INHIBITION BY COLCHICINE OF THE MITOGENIC

STIMULATION OF LYMPHOCYTES PRIOR TO THE S PHASE

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ABSTRACT

Colchicine, vinblastine, and vincristine inhibit the mitogenic stimulation of lymphocytes by concanavalin A as measured by the incorporation of [³H]thymidine and the appearance of blast cells. The inhibitory effect of colchicine could not be accounted for by diminution in cell viability or by metaphase arrest of mitosis in the stimulated cells. Moreover, the inhibition of [³H]thymidine incorporation was not due to blockage of thymidine transport or inhibition of DNA synthesis inasmuch as addition of colchicine had no effect on cells in the S phase of the cell cycle. The time of inhibition was correlated with the kinetics of cellular commitment to lectin activation and the kinetic data indicated that colchicine blocks stimulation early in the sequence of events following addition of the mitogen. These findings support the hypothesis that cytoplasmic microtubular function plays a role in the commitment of resting cells to undergo mitotic division.

Although the detailed mechanism of lymphocyte stimulation by mitogens such as concanavalin A (Con A)[1] is not understood, it is generally assumed that specific interactions between certain cell surface receptors and the mitogen are responsible for the subsequent activation of the appropriate metabolic machinery in the cytoplasm and nucleus (4, 12). On the basis of studies on the modulation of lymphocyte surface receptor mobility by tetrameric native Con A (40), dimeric succinyl-Con A (14), and colchicine and related drugs (8, 41, 42), we have suggested the hypothesis that the membrane receptor-cytoplasmic interactions are mediated by an assembly of colchicine-binding proteins, most likely the microtubules. It was also suggested that direct or indirect interactions between cell surface receptors and microtubules may be con-

nected with the initial events of lectin-induced mitogenesis (8). In accord with this hypothesis, we found that colchicine inhibits the incorporation of [³H]thymidine in mouse splenic lymphocytes stimulated by Con A (8). Subsequent studies on human peripheral blood lymphocytes activated by phytohemagglutinin (PHA) have revealed similar effects of colchicine and pharmacologically related drugs (26).

In the present investigation, a number of experiments have been carried out to analyze the kinetics and biochemical sites of the inhibitory activity of colchicine. The effects of the drug on cell viability, incorporation of thymidine into DNA, blast transformation, and mitosis have been studied in cultures of lymphocytes from man and in some cases from the mouse. Colchicine was found to inhibit both the incorporation of [³H]thymidine and blast transformation that occur after stimulation by Con A. Analysis of the kinetic data suggests that colchicine inhibits mitogenic stimulation early in the sequence of events following addition of the

[1] Abbreviations used in this paper: Con A, concanavalin A; αMM, α-methyl-D-mannoside; PBS, phosphate-buffered saline: 8.00 g NaCl, 0.20 g KCl, 1.15 g Na₂HPO₄, 0.20 g KH₂PO₄ per liter, pH 7.4; PHA, phytohemagglutinin.

lectin. These observations can be interpreted in terms of a recent analysis (13) of the kinetics of cellular commitment during the stimulation of lymphocytes by lectins. This interpretation is consistent with the hypothesis that colchicine acts before the commitment of the cell to enter into the S phase of the division cycle.

MATERIALS AND METHODS

Reagents

Con A and succinyl-Con A were prepared as described previously (7, 14). Lumicolchicine was prepared from colchicine (Sigma Chemical Co., St. Louis, Mo.) by ultraviolet irradiation following the method of Wilson and Friedkin (39). The following materials were obtained commercially and used without further purification: vinblastine sulfate and vincristine sulfate (Eli Lilly & Co., Indianapolis, Ind.), hydroxyurea (Calbiochem, San Diego, Calif.), sodium metaperiodate (Matheson, Coleman & Bell, Norwood, Ohio), Salmonella typhosa lipopolysaccharide (Difco Laboratories, Detroit, Mich.), α-methyl-D-mannoside (αMM) (General Biochemicals, Chagrin Falls, Ohio), [³H]thymidine (Schwarz-Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.), and [³H]colchicine (New England Nuclear, Boston, Mass.). Cell culture media, antibiotics, glutamine, pyruvate, nonessential amino acids, horse serum, and fetal bovine serum were obtained from Microbiological Associates (Bethesda, Md.).

Cell Culture

Human peripheral blood lymphocytes were isolated from the blood of normal donors by density gradient centrifugation (6). Cultures were set up at a cell concentration of 1×10^6 cells/ml in RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum. Experiments were performed in 12 × 75-mm tubes (Falcon No. 2054, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a final volume of 1.0 ml. Con A, when present, was at a final concentration of 20 µg/ml, previously determined to be an optimally stimulating dose. Cultures were maintained at 37°C in a humid atmosphere of 10% CO_2, 7% O_2, 83% N_2.

Rabbit peripheral lymphocytes were prepared as described by Sell and Gell (33) and maintained in modified Eagle's minimum essential medium containing 10% fetal bovine serum. Cultures were set up in 12 × 75-mm tubes containing a final volume of 1.0 ml per tube. The cell concentration was 3×10^6 cells/ml.

Murine lymphocytes were from spleens of NCS mice (The Rockefeller University, New York) and athymic nu/nu mice bred on a BALB/c background. A detailed description of the procedure for culturing mouse splenic lymphocytes has been given elsewhere (13). The methods used for periodate and lipopolysaccharide stimulation of mouse splenic lymphocytes have also been described (25).

Assay of DNA Synthesis

Cells were pulsed with 6 µCi/culture of [³H]thymidine (1.9 Ci/mmol, Schwarz-Mann) between 48 and 50 h after the addition of the mitogen. After the pulse, cells were deposited on Whatman GF/A filters, washed with phosphate-buffered saline (PBS), 5% trichloroacetic acid, and methanol, dried, and counted in a scintillation counter in 5 ml of 0.4% Omnifluor (New England Nuclear) in toluene (13).
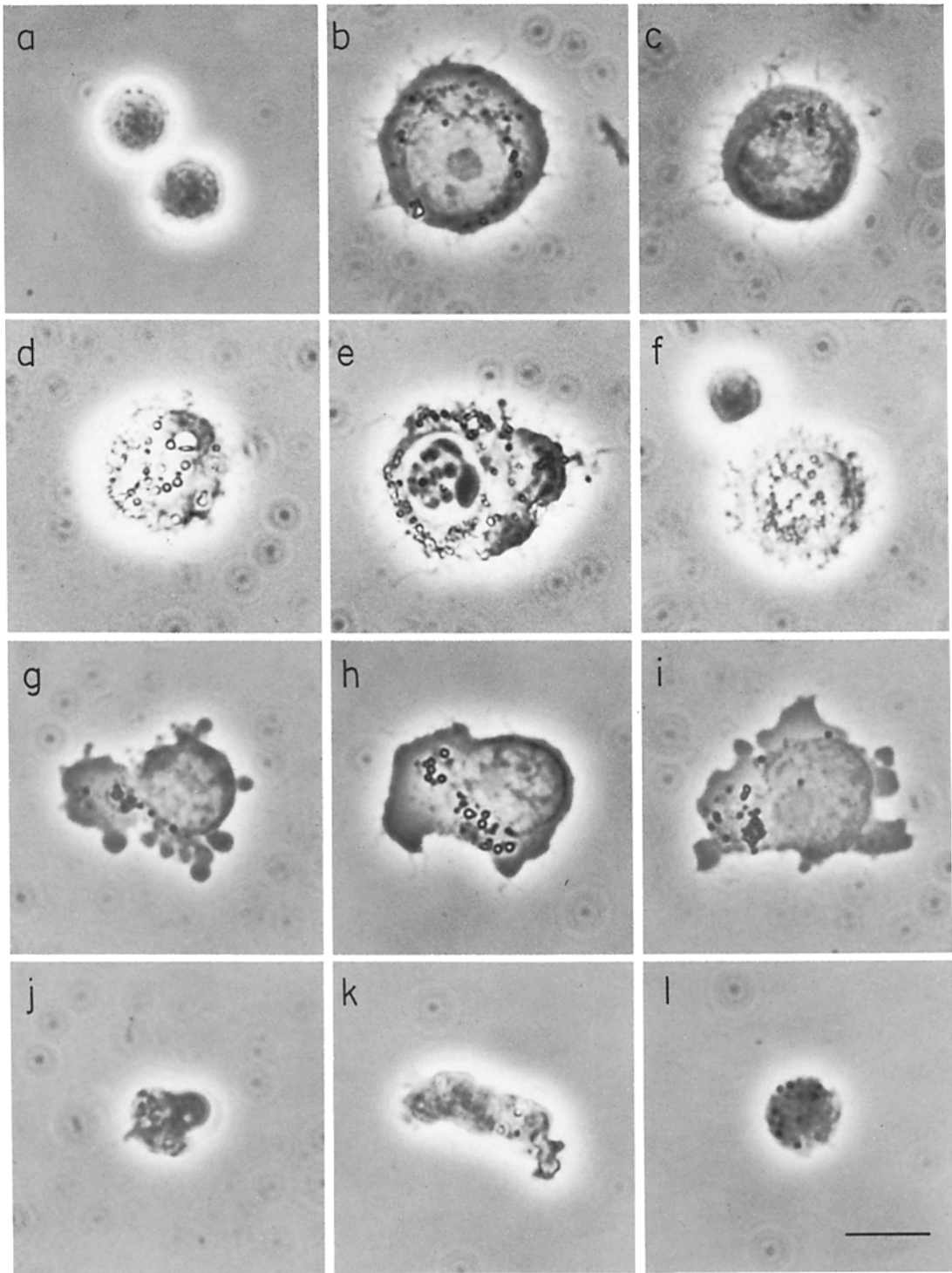
To compare the kinetics of thymidine incorporation in Con A-stimulated cultures in the presence and absence of colchicine, parallel cultures were given a terminal 6-h pulse of [³H]thymidine (6 µCi/culture, 1.9 Ci/mmol) at various times after Con A addition and harvested for scintillation counting. Similarly, the kinetics of thymidine incorporation were also determined in the presence and absence of colchicine on cells stimulated by Con A and collected at the G_1/S boundary using hydroxyurea (see below). In this case, a 1-h pulse of [³H]thymidine (6 µCi/culture, 1.9 Ci/mmol) was used.

Assay of Blast Transformation

Two procedures were used to determine the number of human lymphocytes undergoing blast transformation. In the first method, cells were smeared on gelatin-coated slides, fixed in methanol, stained in Giemsa, dipped in xylene and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.) for observation. Slides were scanned from the leading edge of the cell smear to the end of the slide. Blast cells were identified on the basis of their size (>9 µm), low nuclear-to-cytoplasmic ratio, and in most cases a clearly visible nucleolus.

In the second method, aliquots of cultures were diluted with an equal volume of trypan blue and then loaded in a corpuscle counting chamber (Hausser Scientific, Blue Bell, Pa.). The numbers of leucocytes and blast cells in an area of 5 mm² were counted using the size of the cell and the nucleus-to-cytoplasm ratio to discriminate blast cells from nonresponding leucocytes. Photographs of representative small lymphocytes and blast cells are shown in Fig. 1 a–c. When the percentage of blast cells was determined from the same culture using the two methods described above, it was found that the hemocytometer method overestimated the number of blasts when compared with counts on fixed and stained cells. This may be due to difficulty in determining in the hemocytometer the nuclear-to-cytoplasmic ratio, a criterion important for distinguishing blast cells from large monocytes in the stained smears. It should be noted, however, that using either counting method, the percentage of blast cells in cultures containing colchicine was clearly different from those without the drug. Furthermore, an increase in the percentage of blasts upon stimulation, as detected by the hemocytometer method, was always paralleled by a proportionate increase when assayed by the fixed cell method.

In addition, the method of assaying for blast transfor-

mation using the hemocytometer resulted in three observations which require some explanation. First, cultures with Con A contained small clumps of agglutinated cells. The individual cells in these clumps could not be distinguished so that the absolute number of cells counted in cultures containing Con A was always 30-40% lower when compared to cultures without Con A. Efforts to minimize the agglutination effect using a weaker agglutinin such as succinyl-Con A (14) still resulted in a difference between lectin cultures and cell controls. Inasmuch as this difference was apparent whether or not colchicine was present, we believe that it does not affect the comparisons of the kinetics of appearance of blast cells in Con A-stimulated cultures with and without the drug.

The second observation concerns the presence, in cultures without Con A, of cells large enough to be counted as blasts but which did not resemble the blast cells present in cultures containing Con A. The appearance of these large cells (Fig. 1 d-f) suggested that they were monocytes containing many phagocytized particles. Preliminary experiments have also shown that these phagocytic cells take up colloidal carbon. Because counting these unidentified leucocytes as blast cells would have overestimated the percentage of blast cells in the cell control cultures, we arbitrarily placed these cells in the category of unstimulated cells. It should be noted, however, that these cells were noticeable only in control cultures and they never exceeded 10% of the total population.

Finally, the morphology of cells in colchicine-treated cultures needs special comment. Large blastlike cells, when they do appear, are much more irregular in shape than blast cells in cultures containing Con A alone (Fig. 1 g-i). The size of each of these cells was judged by estimating the diameter the cell would have if it were rounded out to a circular shape. In contrast, some lymphocytes not responsive to Con A stimulation appeared to shrink in size when exposed to colchicine for a long time while others assumed an irregular shape or showed dark granules (Fig. 1 j-l). These effects were only noticeable after 40 h of exposure. Similar alterations in cell shape have been observed in rat peritoneal mast cells

and mononuclear cells treated with colchicine in vivo (28).

Sychronization of Stimulated Lymphocytes at the G_1/S Boundary

Lohrmann et al. (22) have observed that dog lymphocytes stimulated by PHA can be collected at the G_1/S boundary using 4 mM hydroxyurea. We found that 2 mM hydroxyurea completely blocked DNA synthesis in Con A-stimulated human lymphocytes without any effects on cell viability for periods as long as 40 h of exposure to the drug. Cultures stimulated with Con A were therefore made 2 mM in hydroxyurea 30 h after the addition of the mitogen. At 54 h, colchicine was added to half of the cultures at a final concentration of 10^{-6} M. At 60 h, all cultures were washed to remove hydroxyurea. The washing solution was complete RPMI 1640 medium containing 20 μg/ml Con A which had been incubated along with the cultures from the beginning of the experiment and one half of which was also made 10^{-6} M in colchicine at 54 h. The cultures, with and without colchicine, were resuspended in 1 ml of their respective medium. Cell viability and [^{3}H]thymidine incorporation were determined at various times after the removal of hydroxyurea.

Reversibility of the Colchicine Effects

The rates of uptake and efflux of [^{3}H]colchicine in human lymphocytes were determined as follows: cultures containing 20 μg/ml Con A were made 10^{-6} M in [^{3}H]colchicine (3 Ci/mmol) and a set of 10 cultures was immediately washed in complete RPMI 1640 medium containing 20 μg/ml Con A. Two of the washed cultures were deposited on GF/A filters, rinsed with 10 ml of cold PBS, and taken for scintillation counting. Duplicate cultures were also taken for counting at 6, 12, and 24 h after the washing with complete medium and also at 48 h after the addition of [^{3}H]colchicine. The washing procedure was similarly performed on other sets of cultures at various times after the addition of [^{3}H]colchicine and

FIGURE 1 Photographs of representative cells observed in cultures of human lymphocytes stimulated by Con A in the presence and absence of colchicine: (a), unstimulated small lymphocytes in cell control cultures; (b and c), blast cells in cultures stimulated by Con A. Both the nucleus and the nucleolus can be seen; (d-f), large blastlike cells in cultures without Con A with phagocytic particles and dense collections of granules. The nuclei in these cells were not readily discernible; (g-i), blast cells in cultures stimulated by Con A in the presence of colchicine. The cells assume a flatter shape with irregular boundaries. The nucleus can be distinguished from the cytoplasm; (j-l), small lymphocytes in cultures containing Con A and colchicine. These cells appear to be nonresponsive to lectin stimulation. They assume irregular shapes and acquire dark granules after prolonged exposure to colchicine. The photographs were taken on a Zeiss Universal microscope using phase contrast optics and Kodak Panatomic-X film (Eastman Kodak Co., Rochester, N. Y.). The magnification factor for all photographs was × 1,250. The bar represents 10 μm. Con A and colchicine, when present, were used at concentrations of 20 μg/ml and 10^{-6} M, respectively.

samples from each set were taken for counting at the respective times.

To test for the reversibility of the effects of colchicine on human lymphocytes, cells were incubated without Con A in the presence and absence of colchicine for 6 h. At this time, all cultures were washed twice with complete medium and resuspended in the original volume of complete medium without colchicine. 54 h after the beginning of the experiment, cell viabilities were determined for cultures previously exposed and unexposed to colchicine. The cells were then washed once, made up to 1 × 10⁶ viable cells/ml in fresh medium, and cultured with and without 20 μg/ml Con A for another 48 h, at which time they were pulsed with [³H]thymidine (6 μCi/culture, 1.9 Ci/mmol, 2 h) for determination of DNA synthesis.

RESULTS

We have previously observed that colchicine inhibits the incorporation of [³H]thymidine in mouse splenic lymphocytes stimulated by Con A (8). In the present experiments, we found that 10⁻⁶ M

colchicine inhibits the response of human, murine, and rabbit lymphocytes to Con A by about 70-90%, as measured by the incorporation of [³H]thymidine between 48 and 50 h after the start of the culture. A similar inhibitory effect of colchicine was observed in the stimulation of mouse splenic lymphocytes by sodium metaperiodate and in the stimulation of spleen cells from athymic nude mice by lipopolysaccharide. In addition, we found that other antimitotic drugs such as vinblastine and vincristine also inhibited the response of both human and murine lymphocytes to Con A.

Experiments were performed to answer the following questions: (a) Can the inhibitory effect of colchicine be accounted for by diminution in cell viability or by arrest of mitosis at metaphase in stimulated cells? (b) Does colchicine inhibit DNA synthesis or thymidine transport in prestimulated blast cells? (c) Does the inhibition by colchicine of
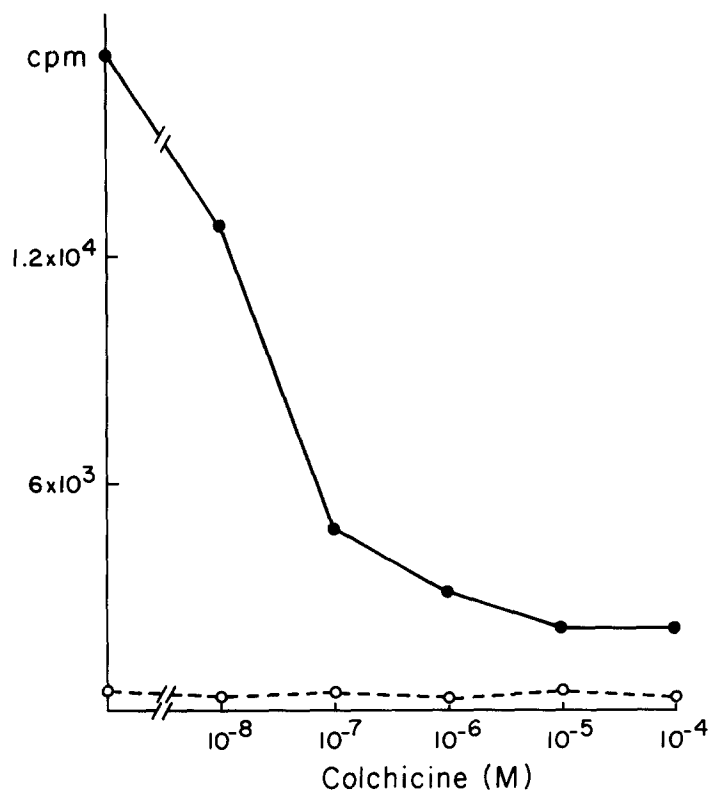

FIGURE 2 The effect of the concentration of colchicine on [³H]thymidine incorporation in human lymphocytes stimulated by Con A. Colchicine was present from the beginning of the experiment. The cultures were pulsed with 6 μCi of [³H]thymidine between 48 and 50 h. ●, incorporation by cultures containing Con A (20 μg/ml); O, incorporation by cultures in the absence of Con A.

the mitogenic response occur at an early point, before the onset of blast transformation and DNA synthesis? (d) Is the effect of colchicine reversible upon removal of the drug?

Because human lymphocytes stimulated by Con A retained a high level of viability under a variety of culture conditions, we chose to study this system extensively to determine the sites of the inhibitory activity of colchicine. The degree of inhibition produced by various concentrations of colchicine in the stimulation of human peripheral blood lymphocytes by Con A is shown in Fig. 2. At concentrations of 10^{-7} M or above, colchicine reduced the response to Con A by about 70%; below 10^{-7} M, the drug had little or no effect. Because of the sharp dose dependence between 10^{-7} and 10^{-8} M (Fig. 2), subsequent experiments were performed using 10^{-6} M colchicine to avoid drastic changes in the extent of inhibition that might result from slight variations in the concentration of the drug.

Inhibition of [³H]Thymidine Incorporation

To test whether the effect of colchicine is due to a shift in the onset of DNA synthesis or to a decrease in the final level of synthesis, the kinetics of thymidine incorporation were compared in Con A-stimulated cultures in the presence and absence of colchicine (Fig. 3). It was found that DNA synthesis in cultures without colchicine began about 30 h after initial Con A binding. In contrast, cultures containing colchicine showed only a low level of DNA synthesis as late as 51 h. These data suggest that colchicine inhibits mitogenic stimulation of lymphocytes by blocking an event occurring as early as the first wave of DNA synthesis in the sequence of events following lectin addition.

After 51 h, the level of DNA synthesis in cultures containing colchicine began to rise, attaining, at 70 h, about 30% of the level seen in cultures without the drug. This small rise in [³H]thymidine incorporation at late times may indicate that
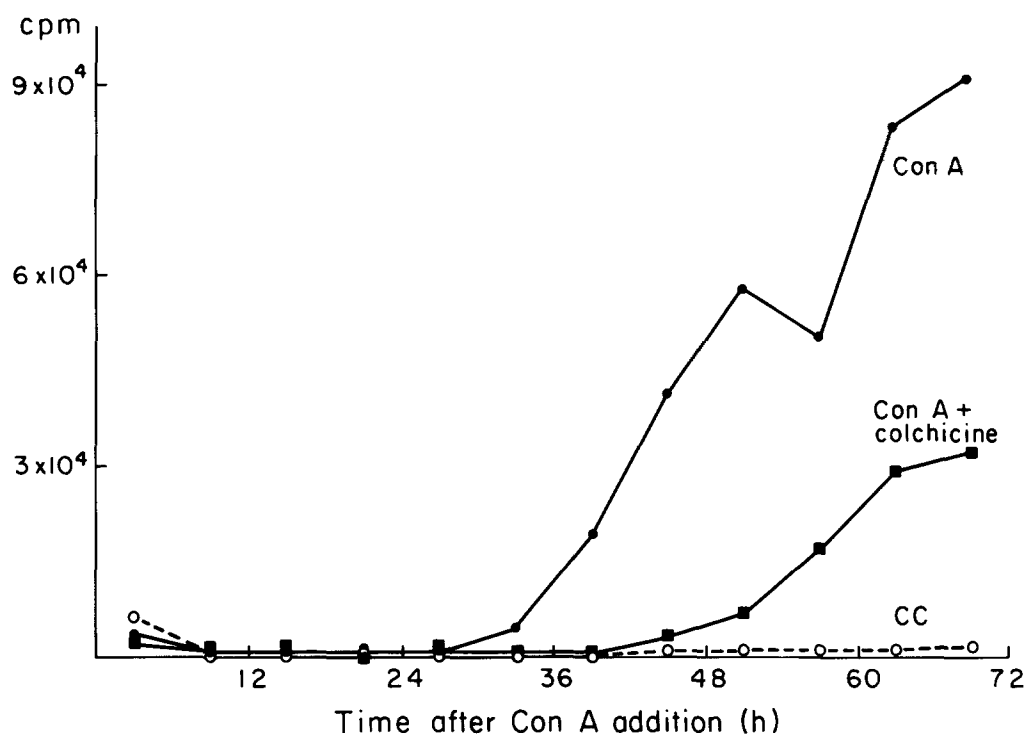

FIGURE 3   The effect of colchicine on the kinetics of [³H]thymidine incorporation in human lymphocytes stimulated by Con A. Colchicine was present from the beginning of the experiment. At various times, parallel cultures were pulsed with 6 μCi of [³H]thymidine for 6 h. Data points are the averages of measurements on duplicate cultures and are plotted at times corresponding to 3 h after the start of each pulse. ●, cultures containing Con A (20 μg/ml); ■, cultures containing Con A (20 μg/ml) + colchicine (10^{-6} M), O, cell control (CC).

colchicine has a delaying effect on the onset of DNA synthesis in Con A-stimulated cultures. Alternatively, it may represent a small population of cells escaping the early colchicine block. After 70 h, the level of DNA synthesis in cultures containing colchicine remained constant. The final level in cultures containing colchicine was about 30% of that observed in the absence of the drug. Further analysis of the difference in the final levels of synthesis between cultures with and without colchicine was not attempted for these late times because of the complexities introduced by the appearance of second generation cells in cultures without the drug.

Inhibition of Blast Transformation

The effect of colchicine on the stimulation of lymphocytes by Con A was manifested not only by a depressed level of [³H]thymidine incorporation but also by a decrease in the percentage of lymphocytes transformed into blast cells. Fig. 4 and Table I show the results of a representative experiment to determine the kinetics of appearance of blast cells in the presence and absence of colchicine. By counting blast cells in Giemsa-stained cell smears taken from cultures at various times, it was found that the percentage of blasts began to rise between 24 and 30 h after the addition of Con A, while the corresponding percentages in cell control cultures or cultures containing colchicine showed no appreciable increase up to about 50 h (Fig. 4). After 50 h, the percentage of blast cells in cultures containing colchicine showed a small rise, paralleling the small increase in the level of DNA synthesis seen in Fig. 3.

Using the hemocytometer counting method to determine the absolute number of blasts and unstimulated lymphocytes, we performed a more detailed analysis on the same cultures from which the data in Fig. 4 were obtained. The results, tabulated in Table I, show that although some blast cells were observed in cultures containing colchicine, the absolute number as well as the percentage of these blasts was lower than in parallel cultures without the drug. A Student's t test on these data showed that the differences in the number of blast cells after 30 h between cultures with and without colchicine were significant to the 1% level. Bearing in mind the differences noted previously on the hemocytometer and cell smear counting methods (see Materials and Methods), we conclude from the combined results of the two

methods that colchicine also inhibits blast transformation.

Using the hemocytometer counting procedure, we also observed that, up to 46 h of exposure to colchicine, the number of viable leucocytes in cultures with the drug was comparable with the corresponding number in cultures containing no colchicine (Table I). After 46 h, the total number of cells in cultures with colchicine was always lower than the number in cultures without the drug. It should be noted, however, that the difference in the total number of cells in the two cultures became significant only at late times when complexities such as the appearance of daughter cells must be considered in the analysis of cell numbers. Inasmuch as the differences in the number and percentage of blast cells in cultures with and without colchicine are clearly discernible at times much earlier than 40 h after the addition of Con A, we conclude that the early effects of colchicine cannot be ascribed to any appreciable decrease in cell viability in the presence of the drug.

Effect of Colchicine on Blast Cells from Stimulated Cultures

In order to test whether the depressed level of blast cells in colchicine-containing cultures was due to selective killing of these cells, lymphocytes stimulated by Con A were synchronized using 2 mM hydroxyurea. Colchicine was added to half of the cultures 6 h before the removal of hydroxyurea. After the release of the hydroxyurea block, the numbers of viable small lymphocytes and blast cells as well as the level of thymidine incorporation were determined. For at least 30 h of exposure to colchicine, there was no appreciable difference in the numbers of blast cells and small lymphocytes (Fig. 5 a and b). Colchicine, therefore, does not exert its toxic effects selectively on blast cells. These results suggest that the difference in the number of blast cells in cultures with and without colchicine (Fig. 4) represents a true inhibition by the drug of the transformation process from a small resting lymphocyte into a blast cell.

In addition, the data on [³H]thymidine incorporation by blast cells formed in the absence of colchicine but arrested at the G_1/S boundary showed that colchicine had no effect on the level of incorporation (Fig. 5 c). Under these conditions, therefore, colchicine appears to affect neither [³H]thymidine transport across the membrane nor the process of DNA synthesis itself. This suggests
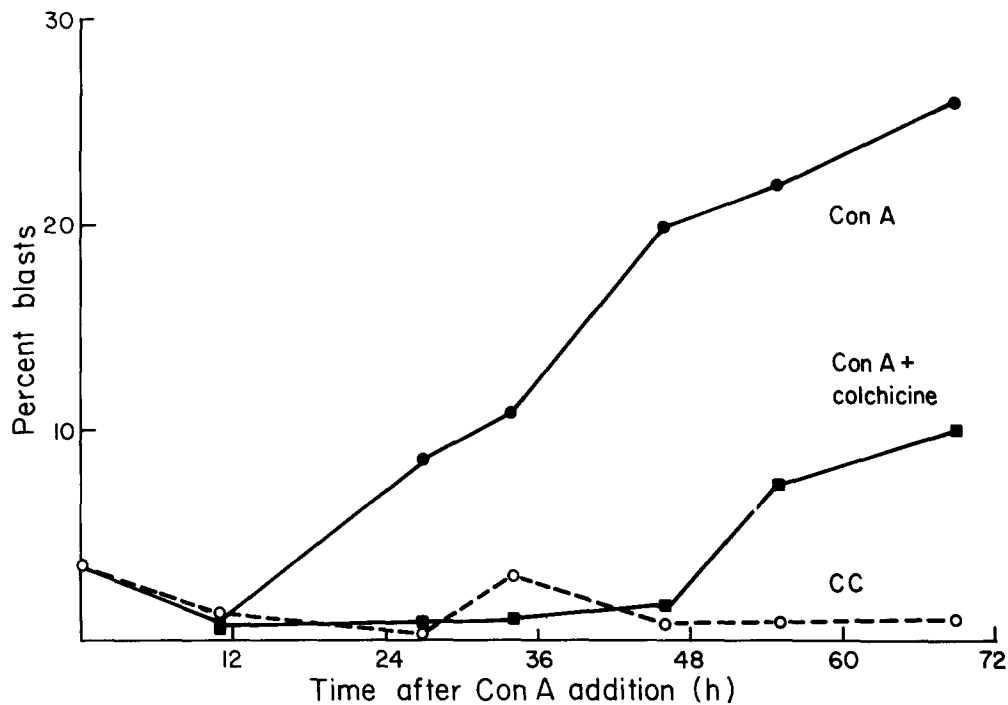
FIGURE 4 The effect of colchicine on the kinetics of appearance of blast cells in human lymphocyte cultures stimulated by Con A. Colchicine was present from the beginning of the experiment. At the indicated times, aliquots of cultures were removed, stained with Giemsa, and the total number of small lymphocytes and blast cells was counted (see Materials and Methods). The data is expressed as the percentage of blast cells present at various times after the addition of Con A. ●, cultures containing Con A (20 μg/ml); ■, cultures containing Con A (20 μg/ml) + colchicine (10⁻⁶ M); O, cell control (CC).

TABLE I

The Effect of Colchicine on the Number of Cells Transformed by Con A*

	Conditions	Hours in Culture						
		0	12	27	34	46	55	67
Cell control	Small lympho-cytes	10.3	11.8 ± 2.0	10.1 ± 1.6	10.5 ± 0.8	11.3 ± 0.7	12.0 ± 1.8	12.0 ± 1.2
	Blasts	0.53	0.53 ± 0.26	0.68 ± 0.26	0.60 ± 0.34	0.28 ± 0.08	0.35 ± 0.20	0.20 ± 0.11
Con A	Small lympho-cytes	—	5.52 ± 0.84	7.00 ± 0.64	4.48 ± 0.68	5.20 ± 0.80	4.20 ± 0.76	4.08 ± 0.56
	Blasts	—	0.16 ± 0.08	1.04 ± 0.72	1.96 ± 0.37	2.60 ± 0.14	3.16 ± 0.96	3.72 ± 0.99
Con A + col-chicine	Small lympho-cytes	—	7.44 ± 0.52	7.56 ± 1.78	6.40 ± 0.72	4.80 ± 1.80	4.48 ± 1.20	3.80 ± 0.84
	Blasts	—	0.16 ± 0.08	0.20 ± 0.26	0.24 ± 0.06	0.40 ± 0.14	0.76 ± 0.25	0.76 ± 0.45

* Number of cells are averages of results from triplicate cultures expressed as: (cells per ml) × 10^{+5} ± 1 SEM.

that the depressed level of [³H]thymidine incorporation seen in cultures containing colchicine (Figs. 2 and 3) represents an inhibitory effect of the drug acting on the lymphocytes prior to their transformation into blast cells.

Comparison of the Inhibitory Effects of αMM and Colchicine

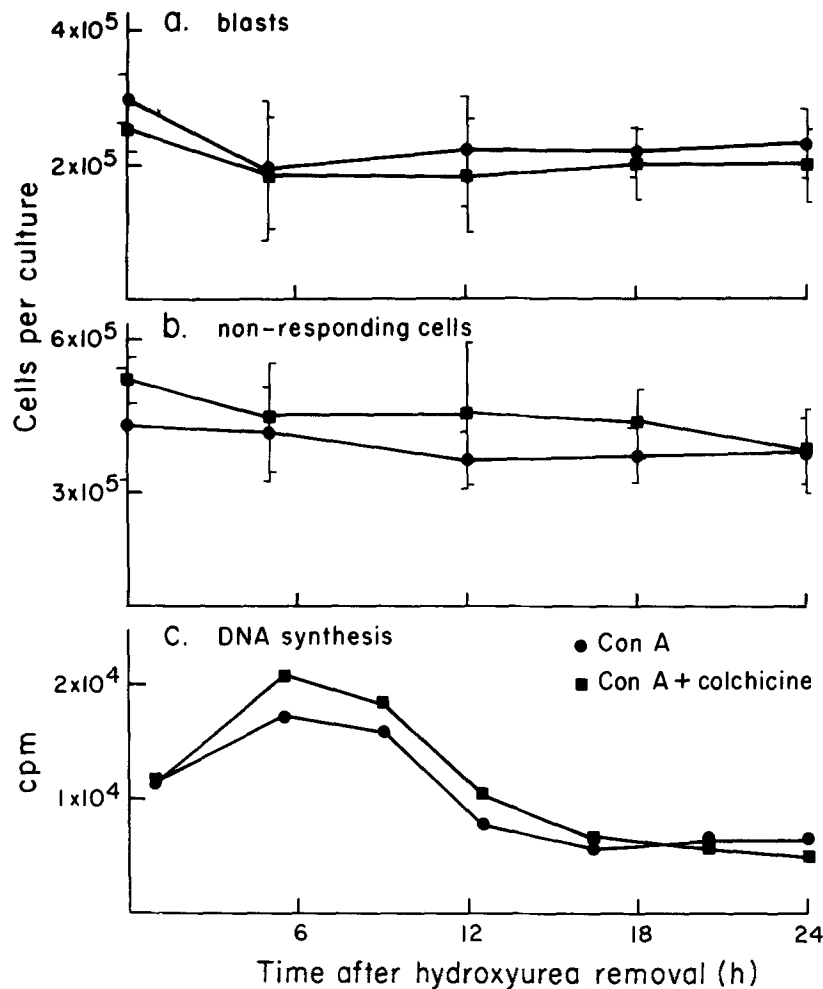In previous studies, we observed (13) that if αMM, a competitive inhibitor of Con A, is

FIGURE 5   The effect of colchicine on the viability and [³H]thymidine incorporation of human lymphocyte cultures stimulated by Con A and arrested at the G_1/S boundary. Blast cells were collected at the G_1/S boundary using 2 mM hydroxyurea, and colchicine was added to half of the cultures 6 h before the removal of hydroxyurea (see Materials and Methods). The data are expressed as the absolute numbers of blast cells (a) and small lymphocytes present (b) and the level of [³H]thymidine incorporation (c) at various times after the removal of hydroxyurea. The cell numbers in (a) and (b) were determined by counting in a hemocytometer. Each point represents the mean of three independent determinations and the vertical bars represent ± 1 SEM. Data points for [³H]thymidine incorporation in (c) (6 µCi/culture, 1-h pulse) are the averages of measurements on duplicate cultures. ●, cultures containing Con A (20 µg/ml); ■, cultures containing Con A (20 µg/ml) + colchicine (10⁻⁶ M).

introduced at various times after the addition of the mitogen to cultures of mouse splenic lymphocytes, the incorporation of [³H]thymidine as measured at 48 h is at least partially inhibited. No inhibition was observed if the saccharide was added 20 h after the addition of Con A; and thereafter, αMM had no effect on the level of the response. In the case of mouse lymphocytes, the

rising level of [³H]thymidine incorporation with later times of αMM addition was proportional to the number of cells committed to lectin stimulation. When αMM was introduced at various times after the addition of Con A to cultures of human lymphocytes, the incorporation of [³H]thymidine at 48 h was inhibited in a fashion similar to that previously observed in the stimulation of mouse

lymphocytes (Fig. 6 a). The minor difference between the two systems concerns the time after which αMM addition has no effect on the level of the response: 24–30 h for the human cells and 18–20 h for mouse cells.

In order to define further the period during which the cells are sensitive to colchicine, experiments were performed in which the drug was introduced into cultures of human lymphocytes at various times after the addition of Con A in a fashion analogous to the addition of αMM (Fig. 6 b). If colchicine was added at the start of the culture, the incorporation of [³H]thymidine as measured at 48 h was inhibited by about 90% when compared to controls. Successively less inhibition was observed the later the addition of colchicine, and no effect was seen if the drug was added 30 h after the addition of the mitogen. Moreover, the
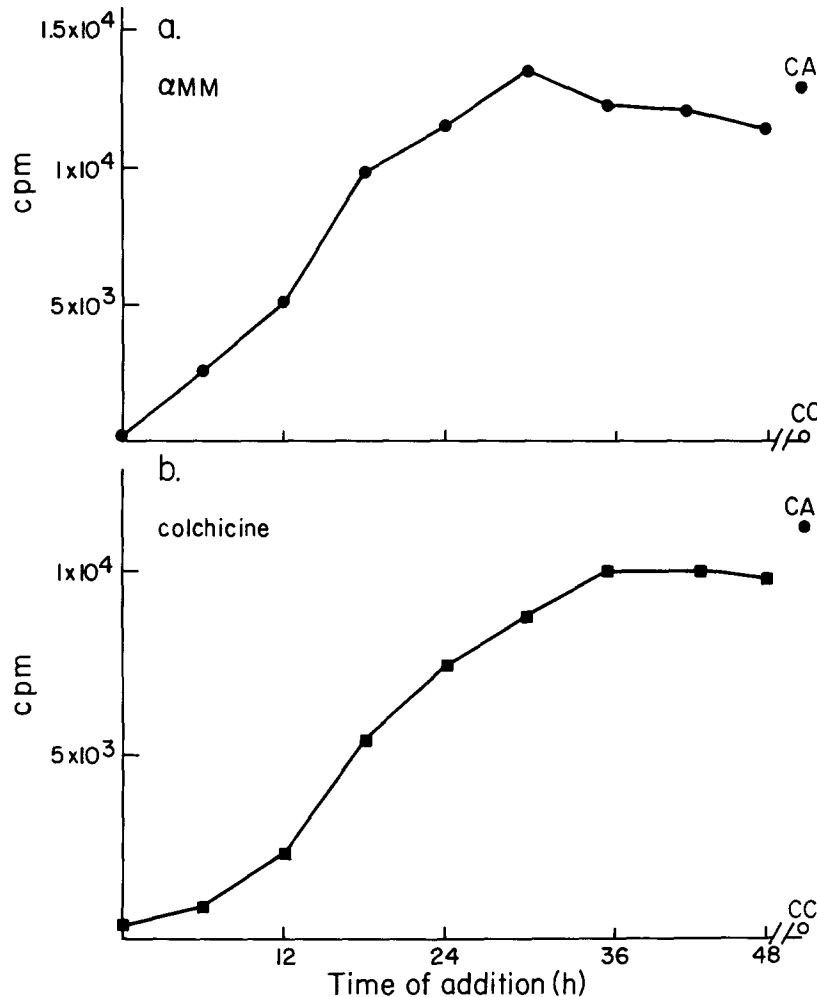

FIGURE 6   Effect of αMM (a) and colchicine (b), added at different times after the start of the culture, on the incorporation of [³H]thymidine in human lymphoctyes stimulated by Con A. Cultures containing Con A (20 μg/ml) were made 0.1 M in αMM (a) or 10⁻⁶ M in colchicine (b) at the indicated time points. The cultures were continued until 48 h at which time they pulsed with 6 μCi of [³H]thymidine for 2 h and harvested. For scintillation counting, 50% and 100% of each of the αMM and colchicine cultures, respectively, were used. In both (a) and (b), the points labeled CA represent the incorporation of [³H]thymidine by cultures containing only cells plus Con A. The points labeled CC represent incorporation by cultures containing cells alone.

effect of colchicine added at various times paralleled the effect of saccharide addition at various times after stimulation (Fig. 6). These results suggest the possibility that the inhibitory effect of colchicine on lymphocytes takes place at or before cellular commitment to lectin activation.

Specificity and Reversibility of the Effects of Colchicine

The specificity of the effects of colchicine on lymphocyte stimulation was studied using vinblastine, vincristine, and lumicolchicine. At concentra-
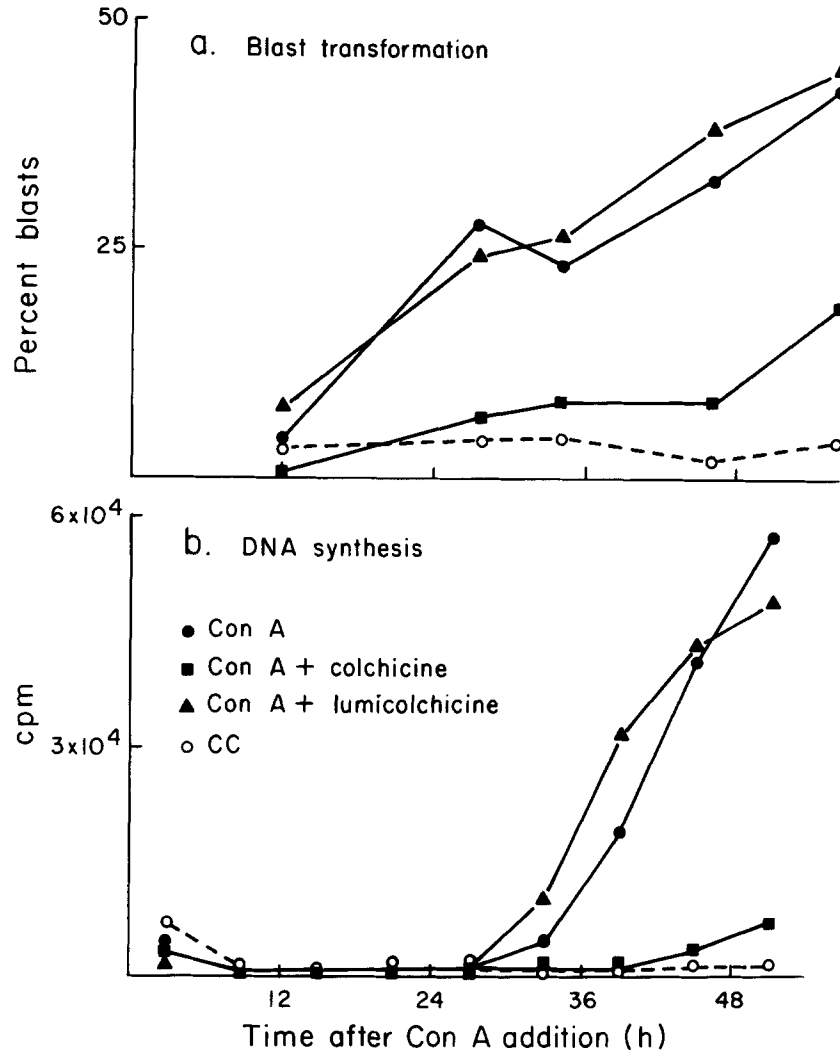

FIGURE 7   A comparison of the effects of colchicine and lumicolchicine on the kinetics of appearance of blast cells and on the kinetics of [³H]thymidine incorporation in human lymphocytes stimulated by Con A. Colchicine and lumicolchicine were present from the beginning of the experiment. At the indicated times, aliquots of cultures were removed and the total number of viable lymphocytes and blast cells were counted in a hemocytometer. The data are averages of determinations on duplicate cultures and are expressed as the percentage of blast cells present at various times after the addition of Con A (a). For measurements of DNA synthesis (b), data points were obtained from the same experiment as in Fig. 3. ●, cultures containing Con A (20 μg/ml); ■, cultures containing Con A (20 μg/ml) + colchicine (10⁻⁶ M); ▲, cultures containing Con A (20 μg/ml) + lumicolchicine (10⁻⁶ M); O, cell control (CC).

tions of 10^{-6} M, the other antimitotic drugs, vinblastine and vincristine, also inhibited the stimulation of human lymphocytes by Con A as assayed by [^{3}H]thymidine incorporation. In contrast, lumicolchicine, a photo-inactivated derivative of colchicine (39), had no appreciable effect on the level of blast transformation or DNA synthesis in lymphocyte cultures stimulated by Con A (Fig. 7). Moreover, the extent of inhibition of [^{3}H]thymidine incorporation by 10^{-6} M colchicine was similar in the presence and absence of an equimolar concentration of lumicolchicine. This suggests that lumicolchicine does not compete with colchicine in binding to the inhibitory site involved in control of lymphocyte stimulation.

To test whether the uptake of colchicine into human lymphocytes can be reversed by washing in culture medium free of colchicine, the binding and the efficiency and rate of removal of [^{3}H]colchicine were determined (Fig. 8). Colchicine binding was

rapid during the first 6 h of exposure to the drug at a concentration of 10^{-6} M; approximately 80% of the maximal colchicine level was reached within this time. A slower rate of uptake was seen over the next 6–10 h. The rate of colchicine efflux was slower than the rate of uptake. 6 h after washing, more than 70% of the initially bound colchicine remained associated with the cells. After an exposure of 6 h to colchicine, not all of the drug can be removed by washing and incubating in colchicine-free medium. The amount of colchicine remaining in the cell even after extensive incubation was about 30% of the maximal level.

Using these data on [^{3}H]colchicine uptake and efflux in human lymphocytes, experiments were performed to investigate whether cells treated with colchicine become irreversibly refractory to Con A stimulation. Cells were exposed to 10^{-6} M or 10^{-7} M colchicine in the absence of Con A for 6 h, washed, and then reincubated in colchicine-free
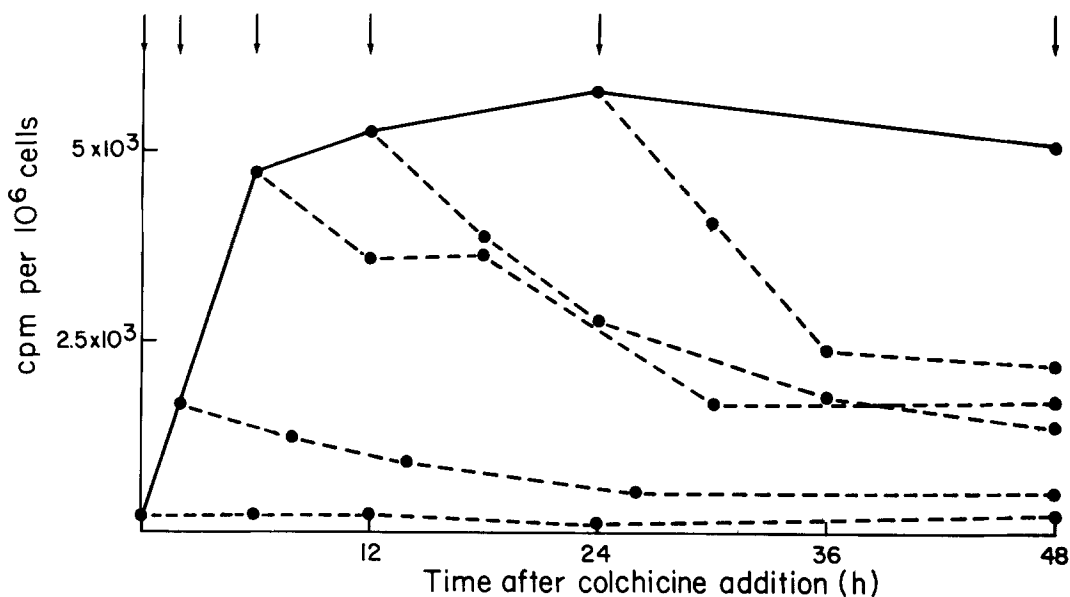

FIGURE 8 The rates of binding and removal of colchicine on human lymphocytes in the presence of con A. Cells (1 × 10^6/ml) were cultured in the presence of Con A (20 μg/ml) and 10^{-6} M [^{3}H]colchicine (3 Ci/mmol). At the times indicated by the arrows, parallel sets of cultures were washed and reincubated in an equal volume of colchicine-free medium containing Con A. The solid line depicts the results of measurements made immediately after the washing was performed. The dotted lines depict the results of measurements made on cultures incubated for 6, 12, or 24 h after washing, and also 48 h after the addition of [^{3}H]colchicine. The experimental procedure used in these measurements is described in Materials and Methods. Data points shown are the average of measurements on duplicate cultures and are expressed as cpm per 10^6 viable cells determined at the indicated times. Assuming a counting efficiency of 20% for ^{3}H-scintillation counting, the maximal level of [^{3}H]colchicine (5.5 × 10^3 cpm/10^6 cells) corresponds to a value of 2.5 × 10^6 colchicine molecules per cell.

The Reversibility of the Effect of Colchicine

Preincubation	Con A	cpm
Control	–	4,300
"	+	28,900
Colchicine (10⁻⁷ M)	–	4,600
"	+	26,900
Colchicine (10⁻⁶ M)	–	5,500
"	+	15,900

* Human lymphocytes were incubated with or without colchicine for 6 h, then incubated in colchicine-free medium for 48 h. The cells were then stimulated with Con A and assayed for [³H] thymidine incorporation (6 μCi/tube, 2 h) 48 h after the addition of the lectin. The cpm shown are averages of results from duplicate cultures.

medium for another 48 h. At this time, cell viabilities were determined for cultures previously exposed and unexposed to colchicine and the cell concentrations were brought to 10⁶ viable cells/ml. The cells were then stimulated with Con A and [³H]thymidine incorporation was assayed in these cultures 48 h after the addition of the mitogen. The results (Table II) show that, while cells exposed to 10⁻⁷ M colchicine were fully responsive to Con A-stimulated DNA synthesis as compared to controls, cells exposed to 10⁻⁶ M colchicine recovered only partially. The response of cells exposed to 10⁻⁶ M colchicine was approximately 55% of the level seen in cells never exposed to the drug. Histologic staining and autoradiographic experiments showed that this corresponds to the percentage of cells responding to stimulation when cultures with and without the drug are compared after colchicine removal. These data indicate that the effect of colchicine on lymphocyte activation must be at least partially reversible, particularly in view of the fact that 10⁻⁷ M colchicine was found to be a potent inhibitor of Con A-stimulated DNA synthesis (Fig. 2).

DISCUSSION

This study was undertaken to investigate in detail the effect of colchicine on the stimulation of lymphocytes by mitogens, particularly in the light of the hypothesis that colchicine-binding proteins play a role in mediating receptor-cytoplasmic interactions (8, 41, 42). We previously reported that the stimulation of mouse lymphocytes by Con A, as measured by [³H]thymidine incorporation in

the period from 48 to 50 h, was inhibited by colchicine at a concentration as low as 10⁻⁶ M (8). We have now observed similar effects of this drug using human and rabbit peripheral lymphocytes stimulated by Con A. The effect of these drugs has also been reported recently by Medrano et al. (26) using human lymphocytes stimulated by PHA.

A satisfactory interpretation of the effects of colchicine on the stimulation of human lymphocytes by Con A rests on the following observations made in the present study: (a) colchicine inhibits the mitogenic response at a very early point, before the onset of blast transformation and DNA synthesis; (b) under the conditions used, exposure of unstimulated lymphocytes or blast cells to colchicine for as long as 30-36 h does not result in any appreciable decrease in cell viabilities; (c) colchicine does not affect DNA synthesis in synchronized, prestimulated blast cells; (d) the effect of colchicine on Con A-stimulated blast transformation and [³H]thymidine incorporation can also be observed using vinblastine and vincristine but not lumicolchicine; and (e) the inhibition by colchicine is at least partially reversible. We shall consider each of these in turn.

The inhibition by colchicine of lymphocyte activation cannot be solely ascribed to the well-known effect of the drug on the mitotic spindle (31). It has been reported previously (32) that the majority of the mitogen-stimulated human lymphocytes enter their first mitosis between 48 and 60 h after the initial binding of the stimulant. We have found, however, that a difference in both the levels of DNA synthesis and blast transformation between Con A-stimulated lymphocyte cultures with and without colchicine can be observed as early as 36 h (Figs. 3 and 4). Therefore, the onset of division in Con A-stimulated cultures occurs too late to support the contention that colchicine acts by metaphase arrest.

It is also clear that the inhibitory effects of colchicine and related drugs cannot be ascribed to their toxicity. Although it has been reported that antimitotic agents such as colcemid, vinblastine, and vincristine are highly toxic to certain mammalian cell lines at concentrations comparable to those used in the present experiments (18, 23), Thompson and O'Connor (37) have found that normal human lymphocytes are resistant to rapid killing by colchicine. In addition, Medrano et al. (26) have reported that colchicine, at concentrations as high as 2.5 × 10⁻⁵ M, does not affect the

viability of human lymphocytes for times as long as 72 h in culture. Our results also show no significant difference in the number of either blast cells or nonresponding lymphocytes between synchronized cultures with and without 10^{-6} M colchicine for exposure times of up to 30 h (Fig. 5).

When asynchronous cultures stimulated with Con A were maintained in the continuous presence of colchicine (Table I), an apparent reduction in the total cell number is seen at 46, 55, and 67 h, as compared to the corresponding number in cultures containing only Con A. Interpretation of this observation is complicated, however, by the fact that cell division commences at about 45 h after the addition of the mitogen (32), resulting in a relative increase in the number of cells in cultures containing Con A alone. Inasmuch as a difference in both DNA synthesis and blast transformation can be detected before the reduction in cell number due to colchicine becomes appreciable, we conclude that the effect of colchicine on mitogenesis cannot be due to cell death induced by the drug.

Two other lines of evidence argue against cell death as the cause of inhibition of mitogenic stimulation by colchicine. First, the observation that the colchicine effect is at least partially reversible suggests that the cells are not permanently damaged. Second, both [³H]thymidine incorporation and blast transformation in cultures containing colchicine increase after 50 h, implying that the cells are still viable and can escape the colchicine block at late times.

This late rise of DNA synthesis and blast transformation in cultures containing colchicine may indicate that the effect of colchicine is to slow the passage of a cell through a given phase of the cell cycle. It has been reported that at high concentrations, such as 10^{-4} M, colchicine can inhibit DNA synthesis (10, 19) or slow the passage of a cell through the S phase (15, 16). Other studies have shown that lower concentrations of colchicine (1–5 × 10^{-5} M) can inhibit thymidine transport in a variety of cell lines (27) and in rabbit alveolar macrophages (5). In our studies, we have found that 10^{-6} M colchicine does not inhibit either DNA synthesis or thymidine transport in blast cells stimulated in the absence of colchicine and collected at the G_1/S boundary using hydroxyurea. A similar conclusion has been made by Medrano et al. (26) for human lymphocytes stimulated with PHA, except that these workers added colchicine only 3 h prior to the measurement of

DNA synthesis. From our binding studies using [³H]colchicine (Fig. 8), we estimate that the intracellular level of colchicine as a result of the 3-h preincubation with the drug would be about 50% of the maximal level. Both studies, therefore, indicate that in colchicine-containing cultures, the inhibition of [³H]thymidine incorporation is not due to an inhibition by the drug of thymidine transport or the process of DNA synthesis itself.

One can now ask the following questions concerning the effect of colchicine: (a) At what stages of the cell cycle does colchicine exert its inhibitory effect on the cell? (b) Are all responding cells susceptible to inhibition simultaneously? To answer these questions, it is first necessary to consider the heterogeneity of the response of the cell population to Con A.

The kinetics of cellular commitment in the stimulation of mouse splenocytes by Con A have recently been analyzed (13). As used here, "commitment" is the point at which a cell becomes stimulated and no longer depends upon the presence of the mitogenic agent. It has been shown that, for mouse lymphocytes, all cells do not become committed to stimulation simultaneously, but rather at various times up to 20 h after the addition of the mitogen. Other workers using human lymphocytes have also observed an increasing response with longer incubations with PHA (43) or Con A (21, 29), although the specific result differs according to the conditions of the assay and the method of removal of the lectin. It is important to emphasize that these conclusions concerning cellular commitment apply only to those cells that have entered their initial S phase by 48 h. There is ample evidence from studies of PHA-stimulated human lymphocytes that the period between lectin addition and the entry of a cell into the first S phase is highly variable (17, 34, 35). Moreover, data on the commitment time for cells entering S phase after 48 h are not easily analyzed due to complications introduced by daughter cell proliferation. In any event, the data presented here (Fig. 6 a) suggest that increasing numbers of human lymphocytes become committed, beginning with the time of addition of Con A and continuing up to 30 h after the addition of the lectin.

Given the facts on the kinetics of cellular commitment, what is the temporal relation between inhibition by colchicine and commitment to stimulation? The two alternatives are: (a) colchicine inhibits mitogenic stimulation near the time

that commitment occurs; or (b) colchicine inhibits either before or after commitment. These alternatives lead to different predictions about the effects of colchicine added at various times to Con A-stimulated cultures (Fig. 6 b). If the first alternative is true, then the addition of colchicine at later and later times will result in an increasing response similar to that observed when αMM is added at various times (Fig. 6 a). If colchicine inhibits before or after commitment, however, then the colchicine-sensitive point and the commitment point can be separated by either a constant time interval from cell to cell, or by a variable time interval. Separation of the two points by a constant time, t, would generate a curve (Fig. 6 b) that paralleled the curve for αMM addition, with a shift of the colchicine curve to earlier or later times by an interval equal to time t. If, on the other hand, the colchicine-sensitive point and the commitment point are separated by a variable time interval, the possibilites become too numerous for analysis to be useful. The fact that the two curves in Fig. 6 a and b are similar is at present consistent with the interpretation that the inhibitory action of colchicine takes place near the time that a cell becomes committed to stimulation by Con A.

The cellular sites of the inhibition by colchicine are not known. We have reported previously (8) that colchicine does not interact with Con A nor does it inhibit the cell-binding activity of the protein. Our present studies using colchicine, vinblastine, vincristine, and lumicolchicine suggest that the inhibition may be due to interaction of the drugs with microtubules rather than to interaction with some component within the cell membrane. While colchicine, vinblastine, and vincristine inhibited mitogenic stimulation, lumicolchicine did not. In processes usually associated with the interaction of colchicine with membrane components, such as inhibition of nucleoside transport, lumicolchicine is just as effective an inhibitor as colchicine while vinblastine does not inhibit (5, 27, 38). Moreover, we have found that colchicine inhibited the stimulation of lymphocytes to the same extent in the presence and absence of equimolar concentrations of lumicolchicine. Inasmuch as lumicolchicine does not compete with colchicine for binding sites on microtubules (38, 39), these results are consistent with the interpretation that the site of action of colchicine on the early events in mitogenesis may be microtubular structures within the cytoplasm.

The observation that the binding of [^{3}H]colchicine to human lymphocytes and the effect of colchicine on cell stimulation are partially reversible is consistent with evidence from the binding of the drug to tubulin subunits in vitro and from experiments on modulation of receptor mobility. If purified tubulin is saturated with colchicine and then incubated for 6 h in the absence of any free colchicine, only 12% of the colchicine-binding sites are unoccupied (38). This is to be compared with a half-life of about 12 h for the loss of colchicine from human lymphocytes (Fig. 8). Yahara and Edelman (41) also found that the effect of colchicine on the modulation by Con A of lymphocyte surface receptor mobility is only partially reversed after 6 h of incubation in colchicine-free medium. It is interesting to note that Taylor (36), using 10^{-7} M colchicine, found that the intracellular level of the drug in a human cell line was reduced by 75% after only 7 h of incubation in colchicine-free medium. This suggests that at 10^{-7} M the degree of reversibility in the binding of colchicine is much higher than at 10^{-6} M. In agreement with this conclusion, treatment of lymphocytes with 10^{-7} M colchicine resulted in a higher degree of reversibility than was observed at higher concentrations (Table II).

All of the present findings, as well as those on the effects of colchicine on the modulation of receptor mobility (41, 42), have important implications for hypotheses on receptor-cytoplasmic interactions and mitogenesis in lymphocytes (8). Inhibition by Con A of cell surface receptor movement and its reversal by colchicine suggests that a colchicine-binding protein system such as the cytoplasmic microtubules may mediate the inhibition process (8, 41, 42). The inhibition by colchicine of the stimulation of lymphocytes by Con A suggests the possibility that mitogenic activation could also be mediated by the same structures which are responsible for the appropriate receptor-cytoplasmic interactions.

Alternatively, the effect of colchicine on lymphocyte stimulation could be due to the control exerted by microtubular proteins on the transport of various ions and metabolites. For example, recent studies have shown that Ca^{++} ion uptake is required for lymphocyte activation (1, 3) and that the Ca^{++}-carrying ionophore A-23187 can stimulate human lymphocytes (J. L. Wang and G. M. Edelman, unpublished observations) and pig lymphocytes (24). Furthermore, one of the early events

after mitogen binding is the increased metabolism of phospholipids, particularly phosphotidylinositol (9), which may be controlled by changes in the intracellular concentration of free Ca^{++} (2).

In view of these observations, it is interesting to consider the possibility that an intact microtubule assembly may be necessary for Ca^{++} ion transport. Colchicine may exert its inhibitory effects by dissociating the microtubules, which regulate the required Ca^{++} ion transport in a fashion analogous to the regulation of other Ca^{++}-sensitive processes such as insulin release by pancreatic islet cells (20) and granulocyte chemotaxis (11). The demonstration of such a formal analogy between the stimulus-secretion coupling system and lymphocyte mitogenesis (30) promises to deepen our understanding of the mechanism of lymphoid cell stimulation.

REFERENCES

1. ALFORD, R. H. 1970. Metal cation requirements for PHA-induced transformation of human peripheral blood lymphocytes. *J. Immunol.* **104**:698-703.
2. ALLAN, D., and R. H. MICHELL. 1974. Phosphatidylinositol cleavage in lymphocytes: requirement for calcium ions at a low concentration and effects of other cations. *Biochem. J.* **142**:599-604.
3. ALLWOOD, G., G. L. ASHERSON, M. J. DAVEY, and P. J. GOODFORD. 1971. The early uptake of radioactive calcium by human lymphocytes treated with phytohemagglutinin. *Immunology.* **21**:509-516.
4. ANDERSSON, J., G. M. EDELMAN, G. MÖLLER, and S. SJÖBERG. 1972. Activation of B lymphocytes by locally concentrated concanavalin A. *Eur. J. Immunol.* **2**:233-235.
5. BERLIN, R. D. 1973. Temperature dependence of nucleoside membrane transport in rabbit alveolar macrophages and polymorphonuclear leukocytes. *J. Biol. Chem.* **248**:4724-4730.
6. BOYUM, A. 1968. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):1-108.
7. CUNNINGHAM, B. A., J. L. WANG, M. N. PFLUMM, and G. M. EDELMAN. 1972. Isolation and proteolytic cleavage of the intact subunit of concanavalin A. *Biochemistry.* **11**:3233-3239.

8. EDELMAN, G. M., I. YAHARA, and J. L. WANG. 1973. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1442-1446.
9. FISHER, D. B., and G. C. MUELLER. 1968. An early alteration in the phospholipid metabolism of lymphocytes by phytohemagglutinin. *Proc. Natl. Acad. Sci. U. S. A.* **60**:1396-1402.
10. FITZGERALD, P. H., and L. A. BREHAUT. 1970. Depression of DNA synthesis and mitotic index by colchicine in cultured human lymphocytes. *Exp. Cell Res.* **59**:27-31.
11. GALLEN, J. I., and A. S. ROSENTHAL. 1974. The regulatory role of divalent cations in human granulocyte chemotaxis. *J. Cell Biol.* **62**:594-609.
12. GREAVES, M. F., and S. BAUMINGER. 1972. Activation of T and B lymphocytes by insoluble phytomitogens. *Nat. New Biol.* **235**:67-70.
13. GUNTHER, G. R., J. L. WANG, and G. M. EDELMAN. 1974. The kinetics of cellular commitment during stimulation of lymphocytes by lectins. *J. Cell Biol.* **62**:366-377.
14. GUNTHER, G. R., J. L. WANG, I. YAHARA, B. A. CUNNINGHAM, and G. M. EDELMAN. 1973. Concanavalin A derivatives with altered biological activities. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1012-1016.
15. GUSTAFFSSON, M. K. S. 1968. Effect of colchicine on DNA synthesis in plerocoids of *Diphyllobothrium dendriticum* (Cestoda). *Exp. Cell Res.* **50**:1-8.
16. HELL, E., and D. G. COX. 1963. Effects of colchicine and colcemid on synthesis of deoxyribonucleic acid in the skin of the guinea pig's ear *in vitro. Nature (Lond.).* **197**:287-288.
17. JASIŃSKA, J., J. A. STEFFEN, and A. MICHALOWSKI. 1970. Studies on *in vitro* lymphocyte proliferation in cultures synchronized by the inhibition of DNA synthesis. *Exp. Cell Res.* **61**:333-341.
18. KLEINFELD, R. G., and J. E. SISKEN. 1966. Morphological and kinetic aspects of mitotic arrest by and recovery from colcemid. *J. Cell Biol.* **31**:369-379.
19. LA COUR, L. F., and S. R. PELC. 1959. Effect of colchicine on the utilization of thymidine labeled with tritium during chromosomal reproduction. *Nature (Lond.).* **183**:1455-1456.
20. LACY, P. E., S. L. HOWELL, D. A. YANG, and C. J. FINK. 1968. A new hypothesis of insulin secretion. *Nature (Lond.).* **219**:1177-1179.
21. LINDAHL-KIESSLING, K. 1972. Mechanism of phytohemagglutinin (PHA) action. V. PHA compared with concanavalin A (Con A). *Exp. Cell Res.* **70**:17-26.
22. LOHRMANN, H. P., C. M. GRAW, and R. G. GRAW. 1974. Stimulated lymphocyte cultures, responder recruitment and cell cycle kinetics. *J. Exp. Med.* **139**:1037-1048.
23. MADOC-JONES, H., and F. MAURO. 1968. Interphase action of vinblastine and vincristine: differences in

the lethal action through the mitotic cycle of cultured mammalian cells. *J. Cell Physiol.* **72**:185–195.

24. MAINO, V. C., N. M. GREEN, and M. J. CRUMPTON. 1974. The role of calcium ions in initiating transformation of lymphocytes. *Nature (Lond.).* **251**:324–327.

25. McCLAIN, D. A., J. L. WANG, and G. M. EDELMAN. 1975. The effects of sodium metaperiodate on T and B lymphocytes.*Cell. Immunol.* **15**:287–293.

26. MEDRANO, E., R. PIRAS, and J. MORDOH. 1974. Effect of colchicine, vinblastine and cytochalasin B on human lymphocyte transformation by phytohemagglutinin. *Exp. Cell Res.* **86**:295–300.

27. MIZEL, S. B., and L. WILSON. 1972. Nucleoside transport in mammalian cells. Inhibition by colchicine. *Biochemistry.* **11**:2573–2578.

28. PADAWER, J. 1960. Effect of colchicine and related substances on the morphology of peritoneal mast cells. *J. Natl. Cancer Inst.* **25**:731–746.

29. POWELL, A. E., and M. A. LEON. 1970. Reversible interaction of human lymphocytes with the mitogen concanavalin A. *Exp. Cell Res.* **62**:315–325.

30. RAFF, M. C. 1975. Role of the plasma membrane in control of differentiated cell functions: an overview. *In* National Institutes of Health International Workshop on Cell Surfaces and Malignancy., In press.

31. SARTORELLI, A. C., and W. A. CREASY. 1969. Cancer chemotherapy. *Annu. Rev. Pharmacol.* **9**:51–72.

32. SASAKI, M. S., and A. NORMAN. 1966. Proliferation of human lymphocytes in culture. *Nature (Lond.).* **210**:913–914.

33. SELL, S., and P. G. H. GELL. 1965. Studies on rabbit lymphocytes *in vitro.* I. Stimulation of blast transformation with an anti-allotype serum. *J. Exp. Med.* **122**:423–439.

34. SÖREN, L. 1973. Variability of the time at which PHA-stimulated lymphocytes initiate DNA synthesis. *Exp. Cell Res.* **78**:201–208.

35. STEFFEN, J. A., and W. M. STOLZMANN. 1969. Studies on *in vitro* lymphocyte proliferation in cultures synchronized by the inhibition of DNA synthesis. *Exp. Cell Res.* **56**:453–460.

36. TAYLOR, E. W. 1965. The mechanism of colchicine inhibition of mitosis. I. Kinetics of inhibition and the binding of ³H-colchicine. *J. Cell Biol.* **25**:145–160.

37. THOMSON, A. E. R., and T. W. E. O'CONNOR. 1974. Selective killing by colchicine *in vitro* of lymphocytes in chronic lymphocytic leukemia. *In* Lymphocyte Recognition and Effector Mechanisms. K. Lindahl-Kiessling and D. Osoba, editors. Academic Press, Inc. New York. 665–670.

38. WILSON, L., J. R. BAMBURG, S. B. MIZEL, L. M. GRISHAM, and K. M. CRESWELL. 1974. Interaction of drugs with microtubule proteins. *Fed. Proc.* **33**:158–166.

39. WILSON, L., and M. FRIEDKIN. 1966. The biochemical events of mitosis. I. Synthesis and properties of colchicine labeled with tritium in its acetyl moiety. *Biochemistry* **5**:2463–2468.

40. YAHARA, I., and G. M. EDELMAN. 1972. Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* **69**:608–612.

41. YAHARA, I., and G. M. EDELMAN. 1973. Modulation of lymphocyte receptor redistribution by concanavalin A, anti-mitotic agents and alterations of pH. *Nature (Lond.).* **246**:152–155.

42. YAHARA, I., and G. M. EDELMAN. 1975. Electron microscopic analysis of the modulation of lymphocyte receptor mobility. *Exp. Cell Res.* **91**:125–142.

43. YOUNKIN, L. H. 1972. *In vitro* response of lymphocytes to phytohemagglutinin (PHA) as studied with antiserum to PHA. I. Initiation period, daughter-cell proliferation, and restimulation. *Exp. Cell Res.* **75**:1–10.