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Limiting dilution analysis: from frequencies to cellular interactions

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Molecular biology techniques have provided the means to pursue the precise definition of molecular components in a single cell type and its clonal progeny. However, to study the workings of the individual components of the immune system as well as the system itself, additional tools and supplementary approaches are required.

Limiting dilution analysis (LDA)[1] has gained widespread acceptance as a tool for quantifying cells that possess observable functional activities. Thoroughly planned titration experiments can produce straightforward and interpretable single-hit kinetics, whereas analyses of unfractionated cell populations over a broader dilution range result in data that deviate from linearity and do not adhere to all-or-none functionality (e.g. virgin and memory $CD4^+$ T cells[2] and others[3–10]). However, by studying the factors that cause the deviation from linearity, the interactions between different cell types in the population can be identified and characterized. As a corollary, it follows that, along with quantification of desired cells, LDA allows an analysis of the regulatory processes that underlie an observed activity.

Complex interactions and their zigzag approximation

Because the immune system is a complex network of cellular and humoral interactions, it is not unreasonable to expect that any unfractionated cell sample that is taken from a functional organism and then dispersed into limiting dilution cultures will yield non-linear titration curves. One of the most frequent nonlinear phenomena that occurs during LD titration of this type is typified by a zigzag curve (Fig. 1). Zigzag relationships have previously been noted in a variety of experimental systems including tests of T-helper-cell function in humoral responses[5,6], mixed lymphocyte responses (MLR)[3,9] and generation of cytotoxic activity[4,7]. Investigators have argued that zigzag curves reflect a superimposition of three distinct types of limiting precursor cells (LPC) with alternative functions: effectors (LPC1), followed by suppressors (sLPC) and then by an additional class of suppressor-resistant effectors or contrasuppressor cells (LPC2)[5,6,11]. Notwithstanding, the existence of three precursor types and the validity of the equation $Z = LPC1 + sLPC + LPC2$ have never been confirmed experimentally. Furthermore, no satisfactory hypothesis has been formulated to create a solid basis for validating this phenomenon. A noteworthy exception, however, is an attempt by Eichman *et al.* to explain LDA data as evidence of a regulatory network existing between T-cell populations[12].

Recently, as a result of reinvestigation of the zigzag phenomenon, a new model has been proposed[8,10]. In short, zigzag-shaped LDA plots were obtained from tests of the following different T-helper-cell functions: (1) proliferative responses against syngeneic or allogeneic feeder cells; (2) interleukin 2 (IL-2) production in the presence of allogeneic cells; and (3) helper function for B cells. To reveal the true titration curve shape, the number of experimental points was increased. The resulting LDA plots were inconsistent with the linear approximation model described above (see Fig. 1a). To approximate the third region of the zigzag curve with a straight line that passed through the origin was especially problematic. The positions of the experimental points between the second and third regions, the kink, usually fell close to or laid on the x-axis such that an approximating line for the third part of the curve needed to be non-linear (see LPC2 curve in Fig. 1b).

LPC types

Based on these results, the whole zigzag curve might represent a competition (possibly for growth factors) between only two distinct cell subpopulations (Fig. 1b). The second subpopulation (LPC2) has n-hit kinetics (where only n or more cells of this type are able to transform the culture into a positive one), and is capable of suppressing the proliferation of LPC1 with m-hit kinetics (where m equals the number of LPC2 cells able to suppress LPC1 activity). From this definition, the proposed model predicts that the zigzag phenomenon will occur when $n>m$. When the conventional model that was based on linear approximation of the curve portions was compared with the newer nonlinear two-cell model, it became evident that the latter was far superior in predicting and conforming with observed results[2,10].

We have also confirmed the validity of the equation $Z = LPC1 + LPC2$ (from Fig. 1b) experimentally in the following manner: (1) the zigzag was split into its elementary constituents – LPC1 and LPC2 – and each of the individual components displayed the predicted kinetics (i.e. LPC1 with single hit and LPC2 with multi-hits); (2) a mixture of two T-cell populations with single-hit and n-hit kinetics produced a zigzag LD curve; (3) when the single-hit kinetics of LPC2 were induced by prolonged cultivation or by supplying necessary growth factors, the zigzag curve degenerated into a straight line (this is the case as the obligatory condition for the zigzag curve appearance is the multi-hittedness of LPC2); and (4) blockade of the lymphokine that is produced by LPC2 (which is capable of inhibiting LPC1) using anti-lymphokine antibodies transformed the zigzag into a straight line[2,10].

Two cell types providing three functions

The two-cell-interaction model uses four parameters to define the zigzag shape: (1, 2) f1

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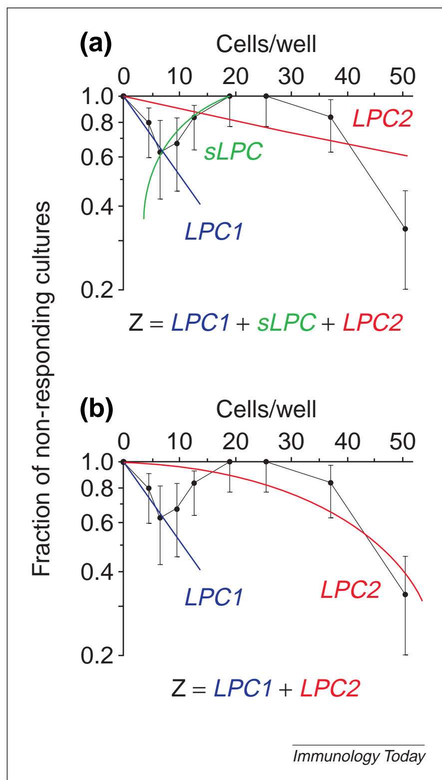

Fig. 1. *Zigzag limiting dilution analysis (LDA) plot. Analysis of the contributing elements using the partial linear approximation method (a) and two-cell model (b) of data from experiments with concanavalin A (ConA)-induced proliferation of mouse CD4*$^+$ *T cells. (a) The resulting zigzag dependence is presented as a superimposition of three distinct cell types all possessing single-hit kinetics: (1) limiting precursor cells with effector functions (LPC1), which are present at the highest frequency; (2) suppressor cells (sLPC), which inhibit LPC1 activity in shared cultures; and (3) suppressor-resistant effectors or contrasuppressor cells (LPC2), which are insensitive to or capable of canceling the inhibitory influence of sLPC. If their activities are considered to possess single-hit kinetics, the frequency of all three precursor cell types can be estimated by traditional methods using approximating straight lines that pass through the coordinate origin. In the case of sLPC, an analogous linear dependence can be obtained if the LDA plot is transformed to reflect the dependence of the fraction of positive cultures (instead of negative cultures) with respect to responder cell input. (b) The resulting zigzag dependence is presented as a superimposition of only two distinctive cell types with different activities and kinetics: (1) LPC1 with single-hit kinetics and high individual activity; and (2) LPC2 exhibiting multi-hit kinetics and an ability to inhibit LPC1 activity in shared cultures.*

and f2, the frequencies of LPC1 and LPC2, respectively; (3) n, the minimal number of cells that promote LPC2 effector function in one culture; and (4) m, the minimal number of LPC2 cells that promote an inhibitory effect on LPC1 activity in a co-habitated culture. The model not only gives a better fit to the experimental data[8,10], but also provides insight into the underlying mechanism of cell interactions, because changes in LDA curve shapes can be monitored as individual parameters that affect either population are altered.

To illustrate this point, four theoretical sets of curves showing the effects of varying the four parameters were created (Fig. 2a–d). Under experimental conditions, real analogs of these curves were produced, thus confirming the practical use of this model (Fig. 2e–g). It should be possible to characterize the regulatory interactions at the clonal level quantitatively from the changes that have been introduced through the participating subpopulations (and through their kinetic parameters). To overcome the gap between the approach of 'theoreticians' and 'practitioners', a set of simplified algorithms[8], which provide more than adequate parameter estimations without the need for any profound mathematical armament, have been developed.

Universality of the competition of alternative activities?

It is astonishing that the zigzag curve shape is obtained from population titrations using a variety of different cell sources and experimental systems. Is it possible that there is something in all cell populations that functions to 'keep a balance'? Although we do not go as far as to suggest that the immune system functions entirely in a 'ying–yang' fashion, the need for homeostatic controls certainly exists in complex biological systems; several immunological examples are provided below.

Th1 and Th2 cell populations

The adaptive immune system is controlled by two subsets of T helper (Th) cells, termed Th1 and Th2, which can be distinguished from each other by mutually exclusive lymphokine production profiles. Strong Th1-like responses (e.g. delayed-type hypersensitivity) are often mutually exclusive of strong Th2-like responses (e.g. strong antibody and allergic responses)[14]. Several lines of indirect evidence have been obtained from testing the responses of Th cells to alloantigen under LD conditions, indicating that the competition between these two cell types might be responsible for creating a zigzag-type dependence[15].

Virgin and memory T cells

The competition between virgin and memory effector B cells was described some time ago for humoral immune responses[16], and was extended to T cells once the Th-cell responses to mitogen activation under LD conditions were investigated[2,10]. Analyses of the corresponding zigzag curves agree very well with the two-cell model. Separated CD4$^+$ virgin and memory cells represent different kinetics of the response: single-hit for virgin cells and multi-hit for memory ones. The inhibitory influence of memory T cells on virgin-T-cell activity can be modeled by adding exogenous interleukin 10 (IL-10) and this, in turn, can be blocked by adding anti-IL-10 antibodies. The multi-hittedness of the response of purified memory T cells results from an IL-2 deficiency and can be converted into single-hittedness through addition of exogenous IL-2. The conversion of multi-hit into single-hit kinetics is – as predicted by our theory – accompanied by the degeneration of the zigzag into a straight line[2].

T cells in young and old mice

Dose-response curves generated for CD4$^+$ T cells from old – but not from young – animals have a zigzag-shaped relationship. Using cell separation methods, treatment with blocking antibody, or exogenously supplied lymphokines, the zigzag phenomenon in T-cell responses from old mice is due to a regulatory interaction between virgin and memory CD4$^+$ T cells[2]. This effect is not seen when T cells from young animals are examined, as the frequency of memory cells that are derived from young mice is insufficient to induce significant nonlinearity in the

dose-response[10,17]. The accumulation of memory cells upon ageing[18] shifts the proportions of virgin and memory cells into the range where the memory cells cause a nonlinearity in the dose-response. Moreover, this zigzag phenomenon can be generated for T cells that are derived from young animals by increasing the proportion of T memory cells experimentally.

Unmasking hidden activities

At a high cell input (e.g. bulk cultures), competition among cell populations might mask the expression of some cellular phenotypes. However, LDA systems can reveal that certain activities are still present (see examples of hidden and unmasked autoreactivities in Refs 9, 19). It is plausible that such an unmasking (of inhibitory activity) *in vitro* has its pendants in certain pathologies *in vivo*. If this turns out to be the case, the two-cell model could provide the basis to estimate the level of pathology, its development and efficacy of treatments.

Concluding remarks

Neither molecular methods nor cellular assays alone offer a full description of the fundamental mechanisms of immunological phenomena. In fact, both approaches are needed, as analysis of immunological responses requires a combination of molecular and cellular techniques to fully understand the participation of a single cell in the context of a much larger and intricate system. Jerne noted 30 years ago that biological science progresses by alternating the effort in studying function and structure[20]. The science of today reveals that the clear distinction between these two worlds has already begun to disappear. For example, LDA is a cellular assay, polymerase chain reaction (PCR) is a molecular one and LD-PCR (Refs 21, 22) is the result of the convergence of these two distinct approaches.

The behavior of cells and their complex interactions are difficult to understand and to monitor *in situ* because only a few of the many parameters can be controlled. By contrast, systems that are controlled strictly, such as work with purified clones or transformed cells, are highly artificial and raise

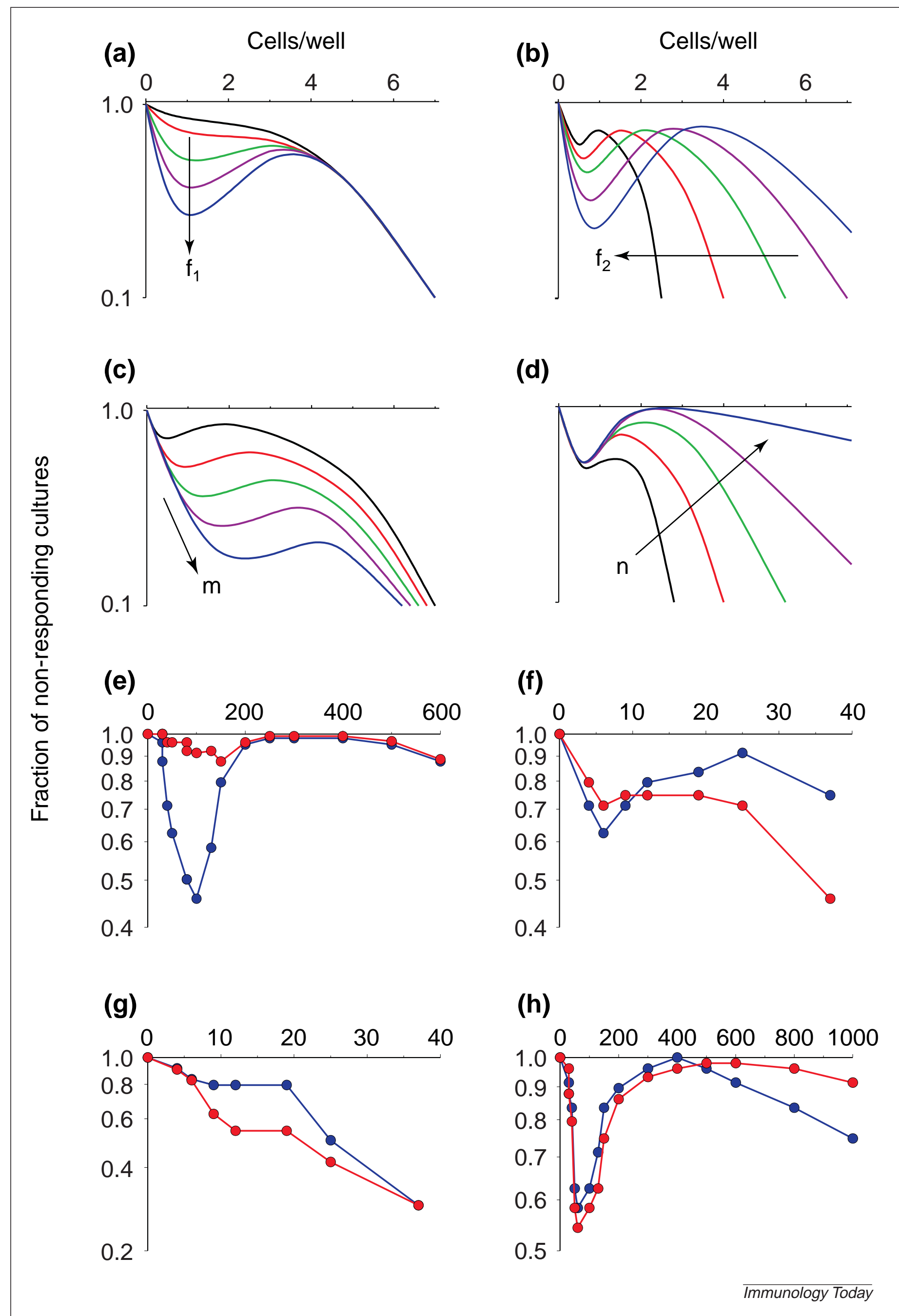

Fig. 2. *Theoretical and experimental sets of limiting dilution analysis (LDA) plots. Theoretical plots (a–d) were generated using the 'two-cell' model by varying one of the four model parameters and keeping the other three fixed. Lower panels (e–h) present experimental data that are analogous to the predicted outcomes and are arranged to correspond with the upper set of panels. (e) Allogeneic mixed lymphocyte reaction (MLR) of non-adherent BALB/c splenocytes against adherent B6 spleen cells, treated (red) or not treated (blue) with all-*trans *retinoic acid before initiation of the LDA culture. Such treatment of antigen-presenting cells (APCs) results in alteration of their membrane structures and an increase of their stimulatory potency for autoreactive T lymphocytes (see discussion in Ref. 13). (f) Concanavalin A (ConA)-induced proliferation of naïve and memory CD4$^+$ T cells at ratios of 9:1 (blue) or 7:3 (red). (g) ConA-induced proliferation of a 1:1 mixture of naïve and memory CD4$^+$ T cells in the presence (red) or absence (blue) of anti-IL-10 antibodies. (h) Autologous MLR of BALB/c non-adherent spleen cells versus mitomycin C-treated BALB/c adherent spleen cells at concentrations of 10^4 stimulators (blue) or 2×10^4 stimulators (red) per well. Data taken, with permission, from Ref. 8.*

some doubts about the applicability of the observations to real processes *in vivo*. LDA does not offer a cheap solution, but it is one of the few methods in which a large number of parameters can be controlled simultaneously, while also allowing a large number of variables to be monitored.

There are many relevant LDA examples that could not be dealt with here because of space constraints. These are given in the new edition of the LDA monograph (see Ref. 1) in which a software package for LDA calculations and LDA simulation is included.

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