rates of transformation of individual cells via such primed or transition states as must exist and this has not yet been satisfactorily achieved. In any case, we might expect large inter-cell variations in the k_i values leading, in the end, to a probabilistic type of interpretation. For purposes of comparison, we could try a model such as the Hill equation in the form:

$$F(t) = Kt^n/(1 + Kt^n) \tag{6}$$

suggested by the sigmoid nature of many of the observed $F(t)$ profiles. Equation (6) has an interpretation only in a very limited sense, i.e. when K is positive and n a positive integer, for then it models the totally cooperative binding of n ligand molecules to a receptor with no intermediate species (Bardsley & Waight, 1978). The only value in such a model is that we can thereby obtain an independent measure of how well experimental data can be fitted by arbitrary models with the same number of disposable constants. Parameter values, as new surface treatments are studied or new media are used, can then be consulted to estimate the significance of any changes resulting. This will be developed in the accompanying paper.

Scaling parameters

A final point stems from the fact that all the expressions for $F(t)$ given so far have the asymptotic property that:

$$\lim_{t \to \infty} F(t) = 1{\cdot}0.$$

In other words, after a sufficient period of time has elapsed, 100 % of the cells become spread. In actual experiments it is unusual for all the cells to be viable and maximum values of 90–95 % of total cells being spread eventually are more likely. To accommodate this we could introduce a scaling factor, λ say, that would normally be of the order of 0·9 and this could be included as a parameter to be estimated. In other words, the experimental data could be fitted by $G(t)$ defined as:

$$G(t) = \lambda F(t)\,. \tag{7}$$

This, of course, raises the problem of how many parameters are needed to fit the data. Clearly, any improvement in goodness of fit resulting from the introduction of such an extra parameter would have to be assessed against the increase in number of degrees of freedom in the model. To discuss this we now formulate a possible experimental approach.

A possible experimental approach

We shall suppose that an experimentalist has layered a suspension of cells onto a surface suitably prepared to promote spreading. At intervals of time, say $t = t_1$, $t = t_2, \ldots, t = t_n$, a number of microscope fields could be counted in replicate, by independent observers if possible, and in this way estimates of $F(t)$ together with standard errors could be determined. If we call these estimates $\mathrm{E}(t_1), \mathrm{E}(t_2), \ldots, \mathrm{E}(t_n)$ then they could be individually weighted by the standard errors, $\mathrm{SE}_1, \mathrm{SE}_2, \ldots, \mathrm{SE}_n$, and the sum of squares of weighted residuals, Q, formed according to:

$$Q = \sum_{i=1}^{n} \{[\mathrm{G}(t_i) - \mathrm{E}(t_i)]/\mathrm{SE}_i\}^2. \tag{8}$$

By non-linear regression analysis, Q could then be minimized and the best fit parameters obtained along with confidence limits on the parameter magnitudes.

Then comes the problem of estimating goodness of fit to experimental data using different models. Where rival models have the same number of parameters, then the best model is simply that with the lowest Q value. However, to decide whether any significant improvement comes with introducing extra parameters as, for instance, a choice of whether to introduce the scaling factor λ referred to previously then we could compute the likelihood ratio test statistic (Lindgren, 1976).

$F(m_2 - m_1, n - m_2)$ defined by

$$F = \frac{(Q_1 - Q_2)/(m_2 - m_1)}{Q_2/(n - m_2)}, \tag{9}$$

where Q_1, $Q_2 = Q$ values for models 1, 2; m_1, m_2 = number of parameters in models 1, 2; n = number of experimental points.

Tables of the F statistic could then be consulted to determine the confidence limit for any improvement in goodness of fit in the event that:

$$Q_1 > Q_2, \; m_1 < m_2, \; n > m_2.$$

Conclusion

In this paper we have argued that there exists a need to provide quantitative measures of cell spreading so that comparisons of different media, cell types and surface coatings can be made numerically. We have shown that simple models with only two or three parameters can be defined in several ways and we have classified alternative approaches as statistical, kinetic or empirical, arguing that in the first two cases a definite interpretation can be given to the numerical estimates of the

parameters of the model. Ways of designing experiments and discriminating between alternative models have been discussed and the use of the F-test statistic recommended for assessing the need for models with increasing degrees of freedom. Parameters obtained in this way could then be studied as functions of other experimental variables such as temperature, concentration of solutes or density of surface coating. In the following paper we shall demonstrate how these theoretical considerations can be put to practical use.

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REFERENCES

APLIN, J. D. & FODEN, L. (1982). A cell spreading factor, abundant in human placenta, contains fibronectin and fibrinogen. *J. Cell Sci.* **58**, 287–302.

APLIN, J. D. & HUGHES, R. C. (1981). Cell adhesion on model substrata: threshold effects and receptor modulation. *J. Cell Sci.* **50**, 89–103.

BADLEY, R. A., WOODS, A., CARRUTHERS, L. & REES, D. A. (1980). Cytoskeleton changes in fibroblast adhesion and detachment. *J. Cell Sci.* **43**, 379–390.

BARDSLEY, W. G. & WAIGHT, R. D. (1978). Factorability of the Hessian of the binding polynomial. The central issue concerning statistical ratios between binding constants, Hill plot slope and positive and negative cooperativity. *J. theor. Biol.* **72**, 321–372.

BEN-ZE'EV, A., FARMER, S. R. & PENMAN, S. (1981). Protein synthesis requires cell surface contact while nuclear events respond to cell shape in anchorage-dependent fibroblasts. *Cell* **21**, 365–372.

BEN-ZE'EV, A. & RAZ, A. (1981). Multinucleation and inhibition of cytokinesis in suspended cells: reversal on reattachment to a substrate. *Cell* **26**, 107–115.

EDSALL, J. T., GILBERT, G. A. & SCHERAGA, H. A. (1955). The non-clotting component of the human plasma fraction I-1 (cold-insoluble globulin). *J. Am. Chem. Soc.* **77**, 157–161.

FOLKMAN, J. & MOSCONA, A. (1978). Role of cell shape in growth control. *Nature, Lond.* **273**, 345–349.

GRINNELL, F. (1978). Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* **53**, 65–144.

GRINNELL, F. & FELD, M. K. (1979). Initial adhesion of human fibroblasts in serum-free medium: possible role of secreted fibronectin. *Cell* **17**, 117–128.

HEATH, J. P. & DUNN, G. A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high voltage electron microscope study. *J. Cell Sci.* **29**, 197–212.

KNOX, P. & GRIFFITH, S. (1980). Distribution of cell spreading activities in sera: a quantitative approach. *J. Cell Sci.* **46**, 97–112.

LINDGREN, B. W. (1976). *Statistical Theory*, p. 543. New York: Macmillan Publishing Co.

SCHOR, S. L. (1979). The effects of EGTA and trypsin on the serum requirements for cell attachment to collagen. *J. Cell Sci.* **40**, 271–279.

STOKER, M. G. P., O'NEILL, C., BERRYMAN, S. & WAXMAN, V. (1968). Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer* **3**, 683–693.

WANG, E. & GOLDMAN, R. D. (1978). The function of cytoplasmic fibres in intracellular movements in BHK21 cells. *J. Cell Biol.* **79**, 708–726.

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