ANALYSIS OF THE STIMULATION-INHIBITION PARADOX EXHIBITED BY LYMPHOCYTES EXPOSED TO CONCANAVALIN A*

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Lectins and various other proteins that bind to cell surface molecules can induce blast transformation in normal lymphocytes and have therefore been used extensively as tools in investigating both antigenic stimulation of lymphocytes and growth control of eukaryotic cells. Although the analysis of lymphocyte stimulation would ideally be carried out using antigens as mitogens, it is presently more feasible to study the detailed kinetics and biochemistry of the commitment to growth using lectins. A typical mitogenic lectin, concanavalin A (Con A),[1] is a tetravalent molecule of known structure and binding specificity (1). Its dose-response curve shows an optimal concentration for stimulating mitogenesis of T lymphocytes; the eventual response as measured by increased DNA synthesis diminishes sharply with lectin concentrations higher than this optimum. We have previously analyzed this unimodal dose-response curve (2) using low molecular weight probes such as phorbol esters, and chemically altered lectins such as succinylated Con A (3), a divalent derivative that shows no inhibitory effects at high doses. It was shown that the stimulatory and inhibitory portions of such a dose-response curve can be dissected and independently modified.

The present study indicates that the action of Con A is paradoxical in higher dose ranges, i.e. it both stimulates and inhibits, a finding in accord with previous evidence (4). Because the relative contributions of these paradoxical effects and their biochemical bases have not been thoroughly explored, we have carried out an extensive analysis of the kinetics of cell commitment, DNA synthesis, and cell interactions. In this paper, we provide evidence that: (*a*) the stimulatory signal is proportional to the concentration of lectin, even in the inhibitory dose range; (*b*) the inhibitory signal can be given both to normal lymphocytes and continuously proliferating lymphoid cells (2, 5–8); (*c*) the inhibition is reversible; and (*d*) the inhibition is not a function of direct cellular interactions as shown by analyzing cultures of cells suspended in agarose. The paradoxical inhibitory phase that occurs despite the stimulation at high doses can be understood in terms of the previously described behavior of surface modulating assemblies (SMA) in lymphoid cells (9–11).

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; αMM, α-methyl-D-mannoside; MEM, minimum essential medium; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline; SMA, surface modulating assembly.

Materials and Methods

Cells. Human peripheral blood leukocytes were isolated from the blood of normal donors by density gradient centrifugation (12) on Ficoll-Hypaque solution (LSM, Litton Bionetics, Kensington, Md.). P388 cells were obtained from the Cell Culture Center of The Salk Institute, San Diego, Calif.

Reagents and Media. Con A and succinyl-Con A were prepared as described previously (3, 13). Fortified 4 × minimum essential medium (MEM) was prepared by mixing 10 ml 10 × Eagle's MEM, 2 ml 50 × essential amino acids, 1 ml 100 × nonessential amino acids, 3 ml essential vitamin mixture, 2.93 ml 7.5% sodium bicarbonate solution, 1 ml 100 mM sodium pyruvate, 1 ml 200 mM L-glutamine, 3.67 ml water, penicillin to 400 U/ml, and streptomycin to 100 μg/ml (all from Microbiological Associates, Inc., Bethesda, Md.). The solution was made 2×10^{-4} M in 2-mercaptoethanol.

Cell Culture and Assay. Lymphocyte cultures in liquid were carried out in 10 × 75-mm plastic tubes with varying cell numbers in 1 ml of medium prepared by adding one part 4 × MEM to three parts sterile water and making it 20% in fetal calf serum (FCS, Microbiological Associates, Inc.) which had been heat-inactivated at 56°C for 30 min.

The procedure for culturing cells with agarose in dishes is schematized in Fig. 1. Cells were cultured in 3-cm tissue culture dishes (Falcon Plastics, Oxnard, Calif.). The bottom layer of agarose filled in the corners and provided a base for the manipulations necessary for autoradiographic processing; it consisted of 0.5 ml of one part 4 × MEM, one part FCS, one part of that amount of 6 × mitogen in MEM-20% FCS to achieve the final designated concentration in the entire mixture, and three parts autoclaved 1.2% agarose (lot 10514, MCI Biomedical, Rockland, Maine) in water. It was found that for maximum efficiency, different concentrations were required for different lots of agarose. The cell layer consisted of 0.4 ml per dish of one part 4 × MEM, one part FCS, one part cell suspension in MEM-20% FCS, and three parts agarose at twice the desired final concentration which was usually 0.5%. To prevent aggregation during the initial plating of the cells, the cell layer contained no lectin; to compensate for this, extra mitogen was added to the overlayer. The overlayer consisted of 0.4 ml of one part 4 × MEM, one part FCS, one part 12 × mitogen in MEM-20% FCS, and three parts of 0.6% agarose. The liquid overlay medium was 1 ml of MEM-20% FCS with 0.09 ml 12 × mitogen. The dish cultures were assembled layer by layer from solutions kept at 45°C; the layers were allowed to harden before the next one was applied. Mitogen and cells were added to the solution immediately before plating. All cultures were kept in a humid atmosphere of 10% CO_2, 7% O_2, 83% N_2 at 37°C. α-methyl-D-mannoside (αMM), when present, was added to cultures as a 1 M solution to a final concentration of 0.1 M. Mixed lymphocyte cultures were made as described above, except that the FCS was replaced by heat-inactivated human AB serum.

Unless otherwise noted, cultures received either 6 μCi [^{3}H]thymidine (1.9 Ci/mmol) or 0.5 μCi [^{14}C]thymidine (54 mCi/mmol; Schwarz/Mann, Div. of Becton, Dickinson, and Co., Orangeburg, N. Y.) at 66 h for agarose cultures or 46 h for liquid cultures. These cultures were assayed 6 and 2 h later, respectively. For studies of the kinetics of DNA synthesis, [^{3}H]thymidine was added at various times in the course of culture and these pulsed tubes were assayed 6 h after addition of the radiolabel. Cells in liquid culture were assayed for DNA synthesis by collecting trichloroacetic acid (TCA) precipitable material on glass filters as described elsewhere (14). The assay for DNA synthesis in agarose cultures was modified from Peters (15). At the end of the pulse period, the liquid phase was poured off and the whole agarose plug was put into 6 ml cold phosphate-buffered saline (PBS) in a conical glass tube. The plug was centrifuged at 300 *g* for 5 min, the supernate was poured off, and 3 ml of 3 M KI-5% TCA added. The agarose was dissolved by agitating the tube in a 65°C water bath for 2 min, then centrifuged at 400 *g* for 15 min. The supernate was discarded, and the pellet was resuspended in 5 ml of cold 5% TCA in water. This was centrifuged at 400 *g* for 10 min, and the supernate was discarded. The pellet was then resuspended in 2 ml Aquasol (New England Nuclear, Boston, Mass.) and transferred to a glass scintillation vial. The centrifuge tube was washed with 2 ml Aquasol, and a further 6 ml were added to the scintillation vial. The vials were incubated at 37°C for 12 h, then cooled and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Autoradiography. Dishes to be processed for autoradiography were pulsed with [^{14}C]thymidine as described. At the end of the pulse time, the whole dish was gently immersed in 200 ml of PBS-3.5% formaldehyde. After 24 h at 4°C, the dish was transferred to distilled water for

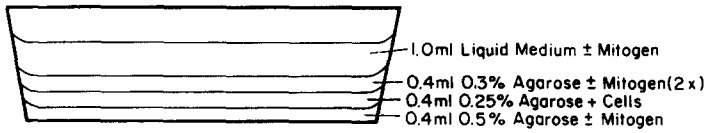

FIG. 1. Schematic diagram of the agarose cultures in dishes. Mitogen, when present, was twice the desired final concentration (2 ×) in the agarose overlayer to compensate for the lack of mitogen in the cell layer.

another 24 h. Then, with the dish still immersed in water, the agarose plug was gently loosened, floated free of the dish, and picked up on a 2 × 2-inch glass slide that had been coated with a thin layer of 1% gelatin and dried. The agarose plug was allowed to air dry, fixed for 1 h in methanol:acetic acid:water (89:1:10), washed for 1 h in distilled water, and dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.). After a 24-h exposure, the slides were developed for 2 min in Kodak D-19 developer, fixed in Kodak Rapid Fixer for 4 min, and washed in running water for 1 h. After air drying, the slides were fixed for 30 min in absolute methanol, dried, stained 20 min in Giemsa solution, washed in three changes of water for 30 min, and air dried. Coverslips were applied with Permount.

Results

Stimulation in the Presence of High Doses of Con A. The typical dose-response curves for human lymphocytes cultured in the presence of Con A or succinyl-Con A are presented in Fig. 2*a*. The curve for Con A shows inhibition of DNA synthesis at higher doses, whereas that for succinyl-Con A shows stimulation in the same dose range. The same dose-response curves occur in agarose cultures (Fig. 2*b*). To investigate the falling limb of the dose-response curve in more detail, parallel cultures were treated with stimulatory (25 μg/ml) or inhibitory (150 μg/ml) concentrations of Con A; DNA synthesis was then monitored at 6-h intervals by pulsing duplicate tubes and assaying incorporation of [^{3}H]thymidine (Fig. 3). It has been shown previously (14, 16) that at optimal doses of Con A most cells are lectin independent after 24 h, i.e. they are already committed to initiate DNA synthesis. In agreement with previous work (14, 17), cells in the stimulated cultures began synthesizing DNA at 24 h, and this level rose as increasing numbers of recruited cells entered the S phase, that part of the cell cycle in which DNA is synthesized. Cells treated with the higher concentration of Con A showed very low levels of DNA synthesis. If, however, the cells that had been in the presence of the same inhibitory concentration of Con A were treated at 24 h with a competitive inhibitor of lectin binding, αMM, they began DNA synthesis after about 6 h and reached a level of [^{3}H]thymidine incorporation comparable to optimally stimulated cultures.

Parallel cultures were set up in the presence of 150 μg/ml of Con A to investigate the kinetics of this release of the inhibited cells. At different times, sets of these cultures were treated with αMM, and the kinetics of DNA synthesis were followed by pulsing duplicate tubes of each set every 6 h with [^{3}H]thymidine. As seen in Fig. 4, even those cells released from the high dose blockade as early as 6 h after beginning the culture did not begin synthesizing DNA until after 24 h; this time period therefore represents a minimum lag between stimulation and entry into the S phase. Cells released after 18, 30, or 42 h showed significant increases in DNA synthesis during the period 6–12 h after the addition of αMM.

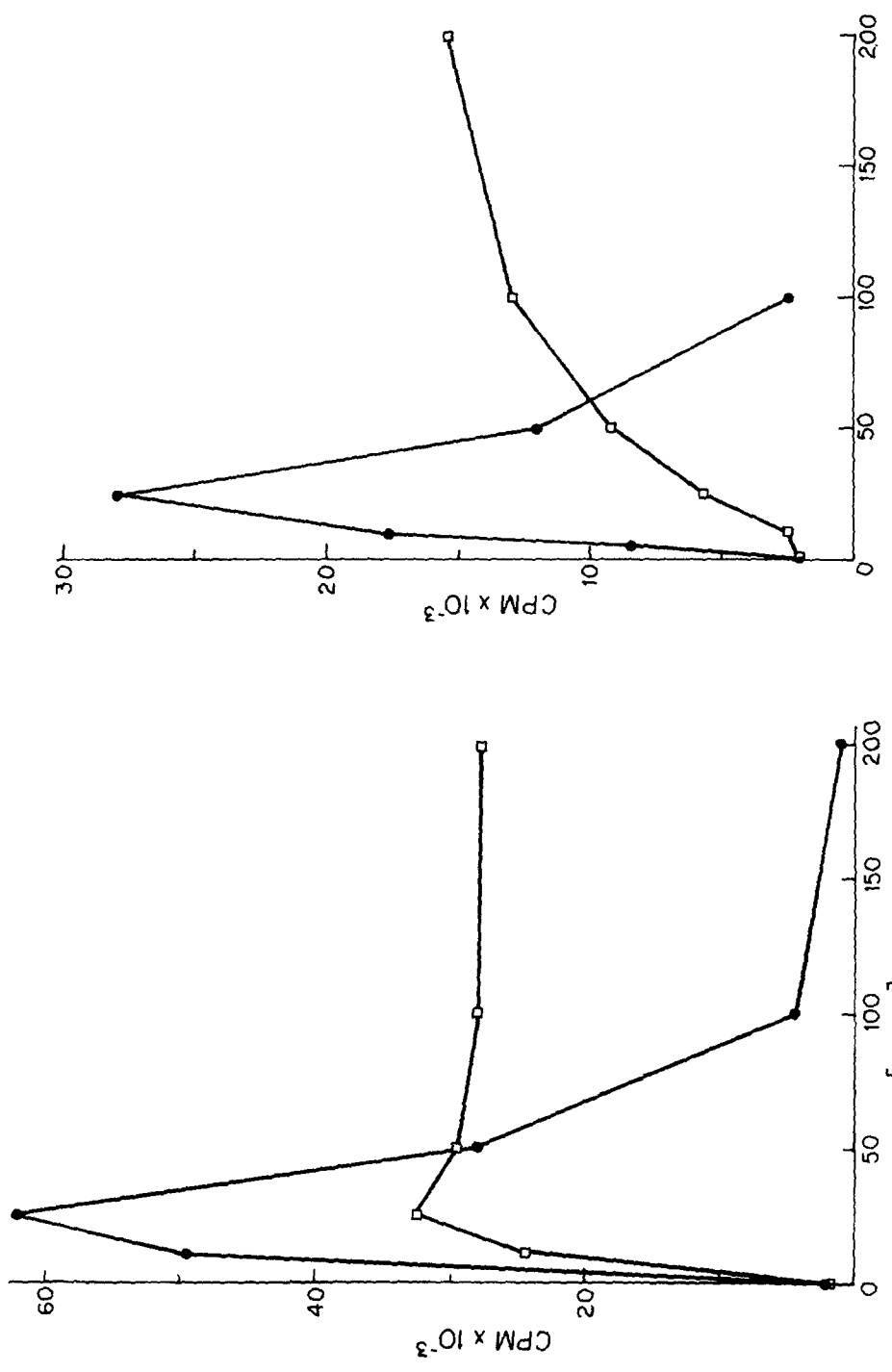

FIG. 2. Dose-response curves of Con A and succinyl-Con A in liquid medium and agarose. (a, left) Cultures of 10^6 human lymphocytes were made in liquid medium with various concentrations of either Con A (●–●) or succinyl-Con A (□–□). Tubes were pulsed with 6 μCi of [^{3}H]thymidine between 46 and 48 h. Results are the means of duplicate tubes. (b, right) Con A and succinyl-Con A dose-response curves in agarose. 4×10^5 human lymphocytes were dispersed in 0.4 ml of agarose as described in the Materials and Methods section and cultured in the presence of various concentrations of Con A (●–●) or succinyl-Con A (□–□). DNA synthesis was assayed at 72 h as described. Results are the means of triplicate cultures.

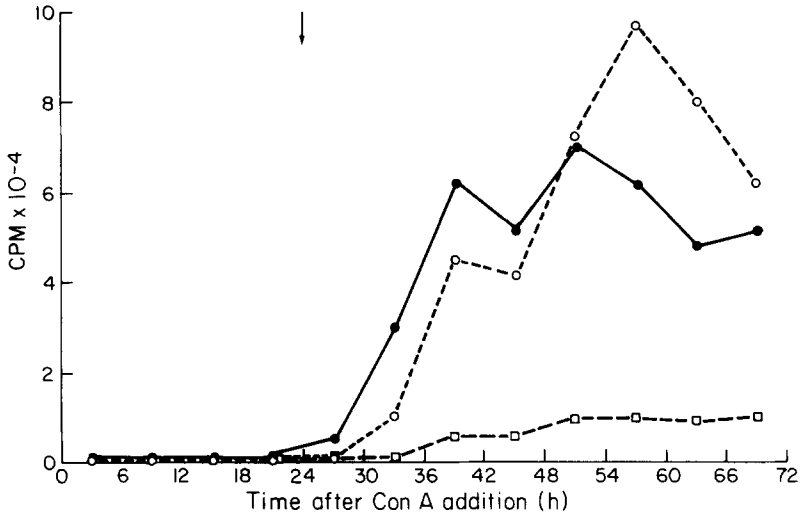

FIG. 3. Kinetics of DNA synthesis in cells treated with high or low concentrations of Con A. 10^6 human lymphocytes were cultured in the presence of 25 μg/ml (●—●) or 150 μg/ml (○---○ and □---□) of Con A. At 24 h, one of the high dose cultures (○---○) received αMM to a final concentration of 0.1 M. Kinetics of DNA synthesis were monitored by 6-h pulses with 6 μCi of [^{3}H]thymidine. Values are the means of duplicate tubes and are plotted at the midpoint of the pulse.

Also noteworthy in Fig. 4 are the different final levels of DNA synthesis achieved by cultures released at different times. The level reached by cultures released at 42 h was lower than that reached by the cells that were treated with αMM at 18 or 30 h. This presumably reflects changes in the number of viable cells; viability as measured by the ability to exclude trypan blue fell to 94% at 6 h, 68% at 24 h, 50% at 48 h, and 14% at 60 h in the high dose cultures. Viability remained approximately 95% in cultures treated with noninhibitory concentrations of Con A. No cells recovered metabolic function after 60 h in the presence of 150 μg/ml of the lectin.

The lower levels of synthesis in cultures receiving αMM at 6 h reflect the fact that fewer cells have become committed to entering the S phase, that is, fewer cells have become lectin independent (14). It has been shown previously (14, 16) that cells in culture with Con A become lectin independent with time; as the Con A is removed later and later by adding αMM, more cells eventually enter the S phase and replicate.

We conclude from the above data that: (*a*) high doses of Con A deliver a positive growth signal; (*b*) this positive signal is sufficient for commitment; (*c*) the entry into S phase of cells treated with high doses of Con A and then released by αMM is not due to residual stimulating doses of Con A, since the 6-h lag before S phase after the release is much shorter than the 24-h lag before S phase seen after an original exposure to stimulating Con A doses; (*d*) the observed unresponsiveness must therefore be due to an additional and sustained negative signal.

The somewhat unexpected result that cells were being recruited to potential mitogenesis by high doses as well as low doses of lectin made it of interest to

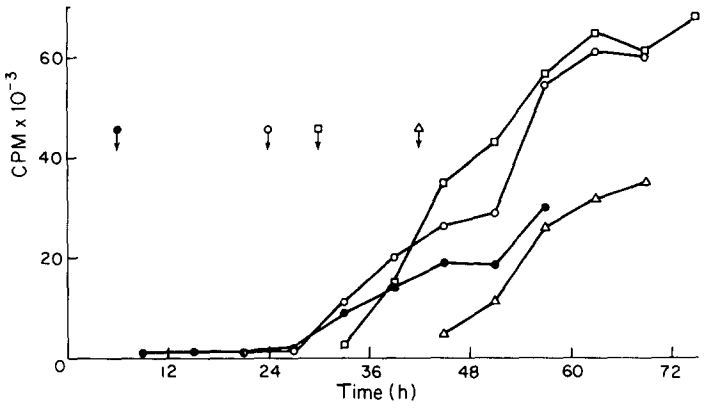

FIG. 4. Kinetics of DNA synthesis in cultures treated with high doses of Con A and released with αMM at various times. Parallel sets of human lymphocyte cultures with 150 μg/ml of Con A were treated with αMM at 6 h (●–●), 18 h (○–○), 30 h (□–□), or 42 h (△–△). The arrows indicate the point at which αMM was added for a given culture. The kinetics of DNA synthesis were monitored with 6-h pulses of 6 μCi of [³H]thymidine at 6-h intervals. Data are plotted as the mean of duplicate tubes at the midpoint of the pulse.

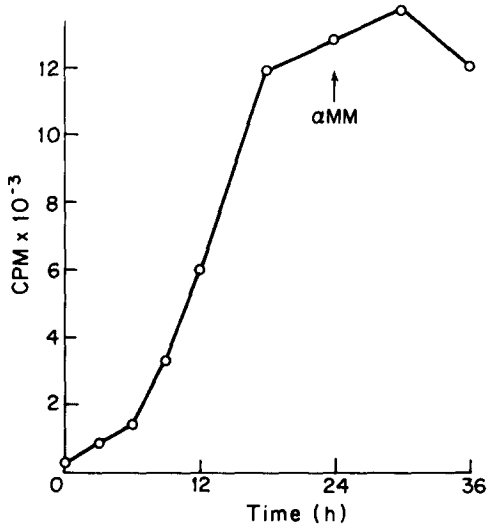

FIG. 5. Effect of colchicine addition to lymphocytes in the presence of Con A. Human lymphocytes (10^6/tube) were cultured with 150 μg/ml of Con A. At 24 h (arrow), all tubes were made 0.1 M in αMM. At the various times, colchicine was added to a final concentration of 10^{-6} M. Tubes were pulsed with 6 μCi [³H]thymidine between 46 h and 48 h. Results of incorporation of label are the means of duplicate tubes.

determine whether cells exposed to high concentrations of Con A were as sensitive to colchicine during the initial inductive phase as cells exposed to lower amounts of the lectin (16, 17). Fig. 5 illustrates that cells treated with high doses were sensitive to the drug. Colchicine had no effect on the DNA synthetic response measured at 48 h if added simultaneously or after the addition of αMM. This represents a time before any cells have entered the S phase, but which is

simultaneous with or after the time that the signal to proliferate has become lectin independent.

Kinetics of Commitment as a Function of Lectin Concentration. We next investigated the kinetics of the commitment event in cultures with different lectin concentrations including concentrations in the inhibitory region. The results (Fig. 6*a*) demonstrated that, if the lectin was removed at early times, the eventual level of incorporation was proportional to the Con A dose; the initial rate of commitment is thus proportional to the lectin concentration even for doses in the inhibitory range of the dose-response curve. It has already been shown that the amount of [^{3}H]thymidine incorporated is directly related to the number of cells stimulated (14), and therefore we conclude that higher concentrations of Con A, even those that are inhibitory when sustained, are driving more cells per unit time through the lectin-dependent phase of stimulation.

The data on kinetics of commitment after 6 h are difficult to analyze using Con A because cell viabilities decrease with time in inverse proportion to the Con A concentration and because cells released after 24 h are delayed in their entry to the S phase. To eliminate these problems, the same experiment was done with succinyl-Con A, which is neither toxic nor inhibitory (Fig. 6*b*); the same relation of concentration and commitment rate was seen at even later times.

Stimulation of Cells Dispersed in Agarose. A basic question concerning the mechanism of high dose inhibition is whether it is a function of intrinsic cellular machinery or whether in addition it depends upon cellular interactions or agglutination. To eliminate direct cell contact, an assay was devised in which cells were stimulated in suspension in agarose (Fig. 1). Direct microscopic examination of these cultures using a water immersion lens revealed that in cultures of 4×10^5 cells per dish, the average distance between cells was 30 μm, and 80% of the cells were clearly isolated from other cells. This number did not increase at up to 60 h in culture. During these observations the larger stimulated lymphoblasts could be distinguished from unstimulated cells, and single, isolated lymphoblasts were seen.

A comparison of the numbers of isolated and contacted small cells and blasts is given in Table I. It can be seen that the cells in agarose did indeed respond to Con A with increases in DNA synthesis. After 60 h in culture, a majority of the blasts counted were isolated, and thus could respond as single cells. Nevertheless, there was a 1.4–1.5 times greater probability for a cell to transform if it were in contact with another cell (see the "contact advantage" row in Table I). Autoradiographic analysis of the cells in agarose confirmed these data and also clearly demonstrated individual labeled blasts in low density cultures that were up to 120 μm from the nearest neighboring cell. The type of analysis performed in Table I could not be done on the autoradiographs because drying a slab of agarose containing suspended cells made more cells appear to be in contact than actually were in contact in the original culture.

Despite the occurrence of many isolated, stimulated cells, there was a lower percentage of blasts in the lower density culture (Table I). In the generation of a mitogenic response in the agarose cultures there is therefore some cellular cooperativity, which may be due either to actual cell contact or to diffusible factors.

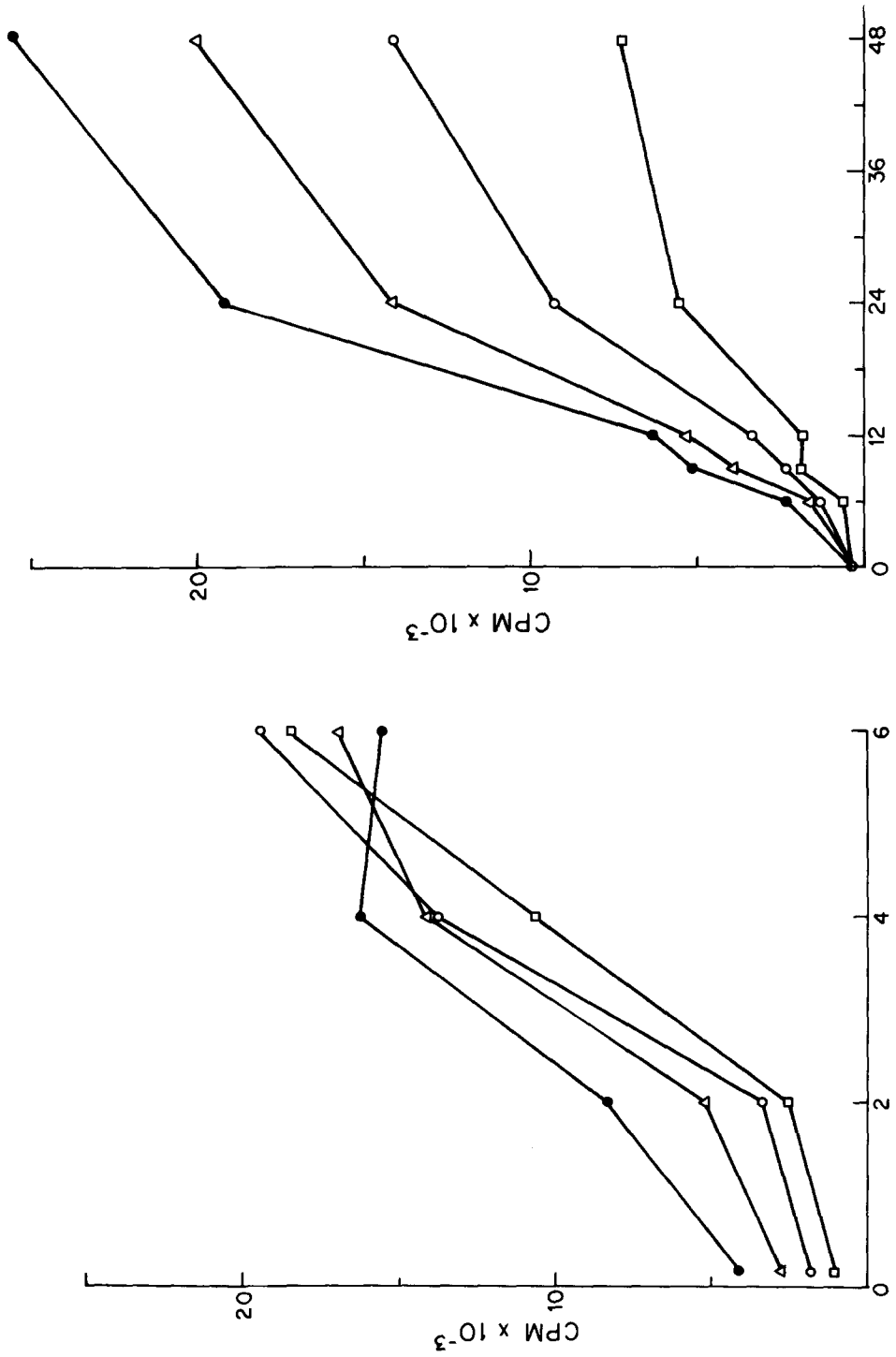

FIG. 6. Rates of commitment to DNA synthesis as a function of lectin dose. Human lymphocytes (10^6/tube) were cultured in the presence of Con A (Fig. 6*a*, left) or succinyl-Con A (Fig. 6*b*, right) at concentrations of 200 μg/ml (●–●), 100 μg/ml (△–△), 50 μg/ml (○–○), or 25 μg/ml (□–□). At different times (abscissa) αMM was added to a final concentration of 0.1 M. DNA synthesis was assayed with a terminal 2-h pulse of [^{3}H]thymidine at 48 h.

TABLE I

A Comparison of the Numbers of Isolated and Contacted Cells and Blasts Observed in Agarose Cultures of Human Lymphocytes at 60 h

	Control culture 4×10^5 cells/dish*	Con A culture 4×10^5 cells/dish*	Con A culture 2×10^5 cells/dish*
cpm‡	700	36,200	8,500
Cells counted§	752	968	78
Percent isolated	88	78	80
Percent contacted	12	22	20
Percent blasts	13	35	15
Percent isolated blasts	9	25	11
Percent contacted blasts	4	10	4
Contact advantage‖	3.3	1.4	1.5

* Cells cultured as described in Materials and Methods. Con A cultures had 25 μg/ml of lectin.

‡ Incorporation of [^{3}H]thymidine, 6 μCi/dish, between 66 and 72 h.

§ Counts of six fields in each of five dishes for columns one and two, and six fields in one dish for column three.

‖ (Percent contacted blasts/percent contacted cells)/(percent isolated blasts/percent isolated cells).

TABLE II

*Occurrence of a MLR in Agarose Cultures**

Cells	Culture medium	cpm minus control background
4×10^5A + 4×10^5B	Liquid in tubes	119,400
4×10^5A + 4×10^5B	Agarose in dishes	0
8×10^5A + 8×10^5B	Agarose in dishes	0
1.6×10^6A + 1.6×10^6B	Agarose in dishes	28,000
4×10^5A + 4×10^5B	Liquid in tubes, transferred to agarose in dishes after 3 days	53,400
4×10^5A	Agarose in dishes + Con A‡	34,900
1×10^5A	Agarose in dishes + Con A‡	150

* Cells from two donors (A and B) were mixed 1:1 in culture conditions similar to those used in the mitogenesis experiments. After 5 days, the cultures were pulsed with [^{3}H]thymidine and assayed for incorporation of the label into DNA.

‡ Con A cultures at 25 μg/ml were assayed for DNA synthesis after 3 days.

To see whether there was any detectable activity that would result from functional cell-cell contacts in the agarose cultures, a mixed lymphocyte reaction (MLR) was attempted in agarose. As shown in Table II, a two-way MLR could be induced in conventional liquid cultures by mixing leukocytes from two donors, whereas in agarose no reaction was seen until there were 3.2×10^6 cells (80% contacted) in a standard culture. In contrast, under the same conditions, a small but reproducible stimulation of lymphocytes with Con A was seen with as few as 10^5 cells. The data also indicate that it was possible to induce the MLR in tubes and then allow it to develop in the agarose cultures, a fact that could be of

TABLE III
Reversibility of High Dose Con A Inhibition in Agarose Cultures

Culture condition*	αMM at 30 h	cpm
No lectin	–	500
Con A (25 μg/ml)	–	24,000
Con A (25 μg/ml)	+	11,000
Con A (150 μg/ml)	–	1,000
Con A (150 μg/ml)	+	10,000

* Cultures containing 4×10^5 cells were made and assayed after 72 h as described.

use in studying whether continuation of that reaction depends on continued cellular contact at early times.

Having established the parameters of the agarose cultures, it was possible to examine the lectin dose-response curves in the different culture conditions. As previously mentioned (Fig. 2*b*), the shapes of the Con A and succinyl-Con A dose-response curves for cells in agarose were the same as those for cells in liquid medium, where they were free to associate and aggregate. The features of these curves in agarose cultures were also independent of cell concentration (data not shown). As shown in Table III, after the addition of αMM, the high dose inhibition is as reversible in agarose as it is in conventional cultures.

Reversible Inhibition by Con A of the Growth of the Lymphoid Cell Line, P388. High doses of lectins and other mitogens are known to inhibit the growth of various nonresting cells (2, 5–8). We have shown, for example, that 50 μg/ml of Con A inhibits the growth of the lymphoid cell line P388. Fig. 7 illustrates that, as found for normal lymphocytes, this growth inhibition is reversed by the addition of αMM and is thus not purely attributable to irreversible cytotoxic effects of Con A. Furthermore, as shown in Table IV, this lectin inhibition is also seen with P388 cells in single cell agarose cultures and is therefore not the result of agglutination.

Discussion

In normal lymphocytes, the inhibition of stimulation due to high doses of Con A has been reported to be reversible (18). Mouse thymocytes show a response when the high doses of Con A are removed as well as an enhanced response to low doses of Con A after treatment with high doses (4). The work described here represents a detailed kinetic analysis of this paradoxical effect of Con A.

The process of lymphocyte blast transformation by Con A can be divided into two phases, one lectin dependent and one lectin independent. Cells do not traverse these two phases synchronously, but instead are recruited to become lectin independent over a time span of several hours. The point at which a cell will go on to enter the S phase of the cell cycle, even if the lectin is removed, has been termed the commitment point (14). In this paper, we have used this distinction between committed and uncommitted cells as a means to help explain the main features of the Con A dose-response curve and some properties of the commitment event itself. We conclude that cells treated with high doses of Con A are committed but "clamped", that is, they have become potentially lectin

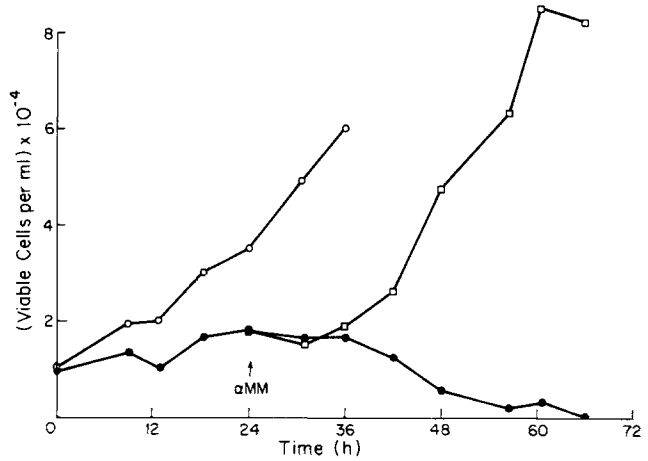

FIG. 7. Con A inhibition of P388 cell growth. P388 cells (5×10^4) were seeded in a 25 cm^2 tissue culture flask in 5 ml of DMEM with 10% calf serum. Parallel cultures received either no Con A (○—○) or Con A at 50 μg/ml (●—● and □—□) at 0 time. One of the sets of cultures with Con A (□—□) was made 0.1 M in αMM at 24 h as shown by the arrow. Cells were counted at various times using trypan blue exclusion as the criterion of viability.

TABLE IV
Inhibition of Growth of P388 Cells in Agarose Cultures

Culture conditions	Cells per dish	cpm*
Control	5×10^4	233,000
50 μg/ml Con A	5×10^4	60,000

* Cells were pulsed from 48–54 h with [^{3}H]thymidine as described.

independent with regard to stimulation, but the bound lectin is preventing the cells from going on to initiate DNA synthesis.

A cell inhibited by high doses of Con A can enter into the S phase if the Con A is removed by αMM. It should be pointed out that this release of the blocked cells is not due to a residual mitogenic dose of Con A left on the cells, inasmuch as the data in Fig. 6 show that, at early times before commitment, the concentration of αMM used can completely inhibit the effect of Con A at concentrations up to 200 μg/ml. Furthermore, the lag time for entry into S phase is much shorter for the cells released after 24 h than for cells exposed to Con A for the first time (Fig. 4). This indicates that the cells exposed to high doses of Con A have in fact progressed from their original resting point in the cell cycle. We have not mapped the precise point in the G_1 phase of the cell cycle at which the cells treated with high doses of Con A are blocked. We have established that it is between 6 and 12 h before the G_1/S boundary, inasmuch as cells blocked and fully committed by high doses of Con A and then released with αMM need that much time before they begin DNA synthesis (Fig. 4). As pointed out above, the block is also past the point at which cells are committed to leave the G_0 or resting state.

The data in Fig. 6*b* show that for any one concentration of succinyl-Con A, the commitment rate remains fairly constant for 24 h. After 24 h, rates for all

concentrations of lectin begin to plateau. This is the pattern that would be expected if resting cells were in a G_0 epicycle (10, 19) and were recruited to enter G_1 and the normal cell cycle through a time window in the epicycle. Increasing the concentration of lectin, it appears, would cause recruitment of a greater proportion of the cells which are passing by that window.

The curves in Fig. 6 also indicate that in the cultures treated with high doses of Con A, commitment is proceeding at an accelerated rate. In fact, analysis of the rates of commitment in the presence of various concentrations of succinyl-Con A (Fig. 6*b*) suggests that the dependence of this rate on lectin concentration is first order at lower lectin concentrations; with higher doses of succinyl-Con A, the rate of increase of the commitment rate decreases and the commitment rate seems to approach a maximal velocity.

These results are particularly relevant to the study of the biochemical changes involved in blast transformation; they clearly show that the initial, precommitment event or events that result from lectin binding occur at high doses of Con A that nevertheless inhibit entry into the S phase. In fact, the rate of any such event appears to be dependent on the Con A dose in the same way as commitment. We suggest that any measured biochemical result of lectin binding that shows a Con A dose-response curve with a falling limb at high lectin concentrations is probably a later event not in the initial stimulatory pathway; it may or may not be involved in the inhibitory mechanism. An example of an event that shows a decreased response at higher Con A doses is the increase in amino acid transport seen in lymphocytes after stimulation (20). This event therefore reflects the blockade by high doses of Con A, rather than the committed state of the cells; the increases in amino acid transport must occur after the point of the blockade, and therefore after commitment. Similarly, the increases in cyclic GMP levels after lectin binding, which have been considered as possible inductive events in mitogenesis (21), follow the pattern of a postcommitment rather than a precommitment event.

The fact that the falling limb of the dose-response curve is seen in cultures dispersed in agarose argues for an intrinsic mechanism of lectin-induced inhibition that is operable in both normal and transformed cells. It should be pointed out that the data on agarose cultures do not rigorously prove that a single, completely isolated cell can be inhibited or stimulated by cell surface probes. There is some cellular cooperativity observed in the stimulation of lymphocytes in agarose, and it has not been determined whether this is due to specific soluble mediators released from some cells, to nonspecific effects of cell density on cell survival, or to actual migration of some cells through the agarose. The last possibility is unlikely because the high doses of Con A inhibit cellular locomotion (22) while facilitating commitment to stimulation. At the very least, Con A stimulation and inhibition are not the results of cellular aggregation.

What, then, is the nature of the inhibitory signal? We have previously hypothesized that the regulation of a growth or commitment signal from the cell surface to the cytoplasm is mediated by a submembranous array, the SMA, which is composed of microtubules, microfilaments, and possibly other contractile proteins (2, 14, 17, 23). High doses of Con A, even when bound locally (24), are known to cause global inhibition of the redistribution of cell surface receptors into patches and caps, and this inhibition is reversible by the addition of

colchicine (9–11), an agent that disrupts microtubules. The dose-response curve of this inhibition of receptor mobility is very closely correlated with the inhibitory limb of the mitogenic dose-response curve (11). Furthermore, in P388 cells, the concentrations of lectin that inhibit growth also inhibit patching of surface receptors (I. Yahara and G. M. Edelman, unpublished data). Thus, the SMA may have a regulatory function in the transmission of signals for growth control; this regulatory activity could be enzymatic, could involve a directed transport function, or could represent a binding activity for enzymes or factors necessary for growth control.

It is pertinent to note here the preliminary observation that high doses of Con A on P388 cells do not affect the level of an activity (25) associated with the initiation of DNA replication in vitro (S. M. Jazwinski, J. L. Wang, and G. M. Edelman, preliminary observations). This activity is normally well correlated with the rate of growth of cells (25). This observation suggests, as do the high dose Con A experiments on lymphocytes reported here, that lectin-blocked and modulated cells are in a committed but arrested state. It has also been shown that the state of the SMA is correlated with the expression of the transforming gene product of Rous sarcoma virus (26). Inasmuch as the relation of the SMA to growth control has not yet been proven to be a causal one, however, it is still possible that the above results may reflect intracellular events along a parallel growth control pathway in which the SMA is only peripherally involved.

It should nevertheless be noted that components of the SMA, particularly microtubules, may also be involved in the transmission of a positive growth control signal as indicated by the inhibition by colchicine of lectin-induced lymphocyte transformation (16, 17). This inhibition has been shown to be before the S phase and its kinetics are, in fact, indistinguishable from the kinetics of commitment to stimulation; the inhibition is not accounted for by cell death or inhibition of thymidine transport (16, 17). Similar results have been obtained with serum-starved fibroblasts, which are inhibited by various antitubulin drugs from entering S phase after readdition of serum (D. A. McClain and G. M. Edelman. Manuscript in preparation).

The present results indicate that Con A can simultaneously deliver both positive and negative signals for growth control to a lymphocyte and can also deliver a negative signal to continuously dividing cells. Work is now in progress to define further the various signals generated by different concentrations of lectins of different valences and to relate these signals to various states of the SMA, particularly in terms of their morphology, component interactions, and biochemical activities.

Summary

High doses of Concanavalin A (Con A), which normally inhibit T-lymphocyte stimulation as measured by increases in DNA synthesis, cause these lymphocytes to become committed to mitogenesis while also generating a dominant but reversible negative growth signal. The observed response to the stimulatory signal as measured by the rate of commitment to enter the S phase (i.e., the rate at which the stimulation becomes lectin independent) increases with lectin concentration even in the inhibitory range. The generation of this positive

signal is prevented by treating the cells with colchicine. Cells that have become committed but are also simultaneously blocked from entering the S phase by the high doses of Con A can begin synthesizing DNA if the lectin is released by adding a competitive inhibitor of binding. Experiments done in agarose cultures in which lymphocytes are kept from contact with each other suggest that the reversible inhibitory signal is mediated by structures in the individual cells rather than as a result of agglutination. Continuously dividing cells of the lymphoid line P388 are also individually and reversibly inhibited by Con A. These findings are considered in terms of the relation of the inhibitory signal to the microtubular components of cell surface modulating assemblies made up of submembranous arrays of microtubules, microfilaments, and associated proteins.

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