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Evidence for Deterministic Chaos in Aperiodic Oscillations of Acute Lymphoblastic Leukemia Cells in Long-Term Culture

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Abstract. Biological systems are dynamic and possess properties that depend on two key elements: initial conditions and the response of the system over time. Conceptualizing this on tumor models will influence conclusions drawn with regard to disease initiation and progression. Alterations in initial conditions dynamically reshape the properties of proliferating tumor cells. The present work aims to test the hypothesis of *Wolfrom et al*., that proliferation shows evidence for deterministic chaos in a manner such that subtle differences in the initial conditions give rise to non-linear response behavior of the system. Their hypothesis, tested on adherent Fao rat hepatoma cells, provides evidence that these cells manifest aperiodic oscillations in their proliferation rate. We have tested this hypothesis with some modifications to the proposed experimental setup. We have used the acute lymphoblastic leukemia cell line CCRF-CEM, as it provides an excellent substrate for modeling proliferation dynamics. Measurements were taken at time points varying from 24h to 48h, extending the assayed populations beyond that of previous published reports that dealt with the complex dynamic behavior of animal cell populations. We conducted flow cytometry studies to examine the apoptotic and necrotic rate of the system, as well as DNA content changes of the cells over time. The cells exhibited a proliferation rate of nonlinear nature, as this rate presented oscillatory behavior. The obtained data have been fit in known models of growth, such as logistic and Gompertzian growth.
Keywords: Proliferation, deterministic chaos, aperiodic oscillations, non-linearity, CCRF-CEM.

1. Introduction

The scope of the present work is to test the hypothesis posed by *Wolfrom et al*. that proliferation shows evidence for deterministic chaos. Biological systems are dynamic systems. The knowledge on how to determine a present state from the previous ones is critical within many areas, or applications, varying from cancer to insect population control. However, it has been proven a very tedious work to discover laws underlying biological systems, since on one hand it is not easy to model such systems due to their complexity, and on the other hand, biological dynamical systems posses significant adaptation capabilities. Their hypothesis was tested on adherent Fao rat hepatoma cells and it was found that these cells

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manifest aperiodic oscillations in their proliferation rate, giving evidence for deterministic chaos. We tested this hypothesis, adding specific modifications to the previously published experimental setup. We used the acute lymphoblastic leukaemia cell line CCRF-CEM since it provided an excellent substrate for modelling proliferation dynamics. Several studies have been occupied with the complex dynamic behaviour of animal populations [1-4]. However, very little is known about the dynamics of tumour cell proliferation [5] and even less is known about the state of proliferation dynamics during oncogenesis; that is until cells reach an adequate population to be diagnosed. The data that can be collected from tumours, regarding their dynamic nature, can only happen after a tumour has been diagnosed, which usually is too late for the patient, as all the progress-determining steps have taken place. Therefore, *in vitro* systems provide an excellent opportunity to study effects that are impossible to measure *in vivo*. Most importantly, they enable the study of long-term behaviour, which is required when it comes to reaching conclusions with regards to non-linearity and chaotic system behaviour. This, in particular, is impossible to happen, even with primary cultures of cells, since they are short-lived (15-20 days) when untransformed, and the only way is the use of established cell lines obtained from different organisms. For that reason we developed a modelling approach so as to simulate the *in vivo* conditions as best as possible. Cells were seeded at a low initial concentration of 20 cells/μl. Since they grew in suspension we assumed that they reached an even/equal distribution in the media solution. Measurements were taken at least at two-day intervals, thus obtaining more than 80 measurements in total, exceeding the *Wolfrom et al.* protocol, which took around 40 measurements. Also, taking a sample from a liquid culture has minimum effects on the total cell population, since it is not essential to trypsinize in order to take the sample; trypsinization, a requirement for obtaining samples from adherent cell cultures, stresses the cells and changes their proliferation dynamics. Cells were passaged at regular intervals. This practically removed the dead cells from the system and the remaining cells were allowed to grow again in a fresh medium. This allowed modelling of the growth of a tumour, such as leukaemia, in a space with finite capacity. Removal of cells modelled the circulation that removes dead cells from a particular position in the organism. Cells were grown for approximately 150 days (5 month period) while as previously reported, Fao cells were kept in culture for 200-240 days in total.
The nature of proliferation dynamics may give insight into the way that cells not only proliferate but also differentiate.

2. Materials and Methods

2.1. The CCRF-CEM cell line:

The CCRF-CEM (T-ALL) cell line was used as the model, obtained from the European Collection of Cell Cultures (ECACC, United Kingdom). The CCRF-CEM cell line, a $CD4^+$ [6] and $CD34^+$ presenting cell line [7], was initially obtained from the peripheral blood of a 2 year old Caucasian female. She was diagnosed with lymphosarcoma which progressed to acute lymphoblastic leukaemia later on [8]. The child underwent irradiation therapy and

chemotherapy prior to obtaining the cell line. Although remission was achieved at various stages, the disease progressed rapidly.[8] The cell line has been observed to undergo minor changes after long-term culture, except for the presence of dense granules in the nucleoli.[9] Finally, the CCRF-CEM cell line has been reported to manifest autocrine catalase activity, which participates to its mechanisms of growth and progression.[10].

2.2. Cell culture conditions: Cells were grown in RPMI-1640 medium, 10% FBS and 0.1× Streptomycin/Penicillin at 37 ^{o}C, 5% CO_2 and ~100% humidity. Cells were cultured in 75cm^2 in total medium volume of 25ml. Cells were seeded at an initial concentration of 20 cells/μl and ~200 cells/μl and were fed at regular intervals thereafter. Medium changes took place by centrifugation at 1000 rpm for 10min, the supernatant was discarded and the remaining cells were rediluted in 25ml media and were allowed to grow.

2.3. Measurements, experimental setup and model*:* The CCRF-CEM cells grow in suspension and therefore give an excellent model of avascular growth. In addition, the following assumptions have been made for its proliferation: a) extracellular signal transduction takes place autocrinaly, b) the cell distribution at seeding and thereafter is considered to be uniform and c) nutrient supply was considered to be stable since cells were fed at regular time intervals. All measurements have been performed in triplicates.
Wolfrom and collaborators (*2000*), had counted the cell population at the end of a time period varying from 5 to 7 days. At the end of this period, cells were trypsinized, measured and then seeded at an initial concentration of 10^5 cells per flask.
In the present study, before every measurement, flasks were gently shaken in order to assure that the sample taken consisted of a representative, equally distributed population size. For the growth dynamics study of the cell culture system, an experimental setup was developed, where cells were assayed at least every 48h and the media renewed every 3-5 days. For the measurements, 200μl from each flask was taken and measured with a NIHON KOHDEN CellTaq-α hematology analyzer.
In that way more than 80 measurements were obtained in a period of 150 days (5 months).

2.4. Mathematical model and analysis*:* We used a one-dimensional representation based on the assumption that the present state of our system is dependent upon the previous one. So, our system is better described by the logistic equation, as $f(x_{n+1}) = kx_n(1-x_n)$ (**1**) and with respect to time $\dot{x}_n = kx_n(1-x_n)$ (**2**) (the *logistic differential equation*). Both equations belong to the family of logistic equations of the form $f(x) = kx(1-x)$ (**3**), where k is the proliferation constant. For the analysis of the data collected we have utilized phase-space and return maps and used the geometrical representation proposed by *Wolfrom et al (2000)*. In addition, we have tested the dependence on initial conditions by using two different starting population sizes. For testing the

chaotic behavior of the system, we have calculated the *Lyapunov* exponent and searched for strange attractors or sources. Many methods have been proposed for the calculation of *Lyapunov* exponents and it is considered to be a difficult task [11, 12]. In our case, given the function which we have based our model on, we used for the approximate estimation of *Lyapunov* parameters the following definition: Let *f* be a smooth map on $\Re$. The *Lyapunov* number *L(x1)* for the orbit $\{x_1,x_2,\ldots,x_n\}$ is defined as $L(x_1)=\lim_{n\to\infty}\left(\left|f'(x_1)\right|\ldots\left|f'(x_n)\right|\right)^{\frac{1}{n}}$ (4) if the limit exists. In conjunction, the *Lyapunov* exponent $h(x_1)$ is defined as $h(x_1)=\lim_{n\to\infty}\frac{1}{n}\left(\ln\left|f'(x_1)\right|+\ldots+\ln\left|f'(x_n)\right|\right)$ (5).

3. Results

We have studied the proliferation dynamics of an acute lymphoblastic leukaemia cell line by developing a modelling approach, where cells from two different initial populations were allowed to grow with a periodic nutrient supply in order to sustain cell growth.

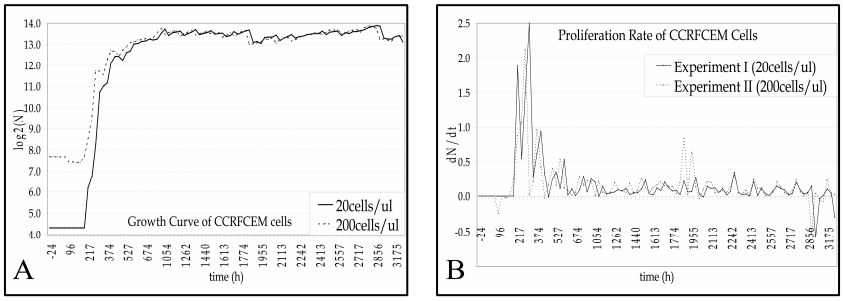

FIGURE 1. *Growth curve of the CCRF-CEM cells as a function of time shows the characteristic pattern of the iterated logistic equation (**A**). Proliferation rate of cells exhibits an almost unpredicted oscillatory behaviour (**B**). N is the actual measured cell population at time t. It is apparent that this oscillatory behavior has a declining tendency, something expected, since cells compete for space during their growth. Nutrients are considered to be abundant and equally distributed among the cells' environment.*

The time series produced from the experimental data showed a characteristic logistic pattern as described in (1), whereas proliferation rate with respect to time manifested an aperiodic oscillatory behavior (Fig. 1A and 1B). In fact, proliferation rate appears to manifest a saltatory pattern where cells after a period of "adjustment" to the environment start dividing rapidly. This was the first evidence that the dynamics of cell population can manifest complex behavior. Interestingly, when calculating the *Lyapunov exponent* of the two curves starting from different initial conditions, these gave different results (Fig. 2A). For the curve describing cell proliferation from 20cells/ul, the *Lyapunov*

exponent h was calculated to be >0, whereas for the curve describing cells starting from 200cells/ul it was <0. Furthermore, for the orbit *Lyapunov exponent* was 0.13 for x_0=20 and -0.09 for x_0=200. The next criterion we investigated was whether the state-space manifested an asymptotically periodic pattern or not. Space-space representation has been proposed by Lorenz [13] and has been used by others in biological time-series [14]. State-space maps showed that irrespectively of the initial population (either 20 cells/ul or 200 cells/ul) both curves converged after a long period of cell culture (Fig. 2).

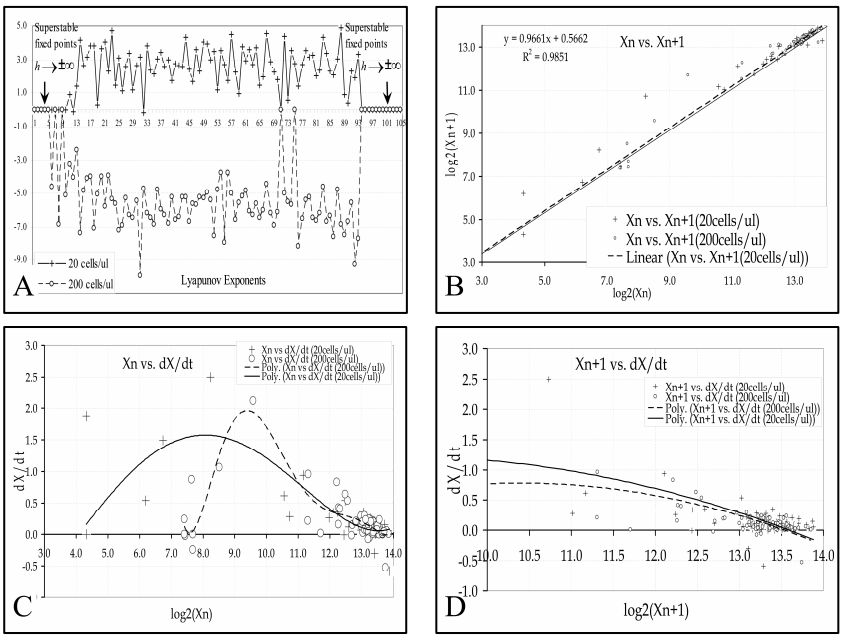

FIGURE 2. State-space of the growth curves of CCRF-CEM cells. *Cells were grown from two different initial populations: 20 cells/ul* (+) *and 200 cells/ul* (o). *Solid lines represent fitting curves for cells started from 200 cells/ul, while dashed lines represent fitting curves for cells started from 20 cells/ul.*
*When calculated for the two growth curves, Lyapunov exponents, gave opposite results (**A**.). Both orbits are asymptotically periodic. However, positive Lyapunov exponents were obtained only for x_0=20 cells/ul, hence it can be assumed that proliferation starting from 20cells/ul has chaotic orbits. Plotting the population x_n vs. x_{n+1} shows that both populations converge to a fixed plane (**B**) which, however, does not manifest asymptotical periodicity. Treating equation (2) as a two-dimensional function shows that the proliferation rate converges to a fixed plane as a function of x_n (**C**) and x_{n+1} (**D**).*

Probably our system reaches a steady-state in the long term (Fig. 2) or at least converges. Again, at first quite interesting was the fact that when we drew the return map of our initial data i.e. the population time-series, it appeared that it gave an almost perfect linear pattern. This was noteworthy because such a fact would mean that oscillatory behavior observed in the experimental measurements of the time-series was probably due to noise. To test this we

performed a *Fast Fourier Transformation* (*FFT*) analysis (data not shown) which did not show the prevalence of noise. On the other hand, how does nonlinearity emerge? The logistic equation, on which we based our model, assumes that a population grows and then decays due to a limited amount of nutrients. In our model this was not the case since we kept a constant supply of nutrients in order to keep cells growing. This led us to the conclusion that the nonlinear factor was not the population *per se* but rather the proliferation constant *k*. In our initial assumptions we assumed that nutrients were abundant and constant, whereas the changing environmental factor was space for growth. Space becomes limited, as time progresses, activating inhibitory mechanisms for growth. Therefore, when drawing the populations at time t_n and t_{n+1} as a function of the proliferation rate (which is equal to $f'(x)$ for discrete time points) we observed that although the system converges towards its possible steady-state it manifests nonlinear behavior (Fig. 2B, 2C). Therefore, we analyzed the proliferation rates calculated for each discrete point in the same way. In other words, we used the return map of the proliferation rates, the first derivative of the proliferation curve at each discrete point. So, let $k_n=\{f'(x_0), f'(x_1),\ldots,f'(x_n)\}$ and $k_{n+1}=\{f'(x_1), f'(x_2),\ldots,f'(x_{n+1})\}$ where k is the proliferation rate from equations (1) and (2). K can also be written as $\frac{dX_n}{dt_n}$ and $\frac{dX_{n+1}}{dt_{n+1}}$ for t at n and $n+1$ respectively with $n \in \mathrm{N}$ as presented in Figure 3B. Interestingly, drawing k_n as a function of k_{n+1} gives us a curve with at least two fixed points for both initial cell populations indicating a period-3 orbit (Fig. 3A). This period-3 orbit implies chaos as it has been previously reported [15].

4. Conclusion

It has been reported previously that biological systems exhibit very complicated dynamics [3, 5]. The present work addressed the question as to whether chaotic dynamics could be detected in the proliferation of acute lymphoblastic leukaemia cells in long-term culture. This had been proposed previously for adherent cell lines [5]. To the best of our knowledge no reports have been published on the proliferation dynamics of suspension cell cultures and in general studies on proliferation dynamics *in vitro* are scarce. The question that arises at this point is whether such studies are meaningful, since *in vitro* systems represent *in vivo* systems only in part.

In vitro systems offer the capacity of performing long-term studies, and isolating the system under study to reduce noise, both of which are not possible in *in vivo* systems.

Cell cultures *in vitro* are considered to manifest a linear pattern of growth. Given the fact that the logistic equation takes into account limited nutrients and space, it predicts that a cell population would reach a steady-state within a certain time and eventually die out.

We have introduced a new constant to our model by making nutrient readily available but keeping space, in terms of total volume, constant. Under these conditions we draw the proliferation curve, which shows aperiodic oscillations,

whereas the proliferation rate manifests these aperiodic oscillations most clearly. As it was shown in Fig. 2A, the return map of Fig.1A gave an almost perfectly linear function, where a "bell-shaped" geometry was expected.

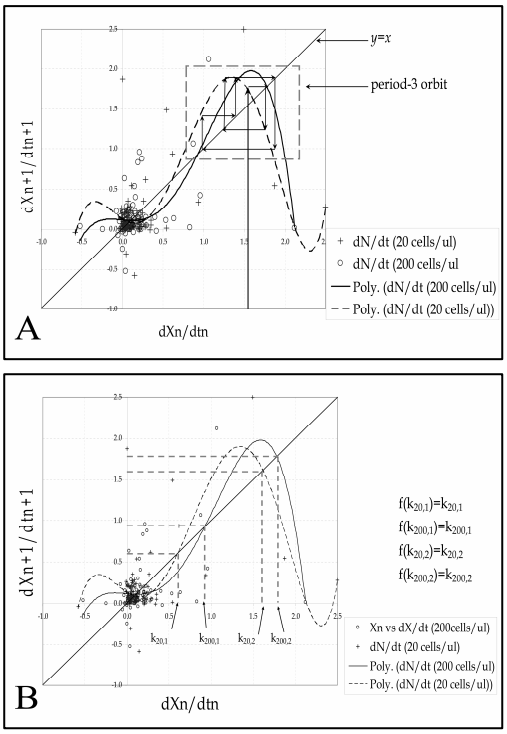

FIGURE 3. *Drawing the constant of proliferation* k_n $\left(\frac{dx_{n+1}}{dt_{n+1}}\right)$ *vs.* k_{n+1} $\left(\frac{dx_n}{dt_n}\right)$ *produces a bell-shaped curve (**B**) with at least two fixed points and period-3 orbit (**A**). The diagonal line is the y=x function, which high lights the fixed points. Solid curve line represents the polynomial fitting of cell population started from 200 cells/ul* (o). *Dashed curve line represents the polynomial fitting of the cell population started from 20 cells/ul* (+).

However, when we expanded our analysis to the return map of proliferation rate (Fig. 3B) we observed a nonlinear behaviour and we found geometrically that the fixed points on this curve manifest a period-3 orbit (Fig. 3A) which implies chaos dynamics. Our work shows evidence for deterministic chaos in the proliferative behaviour of leukaemia cells *in vitro*. Since this is a very complicated phenomenon it requires a lot more effort to understand the mechanisms underlying those dynamics. The implications from the understanding of these systems are tremendous. It will give us insight to the mechanisms of disease progression, such as in cancer, and enable building advanced models for the disease, which combine important features of both *in vitro* and *in vivo* systems. It is known that cancer starts and progresses slowly, at least before clinical presentation. Knowledge on the mechanisms of growth before the clinical symptoms become obvious may contribute to the early treatment of this and other diseases.

References

1. May, RM. Simple mathematical models with very complicated dynamics. *Nature* **261**(5560), p. 459-67(1976).

2. May, RM. Biological populations with nonoverlapping generations: stable points, stable cycles, and chaos. *Science* **186**(4164), p. 645-7(1974).
3. Mackey, MC and Glass, L. Oscillation and chaos in physiological control systems. *Science* **197**(4300), p. 287-9(1977).
4. Romond, PC, Rustici, M, Gonze, D, and Goldbeter, A. Alternating oscillations and chaos in a model of two coupled biochemical oscillators driving successive phases of the cell cycle. *Ann N Y Acad Sci* **879**, p. 180-93(1999).
5. Wolfrom, C, Chau, NP, Maigne, J, Lambert, JC, Ducot, B, Guerroui, S, and Deschatrette, J. Evidence for deterministic chaos in aperiodic oscillations of proliferative activity in long-term cultured Fao hepatoma cells. *J Cell Sci* **113 (Pt 6)**, p. 1069-74(2000).
6. Miranda, L, Wolf, J, Pichuantes, S, Duke, R, and Franzusoff, A. Isolation of the human PC6 gene encoding the putative host protease for HIV-1 gp160 processing in CD4+ T lymphocytes. *Proc Natl Acad Sci U S A* **93**(15), p. 7695-700(1996).
7. Naujokat, C, Sezer, O, Zinke, H, Leclere, A, Hauptmann, S, and Possinger, K. Proteasome inhibitors induced caspase-dependent apoptosis and accumulation of p21WAF1/Cip1 in human immature leukemic cells. *Eur J Haematol* **65**(4), p. 221-36(2000).
8. Foley, GE, Lazarus, H, Farber, S, Uzman, BG, Boone, BA, and McCarthy, RE. Continuous Culture of Human Lymphoblasts from Peripheral Blood of a Child with Acute Leukemia. *Cancer* **18**, p. 522-9(1965).
9. Uzman, BG, Foley, GE, Farber, S, and Lazarus, H. Morphologic variations in human leukemic lymphoblasts (CCRF-CEM cells) after long-term culture and exposure to chemotherapeutic agents. A study with the electron microscope. *Cancer* **19**(11), p. 1725-42(1966).
10. Sandstrom, PA and Buttke, TM. Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium. *Proc Natl Acad Sci U S A* **90**(10), p. 4708-12(1993).
11. de Souza, SLT and Caldas, IL. Calculation of Lyapunov exponents in systems with impacts. *Chaos, Solitons & Fractals* **19**, p. 569-579(2004).
12. Mueller, PC. Calculation of Lyapunov Exponents for Dynamical Systems with Discontinuities. *Chaos, Solitons & Fractals* **5**(9), p. 1671-1681(1995).
13. Lorenz, E. Deterministic Nonperiodic Flow. *Journal of the Atmospheric Sciences* **20**(2), p. 130-157(1963).
14. Strizhak, P, Magura, IS, Yatsimirskii, K, and Masyuk, AI. Return Map Approach to Description of the Deterministic Chaos in Cytosolic Calcium Oscillations. *Journal of Biological Physics* **21**, p. 233-239(1995).
15. Li, TY and Yorke, JA. Period three implies chaos. *American Mathematical Monthly* **82**, p. 985-992(1975).