THE KINETICS OF CELLULAR COMMITMENT DURING STIMULATION OF LYMPHOCYTES BY LECTINS

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ABSTRACT

The kinetics of cellular commitment in the stimulation of lymphocytes by concanavalin A (Con A) has been analyzed by measurement of DNA synthesis, autoradiography, and histologic staining techniques. If the competitive inhibitor α-methyl-D-mannoside (αMM) is introduced into cultures of mouse spleen cells at various times after the addition of Con A, there is a gradual decrease in its capacity to inhibit the lectin-stimulated incorporation of [^{3}H]thymidine. Addition of the saccharide 20 h after exposure of the cells to Con A had no effect on the level of the cellular response to the lectin. With increasing periods of contact with Con A, the percentage of blast cells and the percentage of [^{3}H]thymidine-labeled blast cells increased in parallel with the total radioactive thymidine incorporated while the average number of autoradiographic grains per labeled blast cell remained relatively constant. These observations suggest that the rising level of [^{3}H]thymidine incorporation results from an increase in the number of cells that respond to lectin stimulation and become refractory to inhibition with αMM. Once such cells become committed, they synthesize DNA at a rate independent of the length of exposure to the lectin. The combined results indicate that mouse splenic lymphocytes are heterogeneous in their capacities to respond to Con A and that different cells require different induction periods to be stimulated.

INTRODUCTION

One of the key problems in analyzing the mitogenic stimulation of lymphocytes by various agents such as concanavalin A (Con A),[1] phytohemagglutinin (PHA), bacterial lipopolysaccharides, and sodium periodate is to determine, among the full spectrum of biochemical and morphological alterations, those which lead to cellular commitment and eventual mitosis. Among the numerous studies of this problem, there has been considerable disagreement about the duration of contact with the mitogen that is required to produce irreversible stimulation. For example, various reports on the removal of PHA by washing or on inactivation with anti-PHA serum have produced estimates varying from a few minutes to 48 h (1–5). Some of

[1] *Abbreviations used in this paper:* αMM, α-methyl-D-mannoside; Con A, concanavalin A; FBS, fetal bovine serum; 5% HBSS-FBS, Hanks' balanced salt solution containing 5% fetal bovine serum; MEM, minimum essential medium (Eagle); PBS, phosphate-buffered saline: 8.00 g NaCl, 0.20 g KCl, 1.15 g Na_2HPO_4, 0.20 g KH_2PO_4 per liter, pH 7.4; PHA, phytohemagglutinin-P.

the differences may be ascribed to variation in the preparations of PHA and anti-PHA sera as well as in the precise conditions of incubation and subsequent washing. Although Con A is better characterized in terms of structure (6) and binding specificity (7) than is PHA, similar disparities exist: some investigators report that lymphocytes become irreversibly stimulated after 18–20 h of exposure to the lectin (3, 8, 9) while others indicate that α-methyl-D-mannoside (αMM) or antiserum to Con A can inhibit at even later times (5, 10).

We report here studies on the kinetics of cellular commitment in the stimulation of mouse splenic lymphocytes by Con A. Our data indicate that with increasing periods of contact with Con A, the total radioactive thymidine incorporated and the percentage of [^{3}H]thymidine-labeled blast cells increased in parallel. In addition, the average number of autoradiographic grains per labeled blast cell was found to be relatively constant. These observations suggest that the number of cells stimulated by Con A increases with longer exposure to the lectin and that once a cell is stimulated it synthesizes DNA at a rate independent of the time of contact with the mitogen. Our results indicate, therefore, that members of a given population of resting lymphocytes may be in a number of different states with respect to their capacities to respond to Con A. We have formulated two hypotheses to explain this manifestation of heterogeneity in mouse lymphocyte populations.

MATERIALS AND METHODS

Reagents

Con A, succinyl-Con A, and [^{125}I]Con A were prepared as described previously (11–13). PHA was purchased from Difco Laboratories (Detroit, Mich.), αMM was purchased from General Biochemicals Div., Mogul Corp. (Chagrin Falls, Ohio), and *N*-acetyl-D-galactosamine was purchased from Calbiochem (San Diego, Calif.). Cell culture media, antibiotics, glutamine, pyruvate, nonessential amino acids, and fetal bovine serum (FBS) were obtained from Microbiological Associates, Inc. (Bethesda, Md.).

Cell Culture

Mitogenesis assays were conducted using spleen cells from NCS mice (The Rockefeller University, New York) cultured under the conditions of Mishell and Dutton (14). The spleens were minced and pressed through a large-mesh wire screen into minimum essential medium (MEM). Clumps were removed from the cell suspension by centrifugation at 250 rpm for 30 s in a swinging-bucket clinical centrifuge. The cell suspension was decanted, the cells were pelleted by centrifugation at 2,700 rpm for 2 min, and they were then resuspended in MEM containing 5% FBS, 100 U penicillin-streptomycin/ml, 2 mM glutamine, 1 mM pyruvate, and 1 × nonessential amino acids (complete medium) (14). After the cell suspension was counted for viable lymphocytes using trypan blue dye exclusion, it was diluted and added to the cultures to give a final concentration of 1×10^7 viable lymphocytes/ml. Con A, when present, was at a final concentration of 3 μg/ml, which was previously determined to be an optimally stimulating dose in this system (12). Initial solutions of all nonsterile reagents were passed through Millipore filters (Millipore Corp., Bedford, Mass.). Cell cultures were performed in either small (13 × 75 mm) or large (16 × 125 mm) plastic tubes (no. 2054 and no. 3033, Falcon Plastics, Oxnard, Calif.) in an initial volume of 0.3 or 1.0 ml, respectively. αMM in complete medium was added at the appropriate times in aliquots of 0.05 ml (small tube) or 0.1 ml (large tube) to give a final concentration of 0.1 M. Cultures were kept at 37°C in a humid atmosphere of 10% CO_2, 7% O_2, 83% N_2, and were fed at 24 and 48 h after the start of the experiment as described (14).

For measurement of DNA synthesis, cells were deposited on GF/A filters (Whatman) after incubation with [^{3}H]thymidine (Schwarz/Mann, Orangeburg, N. Y., 1.9 Ci/mmol) washed with phosphate-buffered saline (PBS), 5% trichloracetic acid, and methanol, dried, and counted in a scintillation counter in 5 ml of 0.4% Omnifluor (New England Nuclear, Boston, Mass.) in toluene.

Metaphase Counting

To determine the number of metaphases at different times after the start of the experiment, cultures were given a 6-h terminal pulse of 10^{-6} M colchicine (Sigma Chemical Co., St. Louis, Mo.). Metaphases were counted according to the method of Schindler (15) using siliconized glassware for fixed cells. It was established that decay of metaphases trapped early in the pulse was not a problem, since three contiguous 2-h pulses gave approximately the same number of metaphases as one 6-h pulse.

Autoradiography

For autoradiographic experiments, cultures in small tubes were pulsed with [^{3}H]thymidine (1.5 μCi/tube for 2 h). Cells from 15 small tubes were pooled in each determination. Cells were pelleted, resuspended in 3 ml Hanks' balanced salt solution containing 5% FBS (5% HBSS-FBS) and washed twice through discontinuous

gradients of 5 ml 10% HBSS-FBS layered over 5 ml of 20% HBSS-FBS. After a final wash in 5% HBSS-FBS, the cells were suspended in 1 ml of PBS and smeared on gelatin-coated slides. 0.1 ml of the cell suspension was taken for scintillation counting as described. Autoradiography using NTB-2 Nuclear Track Emulsion (Eastman Kodak Co., Rochester, N. Y.) was performed according to the method of Byrt and Ada (16) using an exposure time of 18 h. Exposing slides for shorter times gave a proportionately smaller average number of grains per cell. Labeling cultures with 1.5 μCi [^{3}H]thymidine per small tube for 2 h gave a satisfactory number of grains per cell. After final fixation, slides were stained in Giemsa, dipped in xylene, and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.).

Grain Counting

Cells were scored as either blast cells or unstimulated lymphocytes and as labeled or unlabeled. Lymphocytes greater than 9 μm in diameter were counted as blasts. Slides were scanned from the leading edge of the cell smear to the end of the slide. Grain counts were made with a hand counter during the initial studies. The error in visual grain counting was estimated by multiple counts of the same cell. This analysis indicates that the standard error of the visual grain counting method is on the order of 5% in each individual measurement.

A photometric method of grain counting (17) was subsequently developed. The system consisted of a Zeiss Universal microscope with epi-illumination through a half-silvered mirror. Reflected light was quantitated by an RCA 1 P 28 Photomultiplier and a Zeiss digital photometer-indicator using a series of three measuring diaphragms. An unlabeled reference lymphocyte was used for the zero setting in the percent transmission mode with the 100% transmittance control set at full clockwise detent. The most convenient apertures were numbers 1 and 2, and calibration curves for these are shown in Fig. 1. The stability of the system was checked periodically during each counting experiment using reference cells of various sizes and grain counts.

The results obtained using the photometric method were checked periodically for agreement with those obtained by visual counting. When both methods were used to obtain grain counts from the same slide, it was found that the two grain count distributions were identical within the error of estimation.

Con A-Pulse Experiments (Table II)

Lymphocyte cultures were treated with Con A (3 μg/ml) for 6, 12, 18, and 24 h in the absence of αMM. At the end of the Con A pulse, the culture was made 0.1 M in αMM and the cells resuspended with a Pasteur pipette. To begin a second pulse in the same culture the medium was removed so as not to disturb the settled pellet. The cells were washed in 1.0 ml prewarmed medium containing 3 μg/ml Con A, and resuspended in 1.0 ml of the same medium. At the end of the second pulse the cultures were again made 0.1 M in αMM. Large tube cultures were used in both experiments A and B. At 48 h each tube received a 2-h pulse of 1.5 μCi [^{3}H]thymidine. The final volume in all tubes was 1.3 ml. For counting, five tubes were pooled for each point. The cells were washed through serum gradients as described above, and resuspended in 1 ml PBS. Counts shown are the average of duplicate 0.1-ml aliquots. To test the

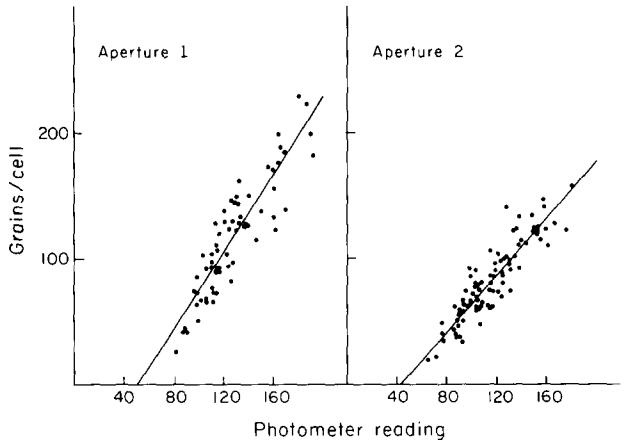

FIGURE 1 Representative graphs showing the relationship between visual grain count per cell and the photometer reading. Photometer readings are expressed as percent transmittance standardized as described in the text. Apertures 1 and 2 refer to photometer diaphragms positioned between the specimen and the photocell. Calibration curves were drawn by a least squares fit of the data to a first order polynomial. Correlation coefficients are 0.86 for aperture 1 and 0.91 for aperture 2.

effects of pooling separate cultures and washing the cells through gradients, multiple parallel cultures were assayed separately, without washing. The results obtained from separate cultures were not significantly different from those obtained with pooled cultures.

RESULTS

Effect of αMM on Lymphocytes Cultured with Con A

The stimulation of lymphocytes by Con A is dependent upon the binding of the lectin to the cell surface inasmuch as prior addition of αMM, a saccharide which binds strongly to the lectin, inhibits the cellular response. If αMM is introduced at various times after the addition of Con A to cultures of mouse splenic lymphocytes, the incorporation of [^{3}H]thymidine as measured at 48 h is at least partially inhibited (Fig. 2). No inhibition is observed, however, if the saccharide is added 20 h after the addition of the lectin. Thereafter, the addition of αMM has no effect on the level of the response. Similar results were obtained by using a divalent Con A derivative, succinyl-Con A (12), as the mitogenic agent. In addition, curves comparable to that shown in Fig. 2 were obtained using three other Con A concentrations, 1, 5, and 10 μg/ml. The effect of αMM is specific for cells stimulated by Con A for it has no effect when added to lymphocyte cultures containing the lectin PHA which binds *N*-acetyl-galactosamine-like sugars. Moreover, we have obtained data similar to that shown in Fig. 2 using PHA-stimulated cells and have found that the action of PHA is inhibited by the addition of *N*-acetyl-galactosamine. In this case, all of the responsive cells also appeared to be totally committed to lectin stimulation by 20 h after the initial binding.

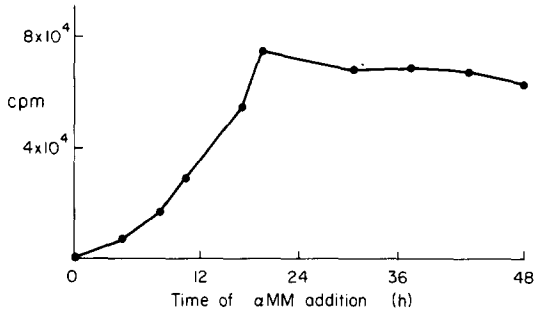

FIGURE 2 The effect of αMM, added at different times after the start of the culture, on the incorporation of [^{3}H]thymidine by Con A-stimulated lymphocytes. Cultures (0.3 ml) containing 3 μg/ml Con A were made 0.1 M in αMM at various time points. The cultures were continued until 48 h at which time 1 μCi of [^{3}H]thymidine was introduced in 0.05 ml of medium. The cells were harvested for analysis at 72 h. Data points shown are the averages of measurements on duplicate cultures.

Experiments were performed to determine whether the difference in the degree of inhibition by αMM as a function of time of exposure of the cells to Con A is due to an increase in the overall level of DNA synthesis rather than to a shift in the onset of synthesis. Particular care was taken to show that the difference did not result from an increase in the number of Con A molecules that have become inaccessible to competitive binding with αMM. Our main efforts were directed to determining whether the level of [^{3}H]thymidine incorporation is proportional to the number of stimulated cells that have become refractory to inhibition by the saccharide.

Kinetics of DNA Synthesis

To test whether the effect of αMM addition seen in Fig. 2 is due to a shift in the onset of DNA synthesis or to an increase in the final level of synthesis, we have compared the kinetics of thymidine incorporation among Con A-stimulated cultures that received αMM at different times (Fig. 3). The data show that the stimulated cultures begin DNA synthesis some 18–20 h after initial Con A binding irrespective of the time of αMM addition. In contrast, the level of DNA synthesis was higher in cultures with late αMM addition than in cultures with early αMM addition. Furthermore, the level of [^{3}H]thymidine incorporation in cultures with αMM added at 24 h was comparable to cultures with no αMM addition at all, in agreement with the notion that all of the responsive cells have already been committed after 24 h of contact with the lectin.

The data presented in Fig. 3 also reveal that by 48 h after addition of Con A, the rate of DNA synthesis has reached a plateau in all cultures, regardless of the time of αMM addition. Incorporation of [^{3}H]thymidine within this plateau period should not, therefore, be greatly affected by changes in either the time of addition or length of incubation with [^{3}H]thymidine. Moreover, except for the absolute level of radioactivity incorporated, experiments using a pulse period of from 48 to 50 h

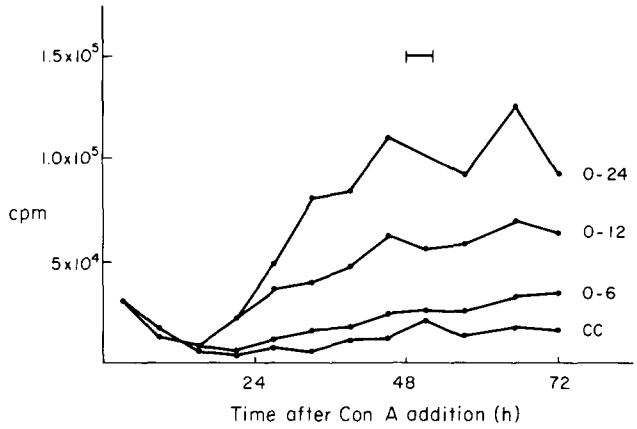

FIGURE 3 The effect of length of exposure to Con A on the kinetics of DNA synthesis in splenic lymphocyte cultures. CC indicates control cultures containing no lectin. 0–6, 0–12, and 0–24 refer to the time periods in which the cultures were incubated with 3 μg/ml Con A in the absence of αMM; at the end of these periods, the cultures were made 0.1 M in αMM. DNA synthesis was measured with a terminal 6-h pulse of [^{3}H]thymidine (1 μCi/culture, sp act 52 Ci/mmol). Data points shown are the averages of measurements on duplicate cultures. The horizontal bar represents the 48–50-h [^{3}H]thymidine pulse used in subsequent experiments (see text).

after Con A addition gave similar results to those shown in Fig. 2, in which the pulse was from 48 to 72 h. We have indicated this shorter 2-h pulse period, chosen for our subsequent experiments, by the horizontal bar marker shown in Fig. 3. This pulse period is short enough so that labeled cells dividing during the pulse do not significantly alter the results of autoradiographic experiments designed to correlate grain counts per cell with thymidine uptake.

We have also examined the kinetics of appearance of metaphase cells in our culture system using the mitotic inhibitor colchicine. The data (Table I) show that prior to 48 h after Con A addition, the percentage of mitotic cells in any given 6-h period does not exceed 1.6% of the total cell population. Furthermore, the total number of cells that have undergone one division and whose daughter cells could be in S phase at 48 h (i.e., those dividing before 42 h) can account for no more than 6% of the viable cells remaining at 48 h. These cells could only contribute significantly to the response seen with very early additions of αMM. The pulse period from 48 to 50 h is, therefore, also early enough to avoid complexities of analysis that might be introduced by mitotic daughter cells, whether labeled or unlabeled with [^{3}H]thymidine.

TABLE I

*Rate of Appearance of Metaphases in Lymphocyte Cultures Stimulated with Con A**

Terminal pulse of 10^{-6} M colchicine	Viable cells per culture at end of pulse	Metaphases per culture	Viable cells in metaphase
			%
Start of culture	1.0×10^7	—	—
18–24	6.7×10^6	8.3×10^3	0.1
24–30	6.4×10^6	2.5×10^4	0.4
30–36	6.9×10^6	6.6×10^4	1.0
36–42	7.3×10^6	1.1×10^5	1.5
42–48	6.1×10^6	1.0×10^5	1.6

* Cultures in large tubes were stimulated with 3 μg/ml Con A and the number of metaphases during the indicated time intervals were determined as described in the text.

Effect of αMM Addition on Con A Binding

The observation that, with increasing length of incubation, an increasing percentage of the Con A-induced response becomes refractory to inhibition by αMM suggests the possibility that more and more Con A molecules may become inaccessible to the action of the saccharide. To test this

hypothesis, we used ^{125}I-labeled Con A in our cultures and measured the average number of Con A molecules on the cells as a function of time both before and after prolonged incubation with αMM. The average number of Con A molecules bound per cell increased as a function of time up to a value of about 3×10^6 molecules per cell at 12 h (Fig. 4). Thereafter, there was no apparent increase between 12 and 24 h and a slight increase between 24 and 36 h of culture. On addition of αMM, the number of Con A molecules bound to cells dropped to about 60% of the original value within 2 h. In no case, however, were all of the originally bound Con A molecules released. More pertinent perhaps is the observation that the release of Con A molecules from the cells by αMM was independent of the time of exposure of the cells to Con A. Addition of αMM at 6, 12, 15, or 18 h reduced the number of Con A molecules to about the same value of 1×10^6 molecules/cell (Fig. 4). Measurements of [^{3}H]thymidine incorporation in parallel cultures yielded results similar to that shown in Fig. 2. These data strongly suggest that the difference in degree of inhibition by αMM as a function of time of exposure to Con A cannot be attributed solely to an increase in the number of Con A molecules that become inaccessible to the saccharide. Instead, it seems probable that with increasing time of incubation in the presence of Con A, the lymphocytes become lectin-independent and their metabolic machinery continues to be active even if the stimulant is removed.

Correlation of the Cellular Response with [^{3}H]Thymidine Incorporation

It was not clear whether the increased response seen with the addition of inhibitor at later times is due to the stimulation to the same degree of more and more cells or to the stimulation to a greater degree with time of a constant number of cells. Are more cells being recruited with time or is each cell being stimulated to a greater extent? To answer these key questions, it is necessary to follow both the number of cells being stimulated at each point and the amount of [^{3}H]thymidine incorporated by each stimulated cell.

To study both parameters, we carried out exper-

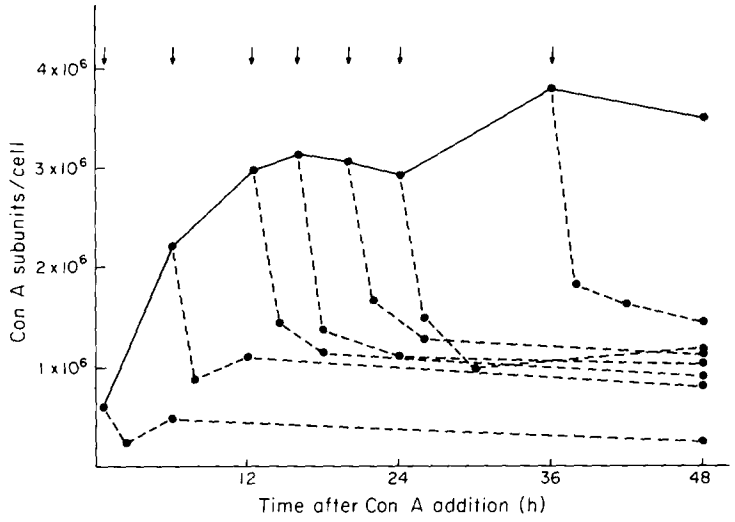

FIGURE 4 The effect of αMM, added at different times after the start of the culture, on the average number of Con A subunits bound per cell. Mouse splenic lymphocytes, purified by density gradient centrifugation (25), were cultured with 3 μg/ml [^{125}I]Con A (sp act 7.5×10^7 cpm/mg) and at the times indicated by the arrows, αMM was added to a final concentration of 0.1 M. The solid line depicts the results of measurements made immediately after introduction of the saccharide. The dotted lines depict the results of measurements made on cultures incubated for 2 or 6 h after the addition of αMM, and also 48 h after the addition of Con A. The experimental procedure used in these measurements has been described (26). Data points shown are the average of measurements on duplicate cultures.

iments similar to the one seen in Fig. 2. At each time point, total incorporation of [^{3}H]thymidine was measured. In addition, cells from the same cultures were smeared on a slide, subjected to autoradiography, and stained for blast transformation. Blast cells and cells labeled with [^{3}H]thymidine were counted and the extent of labeling was estimated by counting the number of grains seen in each labeled cell. The results of a typical experiment are shown in Fig. 5. The data indicate that the percentage of both blast cells and of *labeled* blast cells in the lymphocyte population increases in parallel with the total incorporated radioactive thymidine. In addition, the average number of grains per labeled blast (inset, Fig. 5) was found to be invariant within the error of estimation suggesting that regardless of the time of addition of inhibitor, the stimulated cells synthesize DNA at the same average rate. The total amount of [^{3}H]thymidine incorporated is therefore proportional to the number of cells stimulated. These data support the conclusion that additional cells are being recruited by longer exposure times to Con A, and that once a cell is stimulated it synthesizes DNA at a rate independent of the length of exposure to the lectin.

In the interpretation of these results, it is particularly important to determine whether the distribution of grain counts differs greatly between cells given long and short exposures to Con A. The distributions of grain counts for labeled blasts from the experiment in Fig. 5 are shown in Fig. 6. The profiles look similar, with some variations to be expected due to the relatively small number of blast cells counted in each case (approximately 50). Chi-square analysis of the data represented in Fig. 6 showed that all the distributions are the same at the 90% confidence level (omitting the small population of cells with greater than 200 grains). In addition, chi-square tests indicate that the variance of each distribution about its mean is too great to be accounted for by statistical fluctuation in the rate of decay from a group of cells that are all labeled to the same extent. This wide variation in the number of grains per cell appears to be significant and not determined purely by counting error, as the range covered by the distributions in Fig. 6 is approximately 40 times the standard

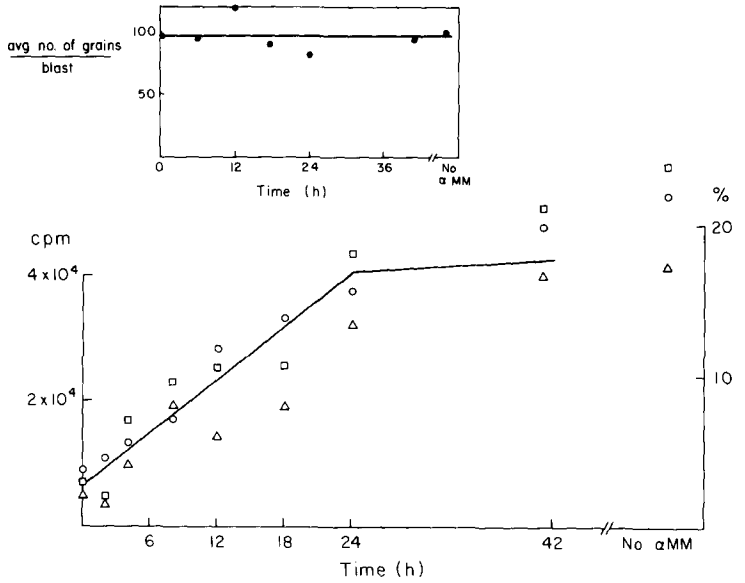

FIGURE 5 Comparison of the total [^{3}H]thymidine incorporated (○, left ordinate), percent blast cells (□, right ordinate), and percent labeled blasts (△, right ordinate) at various times after stimulation of mouse splenocytes with Con A. Con A (3 μg/ml) cultures were inhibited with 0.1 αMM at various times, pulsed with [^{3}H]thymidine (1.5 μCi/culture, sp act 1.9 Ci/mmol) between 48 and 50 h and harvested for autoradiography and scintillation counting as described in the text. *Inset:* The average number of grains per labeled blast cell determined at each time point of αMM addition. (Reprinted by copyright permission from Edelman, G. M., editor. 1974. Cellular Selection and Regulation in the Immune Response. Raven Press, New York. 182.)

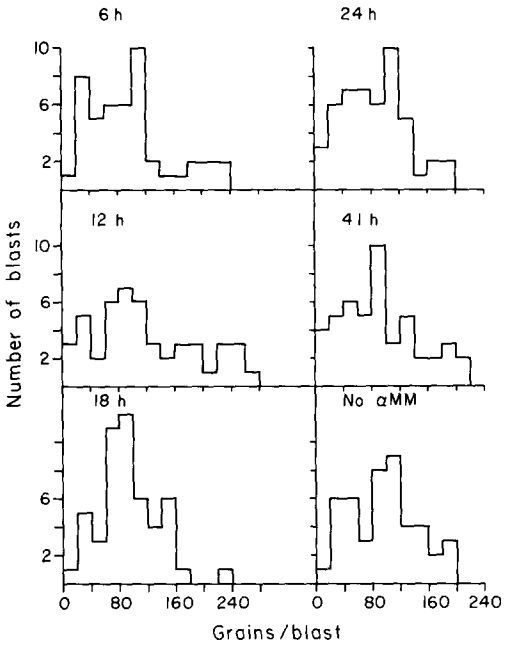

FIGURE 6 Grain count distributions for Con A-stimulated lymphocytes with different times of αMM addition (see Fig. 5).

deviation for each cell count. Whatever the cause of the wide distribution, it must be independent of the time of exposure of the cells to Con A, since early and late additions of αMM produced similar histograms.

Effect of Pulsed Exposures to Con A

Preliminary experiments were also performed to compare the response after a 6-h exposure to Con A to the response after two successive 6-h exposures separated by an interval of 6 h. The scheme of this experiment is outlined in Table II. Within the experimental error, addition of αMM 6 h after the initial binding of Con A yielded the same level of response as addition of αMM at 6 h followed by a washing step at 12 h. This response was about 50% of the level seen with a 12-h exposure to Con A (Table II). If the cells are first exposed to Con A for 6 h, washed, and cultured for 6 h followed by another 6-h contact with Con A, the response in terms of [^{3}H]thymidine incorporation is much greater than two times the value obtained for a single 6-h pulse or the sum of the values for two parallel cultures, one pulsed between 0 and 6 h and the other pulsed between 12 and 18 h. In fact, the level of response of a single culture

TABLE II

Effect of Pulsed Exposure of Lymphocytes to Con A

	Time						
	0 h	6 h	12 h	18 h	24 h	Exp. A	Exp. B
1.*	⊢—					5,300	8,200
2.‡	⊢—		W			6,200	—
3.	⊢—		—			10,900	14,600
4.§	⊢—	W	—			9,600	—
5.	⊢—		—	—		14,000	27,300
6.	⊢—		—	—	—	21,600	—
7.	⊢—		⊢—	—		14,700	25,100
8.‖			⊢—	—		4,900	—
9. Cell control						1,900	5,500

* The horizontal bars represent the time during which Con A was present in the absence of α MM. The results of exps. A and B represent [^{3}H]thymidine incorporation expressed as counts per minute.

‡ At 12 h, cultures were washed once in complete medium and resuspended in 1.0 ml of 0.1 M αMM in complete medium.

§ At 6 h, the cultures were made 0.1 M in α MM, immediately washed once, and resuspended in 1.0 ml complete medium containing 3 μg/ml Con A.

‖ Initially, cultures contained 1×10^7 cells in 0.9 ml. At 12 h, 0.1 ml of 30 μg/ml Con A was added.

pulsed twice (0–6 and 12–18 h) is comparable to the response of cells continuously in contact with Con A for 18 h (Table II). These findings suggest that the initial binding of Con A to cells followed by removal of the lectin may have synergistic effects on subsequent exposure of these cells to Con A.

DISCUSSION

Lymphocyte stimulation induced by Con A can be inhibited by the specific saccharide, αMM, suggesting that the critical initial event in the cellular activation is the binding of the lectin to the cell surface. Our observations that the degree of inhibition by αMM decreases with later times of saccharide addition to the lymphocyte cultures and that addition of αMM 18–20 h after initial Con A binding has no effect on the lymphocyte response are in agreement with the data of Novogrodsky and Katchalski (8) and Inbar et al. (9) using rat lymphocytes. Lindahl-Kiessling (3) also showed that the response of human lymphocytes to Con A was unaffected by addition of αMM after 20 h of

culture, although the effect of early αMM additions, i.e., 1–10 h after Con A addition, differed from that observed here (Fig. 2). It appears that within 20 h of contact with the lectin all of the cells responsive to Con A have been totally committed to transformation.

We have found that 12 h after the initial addition of lectin the number of Con A molecules bound per cell is about the same as that at 15, 18, and 24 h. Furthermore, addition of αMM at any of these times followed by incubation for 2 h reduced the amount of Con A on the cells to approximately the same level (Fig. 4). There were consistently about 1×10^6 irreversibly bound Con A molecules/cell regardless of the time at which washing with αMM was performed. It has been reported that these irreversibly bound Con A molecules are inside the cells and that these molecules are not responsible for the stimulation of the cells (18). The observation that the inhibitory effect of αMM on Con A-stimulated mitogenesis decreases with later times of addition of the sugar cannot be explained, therefore, by any decrease in the ability of αMM to release Con A molecules from the cells.

In apparent contrast to our findings, Powell and Leon (10) have reported that stimulation by Con A of DNA synthesis in human lymphocytes is suppressed by addition of αMM to the culture medium even after 20 h. Using antiserum to Con A, Jones (5) showed that the antibody can inhibit mouse lymphocyte transformation after the 2nd day in culture and ascribed this inhibition partly to the prevention of entrance into the first division cycle and partly to a requirement for mitogen beyond the first proliferation. However, in both of these studies DNA synthesis was measured at times later than 48 h, a time at which a large contribution by daughter cells to the total [^{3}H]thymidine incorporation would be expected. Furthermore, in agreement with the findings presented here, Jones (5) found that a stimulated cell can divide even if the Con A is inactivated by antiserum during part of the S phase and all of the G_2 phase.

Our analysis of Con A-stimulated lymphocytes by autoradiography and histologic staining taken in conjunction with the results of bulk measurement of thymidine incorporation indicates that in lymphocyte cultures stimulated with Con A and inhibited by addition of αMM at various times, the rising level of [^{3}H]thymidine incorporation is associated with an increasing number of labeled blast cells present at 48 h (Fig. 5). This shows that the amount of [^{3}H]thymidine incorporated at all times is proportional to the number of cells responding to lectin stimulation. Thus, cells become committed as early as 2 h after exposure and increasing numbers of cells become committed to DNA synthesis with longer exposures to Con A. Inasmuch as the average number of [^{3}H]thymidine grains per labeled blast cell remains relatively constant, the data suggest that once the cells become committed, they synthesize DNA at a rate independent of the length of exposure to the lectin. Finally, these experiments indicate that mouse splenic lymphocytes may be heterogeneous with respect to their capacities to respond to Con A and, therefore, different cells may require different induction periods to be stimulated. In view of these findings, the results of kinetic studies of biochemical events in stimulated lymphocytes must be reexamined, for they may reflect average properties of the population rather than events in a single cell.

In our autoradiographic experiments, we have found that the spread of the distributions about the mean number of grains per labeled cell was too wide to be accounted for by statistical variations in the rate of decay among cells which are labeled to the same extent. Other factors which could contribute to a wide range of grain counts are variations in the thickness of the photographic emulsion, the presence of activated lymphocytes of higher ploidy, which would synthesize proportionally more DNA in a given time period than the diploid cells and show higher grain counts, and the presence of cells that are in S phase for only a portion of our 2-h labeling period, yielding lower grain counts. The emulsion thickness was judged to be adequate since examination of slides prepared under the experimental conditions used and mounted with glycerin jelly showed very few grains on the upper surface of the emulsion (19). The contribution by cells of higher ploidy is probably minimal as well, as is the case in human thoracic duct lymphocytes (20). From examination of the data on DNA synthesis (Fig. 3) and the fact that the duration of S and G_2 periods for mouse lymphocytes cultured with Con A have been estimated to be 10 and 2 h, respectively (21), we conclude that cells that have been labeled for only a portion of their S phase must be present and could account for the skewing of the distributions toward lower grain counts. It is also possible that

the wide distribution of grain counts is a result of differences in the rate of DNA synthesis from cell to cell, as has been observed in PHA-stimulated human peripheral lymphocytes (22). It must be emphasized, however, that the wide distribution of grain counts observed cannot account for the increasing response seen with longer incubation with Con A, since early and late αMM additions produce the same average number of grains per labeled blast and comparable distribution curves (Figs. 5 and 6).

Our findings on the heterogeneity of lymphocytes, manifested in the variable induction periods required for stimulation by Con A, suggest an interpretation analogous to that proposed by Smith and Martin (23) for cell cycle kinetics in general. We suppose that lymphocytes may be in two states—resting and activated. Cells in the resting state may be challenged by exogenous stimulants such as antigens or lectins and move into the activated state. In the activated state, the cellular activities are deterministic and pass through the classical cell cycle phases toward division. If the transition from the resting to the activated state on stimulation occurs with a constant probability but in a random fashion, then a wide intrapopulation variability would be observed in the kinetics of activation.

A simple mechanism to account for the randomness in the kinetics of activation is to postulate that the rate-determining step for cellular commitment in the series of biochemical reactions resulting from lectin binding follows first order kinetics. This would require, however, that the activation kinetics be described by the usual logarithmic curve for the appearance of product in a first-order reaction and, therefore, the mechanism might have to be modified to fit the slightly concave curve for activation shown in Fig. 2.

We have also considered two different hypotheses to account for the variability in the induction times required for lymphocyte stimulation (Fig. 7): (*a*) the "time of exposure" hypothesis and (*b*) the "autonomous receptive state" hypothesis. In the first hypothesis, we suppose that lymphocytes are heterogeneous and therefore that different cells differ intrinsically in the length of time that they must be continuously exposed to the mitogen before they are irreversibly stimulated. An obvious example of this intrinsic difference between cells is the accumulation of a critical metabolite after Con A binding. If a cell of type C required that the metabolite reach a higher level than in a cell of type B before becoming committed, it would require a longer exposure to Con A (Fig. 7, model I). The autonomous receptive state hypothesis implies that identical cells are distributed among several temporal states (Fig. 7, model II). In this model, cells may pass from one state to another independent of Con A, but they can only become committed to stimulation when in a particular state. When cells are in this Con A-receptive state, they become stimulated if and only if Con A is present. Since, in general, cells will be initially distributed in different states, they will become committed to stimulation at different times after

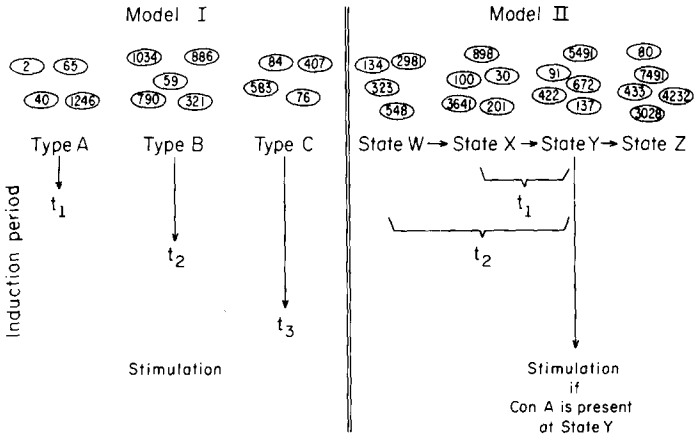

FIGURE 7 Schematic representation of hypotheses to account for variable induction periods required for Con A stimulation.

Con A addition and early additions of αMM will prevent cells in nonreceptive states from becoming stimulated even when they move into the receptive state. A similar hypothesis has been presented (24) to explain the stimulatory effects of serum on stationary cultures of chicken fibroblasts. The combined results suggest the possibility that the dispersion of states in G_1 maybe a general phenomenon.

It is useful to distinguish the two hypotheses (Fig. 7) from an operational point of view. The first hypothesis predicts that the heterogeneous cells may be fractionated to yield cells that are more homogeneous in the kinetics of their response to Con A. The second hypothesis assumes that there is ready interconversion between cells in different states and that any homogeneity achieved by cell fractionation would be altered depending on the time between fractionation and Con A stimulation. Cell fractionation might, therefore, provide the most direct test of these two hypotheses.

It is also important to point out that these two models may exist with or without the "randomness" considered in the transition from a resting lymphocyte to an activated one. In the absence of a first order decay process, either one of the two models can completely account for the observed wide intrapopulation variation. If first order decay from the resting state holds, either of these models would serve to modify it by changing the threshold of activation or by assigning different transition probabilities to different temporal states.

In a preliminary test of these hypotheses, the response of lymphocytes to a single 6-h exposure to Con A was compared to the response of two 6-h pulses of Con A separated by an interval of 6 h (Table II). The time of exposure model predicts that the second 6-h pulse of Con A will stimulate no additional cells since all of the cells requiring 6 h or less continuous exposure to the lectin have already been stimulated by the first pulse. On the other hand, the autonomous receptive state hypothesis would predict that additional cells will be stimulated as they pass through the Con A-receptive state during the second pulse. The results (Table II) show that two pulses of Con A stimulate about three times the response as the first pulse alone, ruling out any simple time of exposure hypothesis in which memory is not included.

A third alternative, which cannot be ruled out by the experiments presented here, is that initially committed cells may facilitate the subsequent triggering of other cells, perhaps by secretion of a soluble factor. Such a mechanism probably cannot involve a factor that is mitogenic by itself, however, since supernates from Con A-stimulated cells produced no response when transferred to unstimulated cells.[2] One possible mechanism of this type involves cells that require two stimuli for commitment, one supplied by Con A itself, the other supplied by a previously committed cell. The participation of phagocytic cells in these events is also a possibility. Experiments on the mitogenic stimulation of single, isolated cells should elucidate the role of cell-to-cell interaction in commitment.

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