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Cloned T-cell proliferation and synthesis of specific proteins are inhibited by quinine

(interleukin 2/potassium channel/DNA content)

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ABSTRACT **Recombinant human interleukin 2 (rIL-2) drives the proliferation of the cloned murine T-helper line L2. The initial G_1 activation occurs during the first 20 hr after stimulation, with DNA synthesis (S phase) beginning approximately 20 hr after rIL-2 stimulation. Three patterns of protein synthesis were observed during G_1 activation. Type I proteins (e.g., p72 and p66) were synthesized at near maximal rates as early as 4 hr after stimulation, with little change in rates of synthesis through the G_1 to S phase transition. Type II proteins (e.g., p52 and p36) were detectable early after stimulation, but their rates of synthesis continued to increase throughout G_1 activation, becoming maximal 24–28 hr after stimulation. Type III proteins (e.g., p93, p89, and p63) were synthesized maximally 4 or 8 hr after rIL-2 stimulation, then their rates of synthesis declined markedly to prestimulation levels. Type II proteins, p52 and p36, were shown to be correlated with cell proliferation, since their rates of synthesis were maximal while L2 cells were proliferating and declined as the cells returned to a quiescent state. The potassium channel blocker quinine inhibited cell growth and the synthesis of p52 and p36 when added 0 or 2 hr after rIL-2 stimulation but not when added 6 hr after rIL-2 stimulation. Thus, a quinine-sensitive event occurring in L2 cells between 2 and 6 hr after rIL-2 stimulation is necessary for synthesis of type II proteins, DNA synthesis, and cell proliferation.**

The activation and amplification of antigen-specific T lymphocytes from a resting G_0 state usually require two signals (1). The first signal involves the interaction of antigen in the context of a class II major histocompatibility complex (MHC) molecule with the antigen receptor (2). Stimulation of the antigen receptor causes the expression of interleukin 2 (IL-2) receptors and the production of lymphokines, which usually include IL-2 (3, 4). This initial antigenic stimulation leads to the transition from the G_0 to the G_1 state as measured by increases in RNA and protein synthesis (5). Under most conditions, further G_1 activation and the transition from G_1 to S require a second signal, provided by the interaction of IL-2 with its receptor (6, 7). T lymphocytes that have been stimulated with antigen and factors but are no longer proliferating are in a postmitotic G_1-transition state (8) characterized by the presence of IL-2 receptors and the ability to proliferate in response to IL-2 alone (6, 9). Cloned T lymphocytes possess many of the characteristics of normal activated T lymphocytes in that they maintain their specific antigen reactivity, they produce lymphokines in response to stimulation with antigen, and they proliferate in response to IL-2 alone (10, 11). Since cloned T cells are homogeneous, easily produced in large numbers *in vitro*, and karyotypically normal (unpublished observations), they provide an excellent model system in which to examine the sequence of events following normal T-cell activation by antigen or IL-2.

Some of the early events following IL-2 activation of T cells include translocation of protein kinase C from the cytoplasm to the membrane (12) and the transcription of genes expressed in the first few hours after stimulation (13). Later events include an increase in voltage-gated potassium conductance (14) and the expression of transferrin receptors (4). There is an ordered sequence of events following IL-2 stimulation, but the regulatory factors controlling this sequence are not known. We describe here the time course of specific protein synthesis initiated by stimulation of the cloned murine T-helper cell line L2 with recombinant human IL-2 (rIL-2). Since it has been shown that the potassium channel blocker quinine inhibits rIL-2-driven growth of L2 cells (14), we examined the effects of quinine on the synthesis of specific proteins to determine at what point after IL-2 stimulation quinine has its effects. We have identified two proteins, p52 and p36, that were synthesized at high rates during late G_1 and the G_1 to S phase transition. When cell proliferation was inhibited with quinine, the increase in rates of synthesis of these proteins was inhibited as well.

MATERIALS AND METHODS

Cell Lines. The derivation and maintenance of the murine L2 cell line has been described (15). Cells were used 6–8 days after previous exposure to antigen and growth factors.

Human rIL-2. Purified human rIL-2 was a gift of Cetus (Emeryville, CA). This substance, produced by recombinant DNA technology in *Escherichia coli* (16), is 98% pure by $NaDodSO_4$/PAGE analysis and contains 0.02 ng of endotoxin per 2×10^5 units of IL-2 as determined by the Limulus assay. An excipient control was supplied by the manufacturer. Specific activity of rIL-2 was assessed by the manufacturer, using a standard bioassay for IL-2 (17) and is nearly identical to native human IL-2 purified to homogeneity from culture supernatants of mitogen-activated T cells (18).

Cell-Cycle Analysis. Propidium iodide staining and fluorescence-activated cell sorter (FACS) analysis of rIL-2-stimulated L2 cells was done as described (14). Histograms of linear fluorescence intensity vs. cell number were deconvoluted into 10 Gaussian curves by the method of Fried (19), and curve-fitting was performed using a simplex algorithm (20). The area under the first curve represents the cells in G_1, the area under the last curve represents the cells in G_2 plus M, and the eight intermediate curves represent various stages of S phase. In this paper we refer to the first two S phase curves

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Abbreviations: r, recombinant; IL-2, interleukin 2; FACS, fluorescence-activated cell sorter; 2D, two-dimensional; 1D, one-dimensional.

as early S, the next three curves as middle S, and the last three curves as late S phase.

Analysis of Protein Synthesis. L2 cells were purified by density gradient centrifugation on Ficoll/Hypaque (21), and 10^6 cells were cultured in 1 ml of Dulbecco's modified Eagle medium (DMEM) (15) supplemented with 10% (vol/vol) fetal calf serum. rIL-2 (100 units/ml) was added, and the cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air. At various time points, 10^6 cells were transferred to methionine-free DMEM containing [^{35}S]methionine at 50 μCi/ml (specific activity 800 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and incubated for 1 hr. The cells were then washed in phosphate-buffered saline (pH 7.4) and lysed with extraction buffer [9.5 M urea, 2% (vol/vol) Nonidet P-40, 5% (vol/vol) 2-mercaptoethanol]. Two-dimensional (2D) gel electrophoresis was carried out as described (22). For one-dimensional (1D) gels, the cells were lysed in 0.01 M Tris·HCl (pH 7.4), 0.15 M NaCl, 0.5% Nonidet P-40, and cellular proteins were separated on a 12% polyacrylamide gel containing 0.04% bis-acrylamide and 0.1% NaDodSO$_4$. Gels were stained with silver or Coomassie blue, dried, and exposed to x-ray film for 2–7 days. The total [^{35}S]methionine incorporation was measured by precipitating cellular extracts onto filter paper circles with 5% (wt/vol) trichloroacetic acid and assaying radioactivity using standard liquid scintillation techniques.

Densitometry. 1D gel autoradiographs were scanned with a Hoefer model GS300 densitometer, which was connected to a Waters M840 data reduction system. 2D gels were scanned with a Photoscan system P-1000, and data analysis was done using a VAX 11/750 and an AED 767 graphics terminal.

RESULTS

L2 cells were maintained in culture by weekly restimulation with antigen and growth factors. At the end of this weekly cycle, 92% of the cells had G_1 DNA content as measured by propidium iodide fluorescence and FACS analysis. When stimulated by rIL-2, L2 cells initiated DNA synthesis within 20 hr of stimulation. At this time, 22% of the cells were in S phase, and 95% of these were in early S phase. Seven hours later, 61% of the cells were in S phase, and the majority of these were now in middle S phase. These results indicate that the cells are relatively synchronized during the first 27 hr of IL-2 stimulation. However, by 46 hr, the cells were cycling asynchronously; they were in all stages of the cell cycle with a relatively even distribution throughout S phase. By 68 hr, they were returning to a resting DNA content distribution. These results are comparable to those we have reported (14). In other experiments using the mitotic inhibitor colchicine, at least 85% of rIL-2-stimulated L2 cells had entered S phase of the cell cycle within 48 hr of stimulation (13). Thus, our observations characterize the majority of rIL-2-stimulated cells, and not a select population. Since the initial passage through the cell cycle is synchronous, L2 cells provide an excellent model for studying the sequence of gene expression during the first 24 hr of rIL-2-stimulated proliferation.

We thus examined rIL-2-stimulated protein synthesis during the initial G_1 activation and G_1 to S phase transition. Initial experiments showed that, in rIL-2-stimulated L2 cells, the majority of cellular proteins exhibited a 5-fold increase in the rate of synthesis, as determined by a 1-hr pulse-labeling with [^{35}S]methionine. In these experiments, a small number of proteins (subsequently referred to as type II proteins) had large increases in rates of synthesis around the time of the G_1 to S phase transition. Additionally, by pulse-labeling cellular proteins and then incubating the cells in medium containing nonradioactive methionine, the half-lives of these late G_1 proteins were shown to be greater than 8 hr, indicating that the incorporation of [^{35}S]methionine reflected rates of synthesis. To further characterize rIL-2-induced proteins, pulse-labeled cellular extracts of rIL-2-stimulated L2 cells were analyzed by 2D gel electrophoresis. The results of such an experiment are shown in Fig. 1. The area and intensity of the spots on these gels are directly related to the rates of synthesis of the cellular proteins. The autoradiographs were scanned on a 2D-gel scanner, and the synthetic rate data for seven representative proteins are shown in Table 1. The calculated rates of protein synthesis represent the relative synthetic rates per cell, since equal numbers of cells were loaded on each gel, and since the cells were cycling synchronously at this time.

Three distinct patterns of rIL-2-induced protein synthesis were observed. The majority of proteins (type I), represented by p72 and p66, were synthesized at increased rates as early as 4 hr after stimulation and had reached maximal rates of synthesis by 8 hr with little change from 8 to 24 hr after stimulation. A second pattern of protein synthesis (type II), represented by p52 and p36, was characterized by relatively low rates of synthesis at 4 hr after stimulation, with major increases in synthetic rates occurring between 4 and 8 hr. Unlike the type I proteins, synthetic rates of type II proteins continued to increase between 8 and 24 hr after stimulation. A third group of proteins (type III), represented by p93, p89, and p63, had high rates of synthesis at 4 or 8 hr after

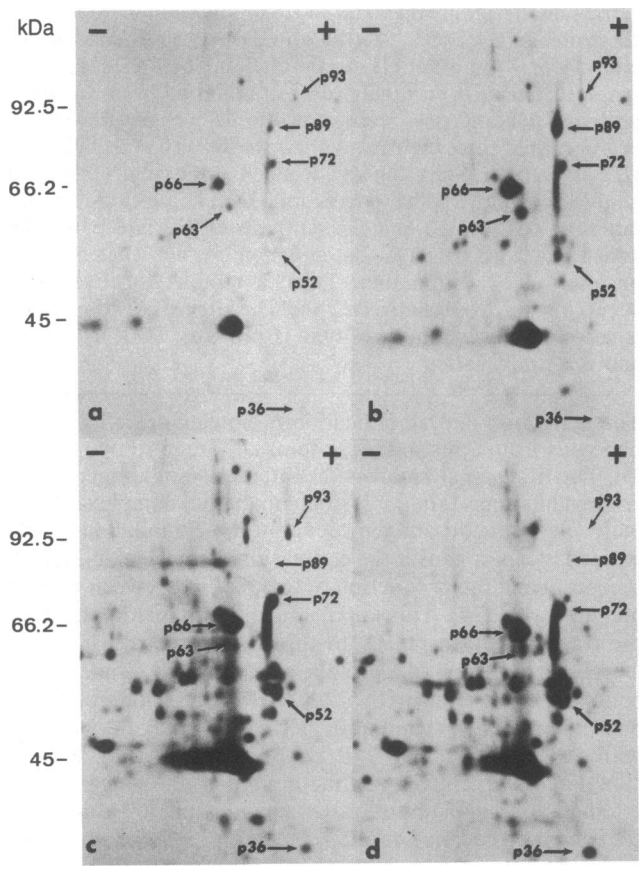


FIG. 1. 2D-gel electrophoresis of [^{35}S]methionine pulse-labeled proteins from rIL-2-stimulated L2 cells. L2 cells (10^6 cells) were stimulated with rIL-2 (100 units/ml) for 4 (*b*), 8 (*c*), or 24 hr (*d*). At these time points, the cells were transferred to methionine-free medium containing [^{35}S]methionine (50 μCi/ml) for 1 hr. Unstimulated cells at time 0 were pulse-labeled as well (*a*). The cells were then washed in phosphate-buffered saline, the proteins were solubilized in lysis buffer, and lysates from 4×10^5 cells were separated by 2D-gel electrophoresis. The gels were exposed to x-ray film for 7 days. Type I proteins (p72 and p66), type II proteins (p52 and p36), and type III proteins (p93, p89, and p63) are indicated in each panel.

Table 1. rIL-2-stimulated protein synthesis

Protein*	Relative rate of synthesis after stimulation†				
	0 hr	4 hr	8 hr	24 hr	Type
p72	16,600	38,600	101,000	102,000	I
p66	41,800	210,000	250,000	220,000	I
p52	100	1,300	77,300	149,600	II
p36	10	100	11,500	32,600	II
p93	2,600	5,200	26,000	60	III
p89	10,400	111,200	10	10	III
p63	4,200	32,600	2,300	1,500	III

*The protein name is based on its molecular size: i.e., the molecular size of p72 is 72 kDa.
†Relative rate of synthesis calculated by 2D densitometry where rate of synthesis = area × average density.

stimulation and had markedly reduced synthetic rates (93% or greater reduction) at later time points.

To link the expression of type II proteins with cell growth, a detailed profile of protein synthesis and DNA content was obtained over 72 hr. L2 cells were pulse-labeled every 4 hr as above, the cellular proteins were separated by 1D polyacrylamide gel electrophoresis, and autoradiographs of the gels were scanned with a densitometer to quantitate rates of synthesis. Since on 2D gels there were no other proteins of molecular weights 66, 52, or 36 kDa that showed changes in rates of synthesis, the changes in density of bands on 1D gels accurately reflected the changes in rates of synthesis of p66, p52, and p36. In addition, the overall rate of protein synthesis was measured by assaying trichloroacetic acid-precipitable radioactivity in an aliquot of cellular extract from each time point. Fig. 2 shows the relative increase in total protein synthesis and the relative rates of synthesis of the type I protein p66 and the type II proteins p52 and p36. The relative rate of synthesis is given as the ratio of the area under the densitometry peaks for p66, p52, and p36 in stimulated cells to the area under the peaks for each protein in unstimulated cells.

As reported above, the fraction of the cells in S phase began to increase approximately 20 hr after rIL-2 stimulation, reached a maximum at 28 hr, and declined thereafter. Total [^{35}S]methionine incorporation was maximal at 24–28 hr after stimulation, and the rate of total protein synthesis showed a 4-fold increase over that in unstimulated cells. Fig. 2 shows that p52 and p36 reached maximal rates of synthesis at 24–28 hr after stimulation, coinciding with the time that the largest fraction of cells was in S phase. The type I protein p66 reached near 50% of its maximal rate of synthesis by 4 hr, was synthesized at its maximal rate from 12 through 28 hr, and declined to its baseline rate of synthesis by 40 hr. While p66 had a 5-fold increase in rate of synthesis from baseline, similar to the increase seen in overall protein synthesis, p52 and p36 had 15-fold increases in rates of synthesis. These data demonstrate that type II proteins are synthesized at high rates specifically during cell proliferation.

In a previous report we showed that the potassium channel blocker quinine inhibited potassium conductance in L2 cells and, when added to cultures at the time of stimulation, inhibited rIL-2-induced p52 synthesis and cell cycle progression (14). By adding quinine at various times after rIL-2 stimulation, we hoped to determine the time of occurrence of the critical event inhibited by quinine. L2 cells were stimulated with rIL-2 and quinine was added at the time of stimulation, 2 hr after stimulation, or 6 hr after stimulation. At 24 hr after the addition of rIL-2, the cells were stained with propidium iodide to measure DNA content by FACS. Additionally, the cellular proteins were biosynthetically labeled for 1 hr with [^{35}S]methionine, and the rates of synthesis of total protein, as well as specific proteins, were determined. At 24 hr after stimulation, 80% of rIL-2-stimulated L2 cells in this experiment were in S phase, and of these, 47% were already in middle S phase (Fig. 3*a*). In contrast, smaller percentages of L2 cells, stimulated with rIL-2 in the presence of quinine added at 0 or 2 hr, were in S phase at 24 hr; and 95% of these were in early S phase (Fig. 3 *b* and *c*). By 48 hr, no cycling cells were present in the quinine-exposed populations. In contrast, when quinine was added at 6 hr after stimulation, 53% of cells were in S phase at 24 hr (Fig. 3*d*), indicating that the L2 cells that had been stimulated with rIL-2 for 6 hr were much less sensitive to the effects of quinine added at this time. Minimal cell cycling was observed 48 hr after rIL-2 stimulation in this cell population (not shown), despite the lack of inhibition seen at 24 hr, indicating that a second round of division was not permitted in the presence of quinine.

The effect of quinine on p52 and p36 synthesis is shown in Fig. 3 *e–h* and is quantitated in Table 2. When quinine was added 0 or 2 hr after stimulation with rIL-2, the rates of synthesis of the type II proteins p52 and p36 were inhibited by greater than 99% compared with their rates of synthesis 24 hr after rIL-2 stimulation in the absence of quinine. In

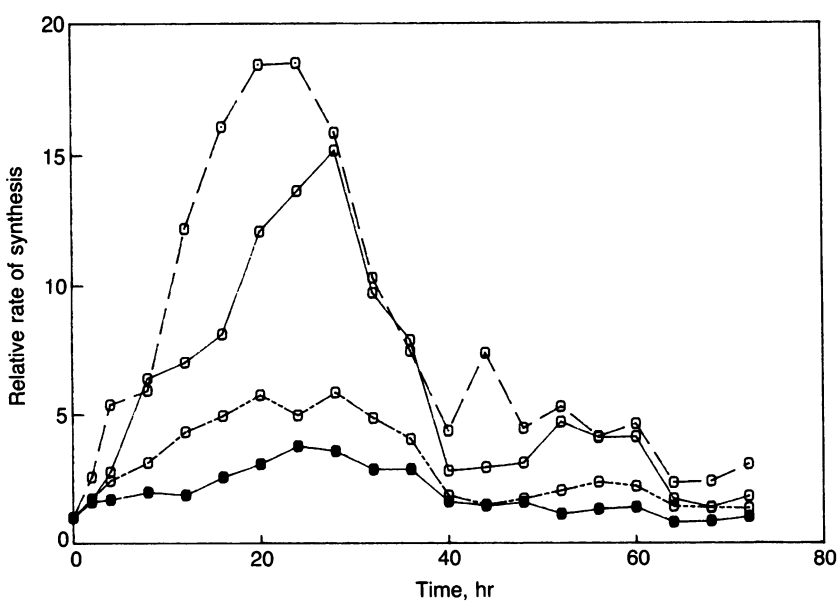
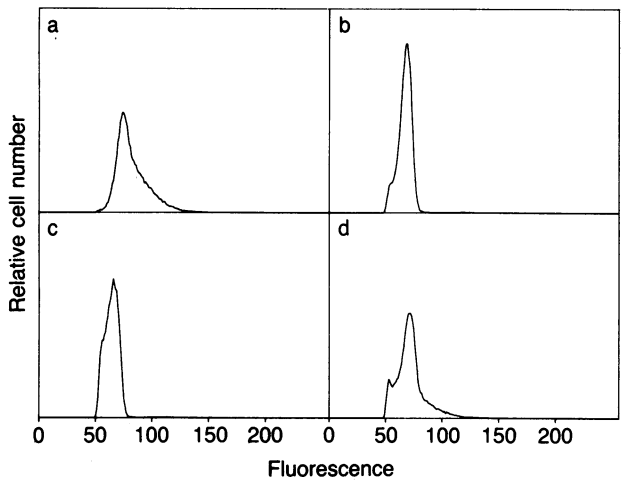


FIG. 2. Relative rates of synthesis of total protein, p66, p52, and p36 after rIL-2 stimulation of L2 cells. L2 cells were stimulated with 100 units/ml of rIL-2 and were pulse-labeled with [^{35}S]methionine at various times up to 72 hr after stimulation. The cellular proteins from 4×10^5 cells were separated by 1D-gel electrophoresis, and the gels were exposed to film. The films were scanned with a densitometer to quantitate the rates of synthesis of p66, p52, and p36 over the 72-hr time course. Relative rates were calculated by dividing the rates of synthesis at the various times by the rates of synthesis in unstimulated cells. The relative rates of total protein synthesis were quantitated by measuring the trichloroacetic acid-precipitable radioactivity from 5×10^4 cells and again normalized to the level in unstimulated cells. ■—■, Total protein; □- - -□, p66; □——□, p52; □— —□, p36.



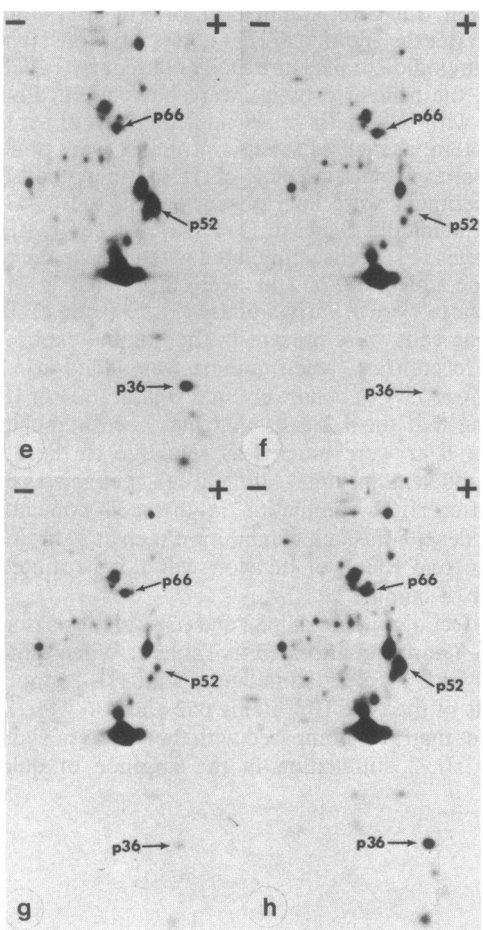


FIG. 3. DNA content and 2D-gel electrophoresis of [^{35}S]methionine pulse-labeled proteins from L2 cells stimulated with rIL-2 (100 units/ml) for 24 hr in the presence or absence (*a* and *e*) of 100 μM quinine added at 0 (*b* and *f*), 2 (*c* and *g*), or 6 (*d* and *h*) hr after rIL-2 addition. (*a–d*) FACS histograms of DNA content of L2 cells stimulated by rIL-2 in the presence or absence of quinine. At 24 hr after rIL-2 addition, cells were stained for DNA content with propidium iodine and analyzed by FACS. Percentages of cells in G_1/early S/middle S/late S/G_2 plus M were as follows: *a*, 17/42/26/12/4; *b*, 69/30/0/0/0; *c*, 86/13/0/1/0; *d*, 46/45/4/5/1. (*e–h*) At 24 hr after rIL-2 addition, the cells were pulse-labeled with [^{35}S]methionine, and the cellular proteins were extracted and separated by 2D-gel electrophoresis as in Fig. 1. The gels were exposed to film for 4 days. Type I protein p66 and type II proteins p52 and p36 are indicated.

contrast, the rate of synthesis of type I protein p66 was inhibited by only 30% when quinine was added at 0 hr and 35% when added at 2 hr (compare Fig. 3 *e*, *f*, and *g*). Overall protein synthesis was inhibited by 59% when quinine was added at 0 hr and 49% when added at 2 hr. Thus, quinine specifically inhibited synthesis of p52 and p36. When quinine was added at 6 hr after stimulation with rIL-2, the rates of synthesis of p52 and p36 were inhibited by only 25 and 35%, respectively. These results again demonstrate a correlation between cell-cycle progression and synthesis of type II proteins.

Table 2. Quinine affects specific protein synthesis

Time of quinine addition,* hr	Total protein synthesis,† cpm	Rate of synthesis, arbitrary units‡		
		p66	p52	p36
None added	28,700	4300	62,700	5300
0	11,800	3100	63	0
2	14,700	2800	270	0
6	27,500	7700	47,000	3500

*Time after rIL-2 addition.
†Total trichloroacetic acid-precipitable radioactivity ([^{35}S]methionine) per 5 μl cell lysate, 24 hr after rIL-2 addition.
‡Arbitrary units calculated by 2D densitometry 24 hr after rIL-2 addition.

DISCUSSION

When T lymphocytes are stimulated with antigen, they produce lymphokines including IL-2 (23) and express receptors for IL-2 (3). IL-2 stimulation results in subsequent DNA synthesis and cell division. Presumably, this process involves changes in gene expression, which result in an ordered sequence of altered protein synthesis, culminating in DNA synthesis. We have used the cloned murine T-helper line L2 to examine the changes in protein synthesis induced by IL-2 stimulation. It should be noted that all rIL-2-induced proteins were detectable in unstimulated cells. rIL-2 stimulation of L2 cells results in protein synthesis of three distinct types. Type I proteins (the majority of cellular proteins) have initial increases in rates of synthesis during the first few hours after stimulation, but the rates of synthesis increase minimally after 4 hr. Type III proteins are synthesized at high rates in the first 4–8 hr of stimulation and then their rates of synthesis decline. Type II proteins are synthesized at maximal rates for 24 hr after rIL-2 stimulation, when the cells are beginning DNA synthesis. These proteins have much larger relative increases in rates of synthesis than type I proteins and total cellular proteins. Quinine, a potassium channel blocker, inhibited cell-cycle progression of L2 cells (14) and specifically inhibited the increase in rates of synthesis of type II proteins, when used under conditions that inhibited cell proliferation.

The presence of voltage-gated potassium channels has been demonstrated in both human (24–28) and murine (14, 29) T lymphocytes. These channels can be blocked with pharmacological agents, including quinine (14, 24–27, 29), which will also inhibit lectin-stimulated proliferation (25–27). Quinine, when added concurrently with, or 2 hr after, rIL-2 addition, had marked effects on L2 cells: it blocked cell-cycle progression and the associated increases in the rates of synthesis of the type II proteins p52 and p36. However, addition of quinine 6 hr after rIL-2 stimulation had minimal effects on both the synthesis of p52 and p36 and cell-cycle progression. Therefore, an event (or multiple events) that regulates later protein synthesis and DNA synthesis and is sensitive to quinine occurs between 2 and 6 hr after rIL-2 stimulation.

While quinine is a potassium channel blocker, it remains to be shown that quinine's effects on cell proliferation and protein synthesis are mediated by the blockade of potassium channels. The more potent an agent is at blocking voltage-

gated potassium currents in human T lymphocytes, the more effective it is at inhibiting phytohemagglutinin-stimulated proliferation (27, 28). While such correlations suggest that potassium currents may be required for proliferation, the doses required for 50% inhibition of proliferation are higher than those needed to block 50% of potassium currents under voltage-clamp conditions. The dose of quinine (25 μM) that inhibits 50% of voltage-gated potassium currents in voltage-clamp experiments does not inhibit IL-2-driven growth (14). Only at 100 μM, when about 95% of the channels are blocked, do we observe quinine inhibition of cell growth. This suggests that proliferation will be inhibited only when a threshold level of channel block is attained (27). These results do not preclude the possibility that potassium channel blockers are inhibiting proliferation by other mechanisms.

In growth-factor-stimulated cells, prior protein synthesis may or may not be required for the expression of certain genes (4, 13, 30–32). In IL-2-stimulated lymphocytes (13) and platelet-derived growth factor-stimulated BALB/3T3 cells (30, 31), the transcription of the oncogene c-*myc* is not inhibited by treatment of the cells with cycloheximide, indicating that the expression of this gene is not dependent on prior protein synthesis. Similarly, in human lymphocytes stimulated with phytohemagglutinin and phorbol ester, the transcription of c-*myc*, IL-2, γ interferon, and IL-2 receptor mRNAs is independent of prior protein synthesis (4). In contrast, platelet-derived growth factor-induced transcription of the gene for the lysosomal major excretory protein in BALB/3T3 cells is inhibited by cycloheximide (32), as is transcription of transferrin receptor mRNA in peripheral blood T cells stimulated by phytohemagglutinin and phorbol ester (4), indicating that some protein synthesis must occur prior to the expression of these genes. The kinetics of expression of type II proteins and the ability to specifically block their synthesis with quinine suggest that expression of p52 and p36 requires prior protein synthesis.

To understand the molecular mechanisms involved in cell proliferation, it is necessary to define the biochemical events leading up to DNA synthesis. By determining a sequential order of protein synthesis following a growth signal, it will be possible to divide the growth process into stages defined by the synthesis of specific proteins. The inhibition of specific early events following a growth signal may result in the inhibition of some, but not all, later protein synthesis. Agents such as quinine, which interrupt the cell proliferation process at specific stages, will be useful for dissecting the mechanism of cell growth. In this way, important regulatory events during proliferation can be identified for further study.

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