A critique of the use of DNA synthesis as a measure of the effect of mitogens on lymphocytes

Marguerite GUIGUET* and Stephen COOPER†

**Laboratoire de Biostatistique, 2 Place Jussieu, Université de Paris, 75221 Paris Cedex 05, France; and †Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A*

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The use of DNA synthesis as a measure of mitogenic activity in lymphocytes is reanalyzed in the light of the Continuum Model, and it is suggested that although lymphocytes may have a linear response with regard to mitogen concentration, the curves using thymidine may show a threshold response at low concentrations and a decrease in activity at high concentrations.

Radioactive thymidine is widely used to measure the proliferation of lymphocytes in the presence of a mitogen. The effect of a mitogen can be measured either by measuring the total precipitable radioactivity or by measuring the number of radioactive nuclei after some suitable period of incubation. Here we merely wish to note that while these methods have numerous advantages (low background, large measurable increase due to mitogens, etc.), there is reason to believe that for some purposes the method may not be suitable. The basic disadvantage is that using DNA synthesis as a measure of the effect of a mitogen does not necessarily produce a result which is proportional to the actual effect of the mitogen. This lack of proportionality may lead to conclusions regarding the mechanism of mitogenesis which would not be considered if it were believed that the stimulatory effect of the mitogen was proportional to the concentration of mitogen. We wish to suggest that it may be better to measure the synthesis of new cytoplasm, rather than DNA synthesis, as a measure of mitogenic potency.

It has been observed by a number of investigators (Wang, McClain & Edelman, 1975; McClain & Edelman, 1976; Archer, Smith, Ulrich & Johnson, 1979; Powell & Leon, 1970; Ling & Kay, 1975; Rao, Schwartz, & Good, 1979; and Kay, 1969, are some examples) that at low concentrations of mitogen there is little mitogenic response, i.e., a threshold is observed. At intermediate concentrations there is an increasing response with increasing concentrations of mitogen, while at much higher concentrations there is a decrease in response with increasing mitogen concentrations. We wish to present a view of mitogenesis which may explain these observations. Crucial to the analysis presented here is the fact that all of these results that we are referring to are performed as 'execution assays'. That is, the DNA synthesis is measured much later in time after the stimulus is added to the cells. In some cases this can be as much as 72 h after

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addition of the mitogen. Prior to addition of the thymidine to measure DNA synthesis, the cells are not incubated in the presence of thymidine.

Our analysis derives from a general view of the regulation of cell growth and proliferation, the Continuum Model (Cooper, 1981a). We will briefly describe the Continuum Model and then apply it to the problem of lymphocyte activation. We do note that the ideas presented here do not depend on the Continuum Model itself, but can stand independently of the Continuum Model.

Brief resume of the Continuum Model

The Continuum Model proposes that the events involved in the regulation of DNA synthesis and cell division are occurring continuously and in all phases of the cell cycle (Cooper, 1979, 1981a,b, 1982). It proposes that there are no G1-specific events, and further that resting cells (such as lymphocytes) are not arrested in a special part of the cell cycle such as G1 or 'G0'. Rather, cells such as lymphocytes are arrested with a G1 content of DNA but not in the G1 phase of the cell cycle. (See reference 8 for a complete explication of this idea, and an analysis of the experiments which have been used to support the notion of G1-arrest or G0.) It has further been proposed that the synthesis of cell mass, and more specifically cell protein, is the primary cause of the initiation of DNA synthesis (Cooper, 1981b). The rate of cell growth and proliferation is therefore regulated by the rate of accumulation of cell mass (Cooper, 1979, 1981b). The stochastic implications of the Continuum Model have now been described (Cooper, 1982), and it has been shown that the distribution of cell interdivision times, sister-cell correlations, and other statistical measures of cell interdivision are explained quite simply by the Continuum Model. It is these concepts of the statistical implications of the Continuum Model which we wish to apply to explain the observed concentration-dependence of mitogenesis.

Application of the Continuum Model to lymphocyte activation

We assume that lymphocytes have been arrested in a condition where they are some way away from achieving the amount of 'initiator' required for the initiation of DNA synthesis. When a mitogen is added to such cells, mass synthesis occurs. It is not proposed that the primary effect of the mitogen is directly on the protein synthetic system, but rather that in all cases protein synthesis, or more generally mass synthesis, is a necessary prerequisite for the initiation of DNA synthesis. Let us now consider a mitogen in which the effect of the mitogen is *directly proportional to the concentration of the mitogen*. That is, let us assume that if the rate of protein synthesis was measured then the rate would be proportional to the concentration of mitogen added to the lymphocytes over a wide range of mitogen concentrations. We now propose the following four assumptions:

1. DNA synthesis is initiated in cells when a unit (arbitrarily defined as 1.0) amount of 'initiator' is accumulated per G1 nucleus. In practice the act of initiation occurs at a mass which is only

approximately 1.0, but which is distributed about this value. For simplicity we shall assume a normal distribution of this 'critical mass' about the value 1.0.

2. Upon addition of a mitogen, initiator synthesis is stimulated at a rate which is proportional to the amount of mitogen present over a wide range of mitogen concentrations. There is no lower limit of mitogen concentration which would give the appearance of a threshold effect. The actual rate of initiator synthesis in a given cell is normally distributed about the mean value. The actual variance in the rate of initiator synthesis is independent of, but may be increased by, any variation in the amount of mitogen bound per cell. That is, if all cells had exactly the same number of mitogen molecules bound per cell there would be a particular variance in the rate of initiator synthesis per cell. Since in practice this does not occur, the actual variance is the result of the inherent variance in the rate of initiator synthesis and the variance due to the inequitable distribution of mitogen molecules over a population of lymphocytes. We further will assume that the synthesis of initiator is exponential, although in practice this is not critical to the argument. Our argument is independent of the mode of synthesis of initiator between a particular state and the final state of initiation.

3. The amount of initiator in a lymphocyte is less than the amount of initiator required for initiation. In particular for lymphocytes, there are few cells which have any amount of initiator close to the amount required for initiation. This is the major assumption which is peculiar to lymphocytes. Previous proposals (Cooper, 1979, 1981a,b, 1982) have emphasized the fact that there was a continuous distribution of initiator amounts in resting cells.

4. The anomalous inhibition of mitogenesis at very high concentrations of mitogen can be explained with the added assumption that lymphocytes can only proceed through only one or a limited number of rounds of replication. This may be due to either limitations in the DNA replicating system or in the ability of the cell to synthesize protein.

The application of these assumptions to lymphocyte activation is diagrammatically illustrated in Fig. 1. Here we illustrate how the use of an 'execution assay' in which the effect of the mitogen is measured by thymidine incorporation at a much later time can lead to the conclusion that there is a threshold level of mitogen activation. We have assumed that once a cell has reached the threshold required for initiation of DNA synthesis, addition of thymidine at any later time will result in either a labeled cell or alternatively a similar amount of label incorporated into the cells. If, as noted in assumption 4, the incorporation of label into DNA following initiation of DNA synthesis does not continue for an extended period of time, then one might consider that at the higher rate of initiator synthesis (that is, higher mitogen concentrations), one might see less incorporation because cells will have initiated DNA synthesis, *and completed such DNA synthesis* by the time that radioactive thymidine is added. This will thus give rise to the apparent 'inhibition' in mitogenesis at higher mitogen concentrations.

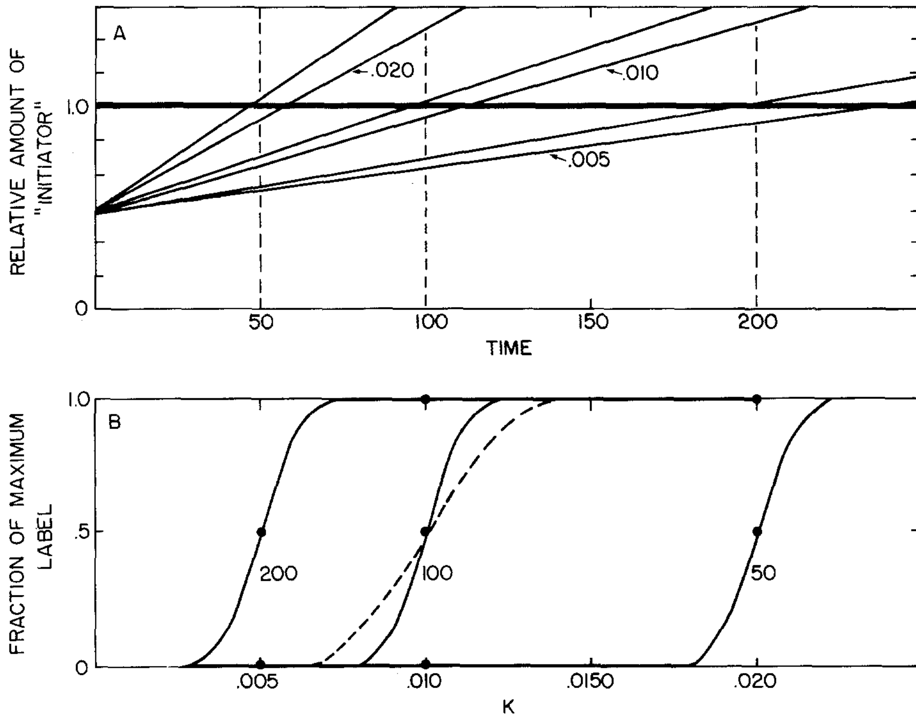

Fig. 1. Illustration of the application of the Continuum Model to the activation of lymphocytes. Consider a population of lymphocytes which have a mean 'initiator mass' of 0.5 which is normally distributed about this value. Consider the addition of three different concentrations of mitogen in the ratios of 1:2:4. As noted in the text, we will assume that the mean rate of initiator synthesis in the lymphocyte population is proportional to the concentration of mitogen. Now consider that at some time radioactive thymidine is added to the populations of cells and either the amount of radioactive label incorporated or the fraction of cells with labelled nuclei is measured. Consider the intermediate time (100) in the upper graph (A) such that at the lowest concentration no cells have synthesized enough initiator to reach the critical initiator mass (normally distributed about the value 1.0), about half of the cells with the intermediate concentration of mitogen will have reached the critical initiator value and will thus have initiated DNA synthesis, and all of the cells with the highest mitogen concentration will have initiated DNA synthesis because they will have synthesized the critical initiator mass. In the lower graph (B) this result is shown by curve 100

with a threshold at the lower concentrations, a rise at the intermediate mitogen concentration, and a plateau at the highest concentration where additional mitogen does not give any further increase in label. The rise in the intermediate range will be determined by the variance in the rate of initiator synthesis. The greater the variance, the shallower the rise as indicated by the dashed line in graph B. Note that an increase in the variance of the rate of synthesis, or the initial 'initiator content', or the critical mass required for initiation in a particular cell will all yield a flatter rise in the labeling curve. It is interesting to consider what would be expected if different times of labeling were chosen as indicated by times 50 and 200. If label were added early one would find a smaller fraction of cells labeled at the highest mitogen concentration and no label incorporated with the lower mitogen concentrations. This produces the labeling curve 50 in graph B. If very late times are used, cells at even the lowest mitogen concentrations may be partially labeled (producing curve 200 in graph B), while the cells with the higher mitogen concentrations will be completely labeled. Now let us consider, as has been proposed in assumption 4, that DNA synthesis may not occur for an extended period after a round of replication has been completed. Since at the highest concentrations of mitogen the initiation of DNA synthesis has begun quite early (around time 50), it may be that by the time the label is added (time 200) no or little incorporation may be found. This would thus give the appearance of an 'inhibition' of mitogenesis at the highest concentrations. There would be less label found at the highest concentration than at some lower concentration. If, in this latter case, incorporation was measured at many time points following stimulation, a different conclusion would be reached regarding the mitogenic ability of the higher concentrations. The major conclusion is that the kind of kinetics of labeling that is obtained from a stimulation experiment may be critically dependent upon the choice of labeling times.

Results

We have performed a number of simulation studies which show in more detail the application of the basic assumptions presented above to the stimulation of DNA synthesis. The methods used have been described elsewhere (Cooper, 1982). Briefly, the Statistical simulation package of the University of Michigan Terminal System (MIDAS) has been used to calculate the expected results using different coefficients

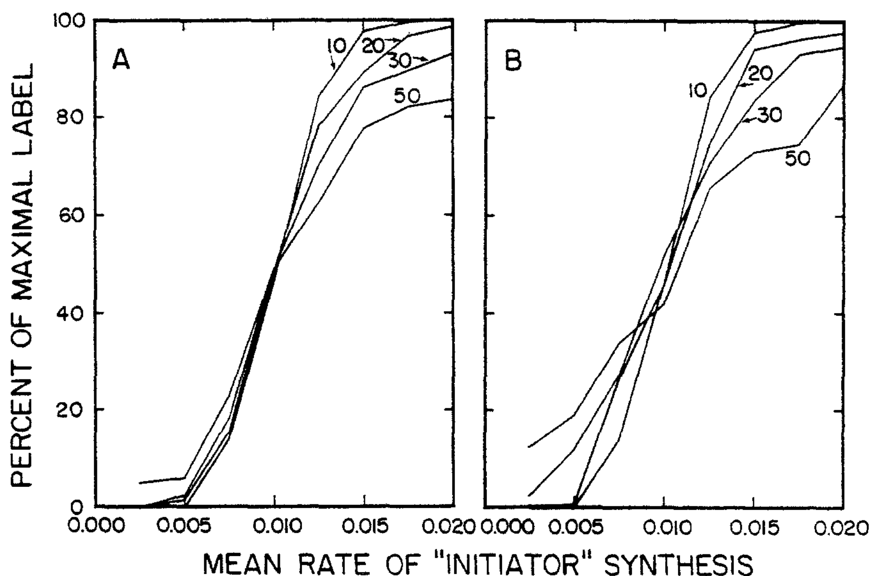

Fig. 2. Simulation of the percentage of labeled cells (or incorporated label) after 100 time units for different values of the rate of initiator synthesis (i.e different values of the concentration of a mitogen assuming that the mean rate of initiator synthesis is proportional to the mitogen concentration). (A) For each line the value of the rate (K) is assumed to be normally distributed but the coefficient of variation (S.D./mean x 100) of the rate over the population is variously 10%, 20%, 30%, and 50%. The initial mass of initiator is assumed to be 0.5 with a coefficient of variation of 10%, and the critical mass required for initiation also has a coefficient of variation of 10%. (B) Stimulation of percentage of labeled cells (or incorporated label) with the coefficient of variation of the initial 'initiator mass' being 10%, 20%, 30%, and 50%. The coefficient of variation of the rates of synthesis and the critical mass required for initiation are set at 10%. Copies of the program and related documentation are available upon request.

of variation for the various input parameters (initial initiator mass, initiator synthesis rate, and critical initiator mass). Typical results are presented in Fig. 2, where it can be seen that the assumptions we have presented can quite easily give the observed 'threshold' effect of mitogenesis, with the threshold decreasing with increased variability in the observed rate of initiator synthesis or in the initial initiator mass postulated in the lymphocytes.

Discussion

The message we wish to convey is a very simple one. We suggest that the complex kinetics of mitogenesis observed in various systems may be merely the result of the use of thymidine as a means to assay mitogenesis, and that the actual mitogenic stimulus is quite possibly a simple and concentration-dependent activator of lymphocytes. Instead of concluding that low levels of activation at low concentrations (threshold effect) and decreasing activation at high levels (inhibition) are unexpected, we suggest that they are actually expected and therefore suggestive of the model of activation upon which the analysis presented here is based. In a broader sense, therefore, lymphocytes are able to be brought into the general class of all 'resting' cells as described by the Continuum Model (Cooper, 1981a). The state in which lymphocytes are found is not a 'G0' but is rather a state brought about by the inhibition of synthesis of cytoplasm or protein.

There are both experimental and theoretical implications of our analysis. On the experimental level we would suggest either that labels be used which are more indicative of the action of mitogens, radioactive amino acids for example, or that when thymidine is used to measure mitogenesis, measurements be taken at a number of time points to ensure that the sampling time is not altering the interpretation of the incorporation data.

A similar critique of the use of 'execution assays' has also been applied to the inhibition of DNA synthesis by antibiotics (Cooper, 1974).

On a theoretical level, our analysis questions the idea that activation of lymphocytes is an 'all-or-none' phenomenon. A survey we have conducted of a number of workers in immunology has indicated that there is a general belief that lymphocyte activation is an all-or-none phenomenon rather than a continuous response. If this belief has been generated by experiments showing a 'threshold' for activation, we suggest that this threshold, as measured by thymidine, is compatible with a continuously increasing activation of lymphocytes by increasing concentrations of mitogen. If the belief in an all-or-one phenomenon is generated by the observation that removal of the mitogen (by the use of various competing sugars, for example) prior to incorporation of thymidine does not stop activation, then we suggest that this can be accounted for by a continued level of protein or mass synthesis after mitogen removal. This continued synthesis, however, would be at a level commensurate with (or proportional to) the original concentration of mitogen. Thus, mitogen may start cells toward initiation of DNA synthesis and this may continue after the removal of the mitogen, but cells will reach the initiation of DNA synthesis at a rate which is variable and proportional to the concentration of mitogen.

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References

Archer DL, Smith BG, Ulrich JT & Johnson HM (1979) Cell. Immunol. **48**, 420.
Cooper S (1974) J. Theo. Biol. **46**, 117.
Cooper S (1979) Nature **280**, 17.
Cooper S (1981a) in *Cell Growth* (Nicolini C, ed), Plenum Press.
Cooper S (1981b) Cell Biol. Int. Rep. **5**, 539.
Cooper S (1982) J. Theo. Biol., in press.
Kay JE (1969) Exp. Cell Res. **58**, 185.
Ling MR & Kay JE (1975) in *Lymphocyte Stimulation,* North Holland.
McLain DA & Edelman GM (1976) J. Exp. Med. **144**, 1494.
Powell AE & Leon MA (1970) Exp. Cell Res. **62**, 315.
Rao KMK, Schwartz SA & Good RA (1979) Cell. Immunol. **42**, 270.
Wang JL, McClain DA & Edelman GM (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1917.