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ARTICLES

Exogenous Glutamine Requirement Is Confined to Late Events of T Cell Activation

Heidi Hörig, Giulio C. Spagnoli, Luis Filgueira, Reto Babst, Harald Gallati, Felix Harder, Antonio Juretic, and Michael Heberer

Departments of Surgery and Research, University of Basel, CH-4031-Basel, Switzerland (H.H., G.C.S., L.F., R.B., F.H., A.J., M.H.) and Central Research Unit, F. Hoffmann LaRoche Ltd. Co. Basel, Switzerland (H.G.)

Abstract Glutamine is required for the proliferation of lymphocytes, but quantitative effects on discrete steps of activation remain unknown to date. Therefore the influence of glutamine (range: 0 mM–1 mM) on the in vitro response of human peripheral blood mononuclear cells (PBMC) to a mitogenic anti-CD3 monoclonal antibody (mAb) was investigated. Expression of surface activation markers by flow cytometry, presence of mRNA of cytokine genes by polymerase chain reaction, release of cytokines by ELISA, and entering into the cell cycle by flow cytometry were sequentially analyzed. Proliferation was measured by a ^{3}H-thymidine incorporation assay.

mRNA coding for IL-2, IL-2 receptor, IL-4, IL-5, GM-CSF, and IFN-γ was detectable independently from exogenous glutamine provision; expression of the cell surface activation marker CD69 was also glutamine independent. In contrast, *later* activation events including the expression of the surface activation markers CD25, CD45RO, and CD71 as well as the production of IFN-γ were found to require exogenous glutamine supply. In contrast, production of TNF-α could be observed in the absence of glutamine and was increased to a limited extent by exogenous glutamine. The *overall lymphocyte response* as reflected by entering into the cell cycle and proliferation was directly correlated with the glutamine concentration of the culture medium. Efficient progression through the cell cycle was found to require at least 0.5 mM glutamine and an increase in glutamine concentration from 0.1 mM to 1 mM enhanced proliferation by 50%. These results were supported by data obtained following anti-CD3 stimulation of a CD4$^+$ T cell clone. Altogether, these data underline that a complete cellular immune response depends on an exogenous glutamine supply. Regarding glutamine requirements, they define early, glutamine-independent and late, glutamine-dependent lymphocyte activation stages.

Key words: lymphocytes, activation, cytokines, cytokine gene transcription, glutamine

Glutamine is considered a nonessential amino acid [Meister, 1956], but its utilization and oxidation were found to be mandatory for rapidly dividing cells, including lymphocytes [Eagle, 1959; Kovacevic and McGivan, 1983; Ardawi and Newsholme, 1983]. Low concentrations of glutamine lead to decreased in vitro proliferation of mitogen-stimulated rat and human lymphocytes [Ardawi and Newsholme, 1983; Szondy and Newsholme, 1989; Parry-Billings et al., 1990; Ardawi, 1988], and to an impairment of human plasma cell differentiation and immunoglobulin synthesis and secretion [Crawford and Cohen, 1985]. Clinically, glutamine depletion below physiological plasma concentrations (0.3–0.6 mM), as caused by major burns, seems to play a role in the associated impairment of immune response [Parry-Billings et al., 1990].

In contrast to these general effects caused by glutamine restriction, little is known of the role of glutamine in the events which are involved in the generation of immune response. Therefore, we set up experiments to evaluate which stages of T cell activation require exogenous glutamine and what concentrations are needed.

Stimulation of T lymphocytes through the antigen receptor complex (TCR:CD3) can be in-

Abbreviations: PCR, polymerase chain reaction; GM-CSF, granulocyte-macrophage–colony stimulating factor; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; PHA, phytohaemagglutinin; TCR:CD3, T cell receptor complex; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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Address reprint requests to H. Hörig, Laboratory of Surgical Research, ZLF, Hebelstrasse 20, CH-4031 Basel, Switzerland.

duced by monoclonal antibodies (mAb). Activated T lymphocytes proceed through a series of steps including signal transduction, transcription of activation genes, expression of specific surface markers, secretion of cytokines, and entering into cell cycle ultimately resulting in proliferation [Alcover et al., 1987; Weiss and Imboden, 1987].

We hypothesized that the immediate events of T cell activation (≤ 30 min), which do not require de novo synthesis of proteins [Crabtree, 1989], would probably not depend on exogenous glutamine, in contrast to early (30 min–72 hr) and late events (≥ 72 hr). Thus, peripheral blood mononuclear cells (PBMC) of healthy volunteers were stimulated with anti-CD3 mAb in the presence of different glutamine concentrations. mRNA encoding cytokines and IL-2R were investigated, expression of early and later cell surface activation molecules on T cells was tested, release of IFN-γ and TNF-α was measured, and the glutamine requirements for PBMC cell cycle transition and proliferation were quantified.

MATERIALS AND METHODS

Cell Preparation

Mononuclear cells (PBMC) were separated from heparinized peripheral blood of healthy donors by Lymphoprep™ (Nycomed, Oslo, Norway) density gradient centrifugation. Cells were washed and the concentration was adjusted to 10^6/ml in glutamine-free RPMI 1640 (Gibco, Life Technologies, Ltd., Paisley, Scotland) supplemented with 5% dialysed and heat-inactivated AB serum (Bloodbank, SRK, Basel, Switzerland), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (complete medium) (both from Gibco). Then, PBMC were cultured overnight in the absence of glutamine to deplete the intracellular glutamine pool.

T Cell Clone

A $CD4^+$ T cell clone (ALP-8), courtesy of Prof. A. Lanzavecchia, Basel, Switzerland, was maintained in glutamine-additioned 5% AB serum RPMI 1640 complete medium in the presence of 100 U/ml rhIL-2 (Hoffmann-LaRoche Ltd., Basel, Switzerland) and periodically restimulated with purified solid phase bound anti-CD3 mAb CB3G (generously provided by Prof. F. Malavasi, Turin, Italy) at 0.5 μg/ml final concentration.

Cell Culture and Schedule of Experiments

After overnight glutamine deprivation, PBMC or ALP-8 were cultured in flat-bottom 96-well plates (Costar, Cambridge, MA) at 2×10^5 cells/well in complete medium supplemented with L-glutamine (Gibco, sterile filtered and dissolved in PBS). Glutamine concentrations ranged between 0 and 1 mM. Cells were cultured at 37°C in humidified 5% CO_2 atmosphere. Cell stimulation was provided by soluble purified anti-CD3 mAb (CB3G) at 0.5 μg/ml final concentration. Purified PHA, 1.5 μg/ml final concentration (Wellcome, London, UK), served as control. As stimuli for ALP-8, solid phase bound anti-CD3 mAb (0.5 μg/ml) or rhIL-2 (50 U/ml) were used. All tests were set up in triplicates and experiments were repeated at least three times.

Surface Activation Markers

Cultured PBMC were washed and stained with fluorochrome labeled mAb recognizing CD3, CD25, CD69, CD71 (Becton Dickinson, Mountain View, CA), and CD45RO (Dako, Copenhagen, Denmark). As negative control purified mouse immunoglobulins labeled with FITC or PE (Becton Dickinson) were used. All reagents were used at saturating concentrations. In order to obtain data concerning the expression of activation markers by $CD3^+$ lymphocytes, cells were simultaneously stained with anti-CD3 mAb and mAb recognizing activation markers, labeled with different fluorochromes (i.e., FITC-labeled anti-CD3 and PE-labeled anti-CD69). After 30 min of incubation at 4°C, cells were washed, resuspended in 0.02% paraformaldehyde, and specific fluorescence was analyzed by FACScan flow cytometer (Becton Dickinson). Data were expressed as percentages of the $CD3^+$ cell population displaying double staining.

Polymerase Chain Reaction Assays

Total RNA was extracted from cells under investigation 8 hr after stimulation with purified anti-CD3 mAb by the guanidinum isothiocyanate method [Chomczynski and Sacchi, 1987]. RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (200 U) in the presence of oligo dT and dNTP (all from BRL, Gaithersburg, MD) according to the producer's instructions. The cDNA obtained was tested for the presence of specific gene sequences in polymerase chain reactions (PCR) (GeneAmp kit, Perkin Elmer Cetus, Norwalk, CT) performed

in 20 μl volumes, by using specific primer pairs for IL-2, IL-4, IL5, IL-2R, IFN-γ, and GM-CSF [Ehlers and Smith, 1991]. As internal standard, primers amplifying cDNA encoding β-actin, a "housekeeping" gene, were employed. In order to exclude possible false positive results due to contaminating genomic DNA, all primer pairs amplified cDNA sequences encoded for in different exons. cDNA amplification was carried out by 25 cycles, each including 40 sec denaturation at 94°C, 40 sec annealing at 62°C, and 1 min extension at 72°C. After PCR, the products obtained were run on 1.5% agarose (Gibco) gels, in Tris-borate/EDTA electrophoresis buffer (0,5×) in the presence of ethidium bromide. Molecular weight markers were included in all gels (Gibco BRL, PhiX174 DNA, HaeIII digested). The identity of amplified cDNA sequences was validated by correspondence to the expected gene size and by restriction mapping (data not shown).

Cytokine Production

Cell culture supernatants obtained 48 hr after stimulation were assayed for INF-γ and TNF-α using ELISA tests [Gallati et al., 1987]. Specific mAb were bound to high binding EIA plates (Costar) and incubated overnight at 4°C. Wells were then washed and saturated with TRIS/HCl buffer, 1% bovine serum albumine (BSA; Sigma, St. Louis, MO), for 24 hr. Peroxidase-conjugated anti–IFN-γ and anti–TNF-α, respectively, recognizing cytokine epitopes different from those identified by the first mAb, were added, together with the culture supernatants, in triplicate samples. After 24 hr incubation, colors were developed by addition of specific substrates and read at 450 nm on a microplate reader (Molecular Devices, Menlo Park, CA).

Cell Cycle Kinetics

Cells were resuspended in citrate buffer (pH 7.6) on days two and three after stimulation. The CycleTest kit (Becton Dickinson) was used. Briefly, a 3% trypsin solution was added to the cells and incubated at room temperature for 10 min with careful shaking of the tubes. Subsequently, 0.5% trypsin inhibitor and 0.1 mg/ml RNAse A (pH 7.6) were added and cells were incubated as before. Propidium iodide (0.5 mg/ml) and spermine tetrahydrochloride (0.1 mg/ml) were added and the incubation was prolonged for 10 min more with the tubes protected from light. Cell cycle phase distributions were analyzed by FACScan flow cytometer (Becton Dickinson) equipped with a Cell-fit software (Becton Dickinson) using S-fit parameters [Vindelov et al., 1983].

Cell Proliferation

"De novo" synthesis of DNA was measured by ^{3}H-thymidine incorporation. Briefly, ^{3}H-thymidine was added (0.5 μCi/well, DuPont, NEN Research, Wilmington, DE) 6 hr before the termination of cultures. Cells were harvested onto glass fiber filters by using an automated cell harvester (Skatron, Lier, Norway). The dried filters were transferred to scintillation vials containing 3 ml of scintillant (Opti-Fluor, Canberra Packard, Warrenwille, IL) and tritium incorporation was measured by a β-counter. Data are expressed as counts per minute (cpm ± SD).

RESULTS

Surface Activation Markers

In all unstimulated cultures low percentages of lymphocytes expressed CD69, CD25, and CD71 (<4%). On the other hand, CD45RO was expressed on 37.7 ± 5.3% of unstimulated lymphocytes [LaSalle and Hafler, 1991]. In activated T cells, CD69 was found to be expressed following 8 hr of anti-CD3 stimulation irrespective of glutamine addition to the cultures (Fig. 1, panel A). In contrast, the percentage of CD3$^+$ cells expressing CD25 (p55 IL-2R), CD45RO (leukocyte-common antigen receptor isoform), and CD71 (transferrin receptor), as measured 3 d after T cell activation, was directly related to the addition of exogenous glutamine (Fig. 1, panel B). In cultures stimulated in the absence of exogenous glutamine, CD71 was expressed on 6.7 ± 3.1% and CD45RO on 53.0 ± 2.2% of T cells. In these conditions, CD25 was expressed on a significantly higher percentage of cells (46.8 ± 7.1) as compared to unstimulated cultures. Remarkably, at this stage of activation, percentages of CD3$^+$ cells were comparable in cultures performed in the presence of different amounts of glutamine (data not shown). Figure 2 shows data concerning double labeling of lymphocytes with anti-CD3 and anti-CD25 or anti-CD69 from one representative experiment.

Detection of Cytokine mRNA

mRNA from β-actin gene was detected under all conditions (positive control). Detection of IL-2, IL-4, IL-5, IL-2R, IFN-γ, and GM-CSF mRNA was confined to anti-CD3–stimulated

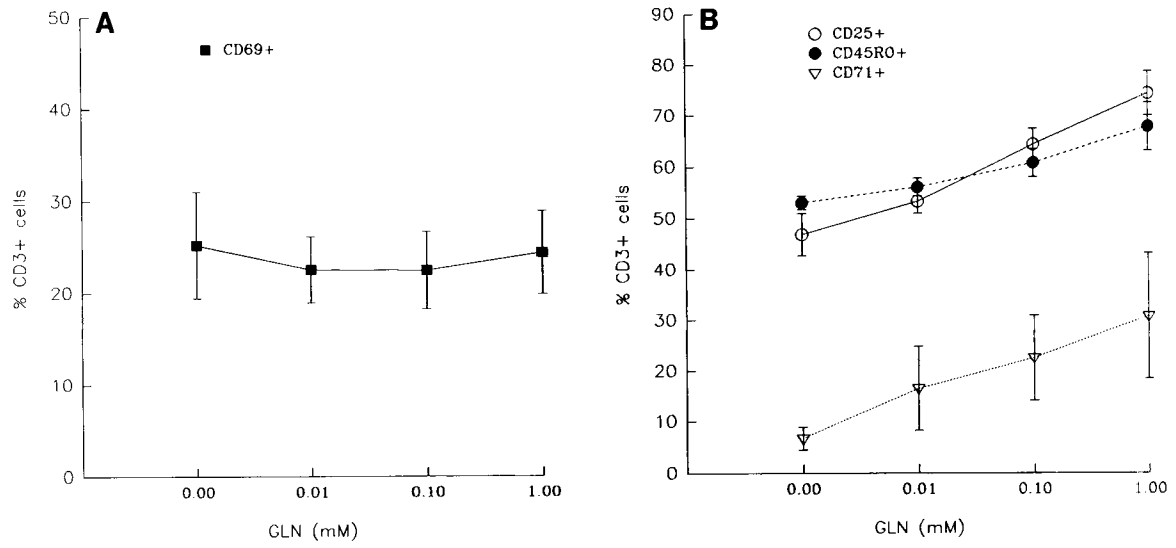

Fig. 1. Effects of glutamine on the expression of the activation marker CD69 on T cells ($CD3^+$) 8 hr after stimulation **(panel A)**, and on the expression of CD25, CD71, and CD45RO on T cells ($CD3^+$) 3 d after stimulation **(panel B)**. CD69, CD25, and CD71 were expressed on a low percentage of unstimulated cells (< 4%), at difference with CD45RO (37.7 ± 5.5%). Data represent the relative percentages of double-labeled cells and are expressed as mean values of three different experiments (x ± SEM).

PBMC but was also observed in the absence of glutamine (Fig. 3). No cytokine transcripts were detectable in unstimulated PBMC, even in the presence of optimal glutamine concentrations (Fig. 3).

Cytokine Production

IFN-γ and TNF-α production of unstimulated PBMC was below detection limits irrespective of the glutamine concentration in the culture medium. The production of both cytokines, upon anti-CD3 mAb activation, was found to be dose dependently related to the concentration of glutamine in the culture medium (Fig. 4). Without addition of glutamine, IFN-γ production reached only 30% of the amount produced in the presence of 1 mM glutamine. In contrast, TNF-α production in the absence of glutamine accounted for 80% of that produced in the presence of 1 mM glutamine. The TNF-α production reached a plateau at 0.1 mM glutamine, while maximal production of IFN-γ required 0.5 mM glutamine.

Cell Cycle Kinetics (Fig. 5)

Two days following activation, the percentage of cells that had entered in the cell cycle (S, G_2, and M phases) was directly proportional to the concentration of exogenous glutamine (Table I, panel A). In anti-CD3–triggered cultures performed in the absence of glutamine, only 7.5% of cells were found to be cycling, as compared with 4.7% in unstimulated cultures. The percentage of cycling cells increased with increasing glutamine concentration reaching optimal transition through the cell cycle at 0.5–1 mM glutamine (30–32%) with more than 5% of cells in G_2/M phase. On day 3 of culture, however, about 20% of cells were cycling even in cultures performed in the absence of glutamine, but only 2% of cells were in the G_2/M phase. This delayed entrance into the cell cycle was also observed in cultures with low concentrations of glutamine (0.01–0.1 mM) (Table I, panel B). In the presence of 0.5 mM and 1 mM glutamine, increased percentages of cycling cells were detectable (> 30%) on day 2 and 3 of culture.

Cell Proliferation

Virtually no de novo synthesis of DNA, as detectable by ^{3}H-thymidine incorporation, could be observed in cultures performed in the absence of exogenous glutamine and anti-CD3 mAb stimulation. Lymphoproliferation directly correlated with the glutamine concentration of the medium: proliferation in cultures performed at 0.1 mM glutamine was about 50% of that of cultures performed in the presence of 1 mM glutamine. Similar results were obtained upon stimulation with PHA (Fig. 6).

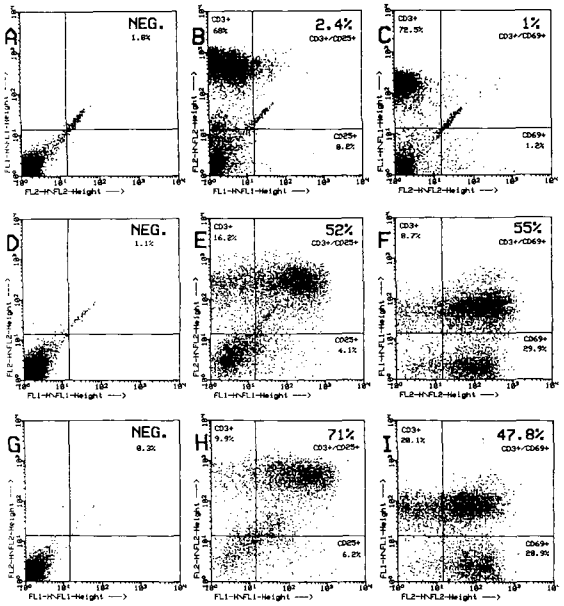

Fig. 2. Effects of glutamine on the expression of CD25 and CD69 in activated T cells. Cells were harvested from unstimulated cultures performed in the presence of 1 mM glutamine (**panels A, B,** and **C**), or from anti-CD3–stimulated cultures performed in the absence of glutamine (**panels D, E,** and **F**) or in the presence of 1 mM glutamine (**panels G, H,** and **I**). Cells were stained with FITC-labeled mouse IgG_1 (x axis) and PE-labeled IgG_{2a} (y axis) as negative controls (**panels A, D,** and **G**); percentages of positive cells never exceeded 3% in these conditions. Panels B, E, and H show data from cells stained with FITC-labeled anti-CD25 (x axis) and PE-labeled anti-CD3 (y axis). Panels C, F, and I report data from cells stained with FITC-labeled anti-CD3 (y axis) and PE-labeled anti-CD69 (x axis). Digits reported in quadrants refer to percentages of cells scoring positive for one or both markers.

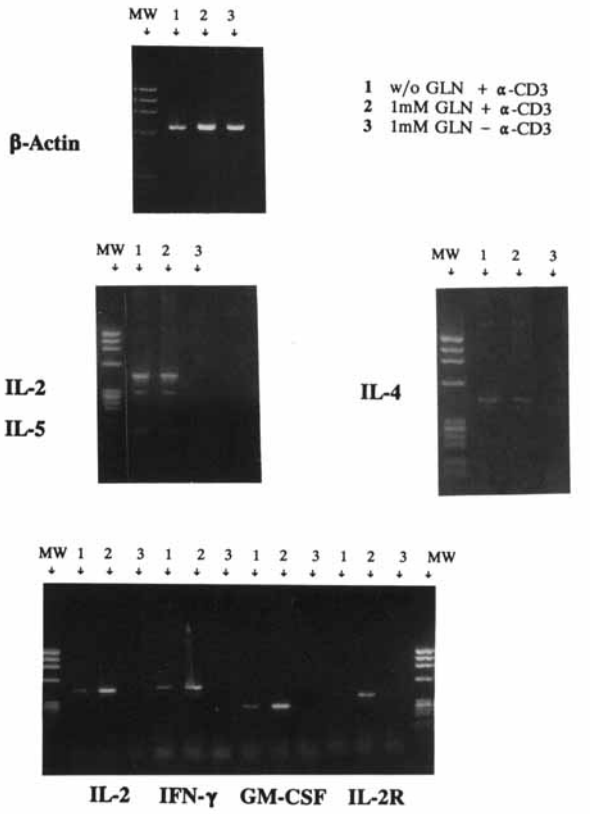

Fig. 3. Effects of glutamine on cytokine mRNA detection in activated PBMC. Total RNA was extracted from PBMC, reverse transcribed, and assayed in 25 cycles PCR by using specific cytokine primer pairs. PCR products were then run on 1.5% agarose gels in the presence of ethidium bromide and pictures were taken upon UV transillumination. For each probe, **lanes 1–3** refer to the following culture conditions: 8 hr culture without glutamine, with anti-CD3 (lane 1), with 1 mM glutamine and anti-CD3 (lane 2), and with 1 mM glutamine, but without stimulation (lane 3). MW refers to the molecular weight markers.

Proliferation of a CD4+ T Cell Clone

Anti-CD3 stimulated blastogenesis of the $CD4^+$ T cell clone ALP8 was dose dependently related to the concentration of glutamine at the initiation of culture (Fig. 7). In addition, IL-2–triggered proliferation was also found to depend on exogenous glutamine (Fig. 7).

DISCUSSION

The aim of this study was to elucidate the glutamine requirements of distinct steps of T cell activation. Glutamine is an important energy fuel for both resting and activated lymphocytes [Kovacevic and McGivan, 1983; Ardawi and Newsholme, 1983; Ardawi, 1988] and other rapidly dividing cells [Reitzer et al., 1979; Eagle et al., 1956]. The high flux of the nonoxidative glutamine metabolism provides glutamate, ammonia, aspartate, and lactate [Newsholme et al., and 1985], and allows precise regulation of biosynthetic processes at any time of the cell cycle [Szondy and Newsholme, 1989]. Furthermore, glutamine is also required for purine, pyrimidine, and protein synthesis [Salzman et al., 1958; Newsholme et al., 1985], as also suggested by the fact that it can be partially replaced by exogenous nucleosides added within 4–6 hr after cell stimulation [Szondy and Newsholme, 1991]. Lymphoproliferation induced by mitogenic PHA stimulation is known to depend on glutamine [Szondy and Newsholme, 1989; Ardawi, 1988; Parry-Billings et al., 1990; Crawford and Cohen, 1985].

Physiological T lymphocyte activation by antigen can be mimicked by anti-CD3 mAb stimulation [Weiss and Imboden, 1987; Alcover et al., 1987]. Subsequent events include signal trans-

duction, transcription of activation genes, expression of cell surface markers, release of cytokines, and finally lead to mitosis [Weiss and Imboden, 1987]. Immediate events (≤ 30 min), which are independent of protein synthesis, as well as early (30 min–72 hr) and late events (≥ 72 hr) of T cell activation, which depend on protein synthesis, have been defined [Crabtree, 1989]. In this work, we focused on early events of T cell activation, beginning with the detection of cytokine mRNA and the release of specific proteins [Crabtree, 1989].

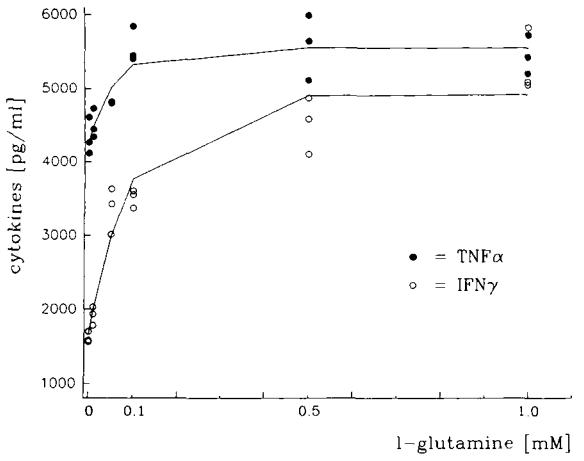

Fig. 4. Effects of glutamine on the cytokine production of anti-CD3–stimulated PBMC (2×10^5/200 µl). Glutamine-dependent IFN-γ and TNF-α production was verified by iterative exponential curve fitting (Sigma Plot, Jandel Scientific, Scientific Graph Systems). TNF-α: $y = 1270(1 - e^{-16.9x}) + 14293$; IFN-γ: $y = 3253\ (1 - e^{-10.3x}) + 1671$.

Immunocompetent cells transcribe most lymphokine mRNA 6–9 hr after stimulation [Ehlers and Smith, 1991]. We have found that the mRNA encoding for IL-2, IL-2R (p55), IL-4, IL-5, GM-CSF, and INF-γ is detectable in the absence of exogenous glutamine 8 hr after initiation of the culture. Furthermore, expression of the cell surface glycoprotein CD69, an activation marker detectable as early as 2 hr after TCR:CD3 complex triggering [Testi et al., 1989a], was also observed in the absence of exogenous glutamine. CD69 has been reported to transduce signals, ultimately resulting in IL-2 and IFN-γ gene expression [Testi et al., 1989a,b]. Therefore, it appears that activated T cells proceed first through a phase which is independent of exogenous glutamine. The glutamine required for de novo protein synthesis during this period could be provided by uptake of exogenous protein and intracellular proteolysis.

Expression of later cell surface activation markers, as exemplified by low affinity p55 IL-2R, CD45RO, and CD71, was clearly dependent on the concentration of glutamine in the culture medium. Activated T cells produce IL-2 and can concurrently amplify the proliferative response by autocrine upregulation of p55 receptor and IL-2 synthesis [Smith, 1988]. In addition, the concentration of IL-2 and the number of IL-2 receptors expressed per cell regulate the entry

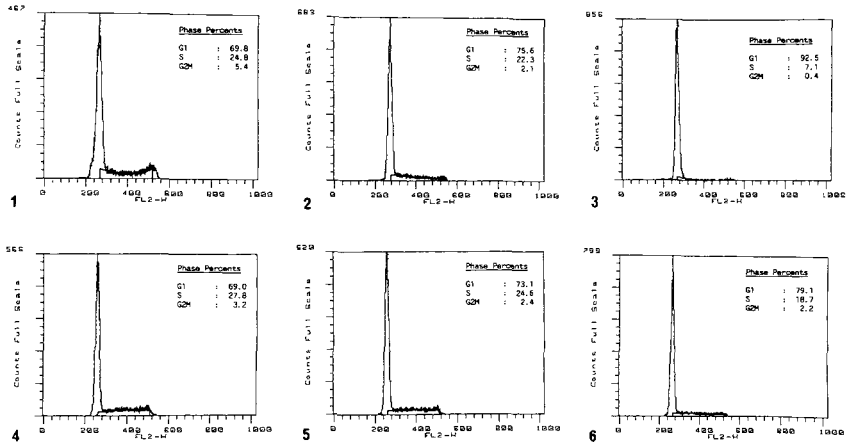

Fig. 5. Cell cycle evaluation by flow cytometric analysis of propidium iodide DNA. Cells in G_0/G_1 resting phase are represented by the narrow left peak of the DNA histogram. The broad through represents cells in the synthesis S phase. The cells in the G_2/M phase of the cell cycle are represented by the smaller peak located on the right side of the distribution. **Panels 1–3** describe cell distributions as observed on day 2, and **panels 4–6,** day 3 of investigation. Data referring to stimulated cells cultured at 0.5 mM glutamine concentration are reported in panels 1 and 4. Data from cultures performed at 0.1 mM glutamine are reported in panels 2 and 5, and data from cells cultured in the absence of glutamine in panels 3 and 6. Values are expressed as percentages of total cells.

TABLE I. Effects of Glutamine Concentrations on Cell Cycle Kinetics in the Presence or Absence of Anti-CD3*

A				
GLN/α-CD3 stimulation	G_0/G_1	S	G_2/M	S + G_2/M
1.0 mM GLN/ w/o α-CD3	95.3	3.4	1.3	4.7
w/o GLN/+ α-CD3	92.5	7.1	0.4	7.5
0.01 mM GLN/ + α-CD3	89.6	10.1	0.3	10.4
0.05 mM GLN/ + α-CD3	81.1	17.8	1.0	18.8
0.1 mM GLN/ + α-CD3	75.6	22.3	2.1	24.4
0.5 mM GLN/ + α-CD3	69.8	24.8	5.4	30.2
1.0 mM GLN/ + α-CD3	67.9	24.7	7.4	32.1
B				
GLN/α-CD3 stimulation	G_0/G_1	S	G_2/M	S + G_2/M
1.0 mM GLN/ w/o α-CD3	95.9	3.9	0.1	4.0
w/o GLN/+ α-CD3	79.1	18.7	2.2	20.9
0.01 mM GLN/ + α-CD3	77.3	21.3	1.4	22.7
0.05 mM GLN/ + α-CD3	73.2	24.5	2.3	26.8
0.1 mM GLN/ + α-CD3	73.1	24.6	2.4	27.0
0.5 mM GLN/ + α-CD3	69.0	27.8	3.2	31.0
1.0 mM GLN/ + α-CD3	67.1	29.5	3.4	32.9

*PBMC were analyzed on day 2 (A) and on day 3 (B). Reported values represent percentages of total cell numbers.

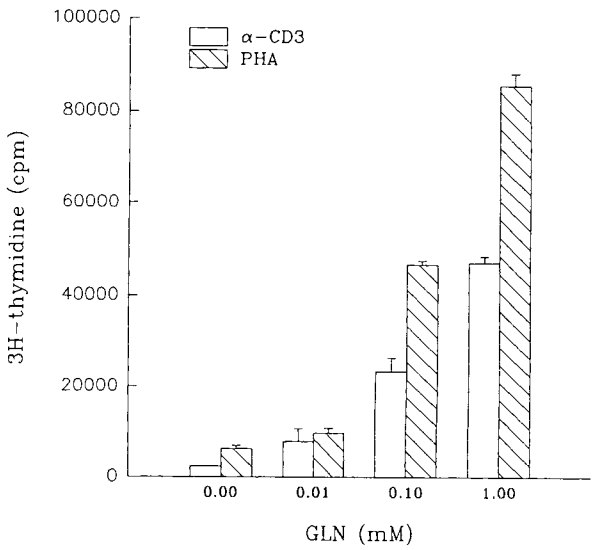

Fig. 6. Effects of glutamine on ^{3}H-thymidine incorporation by PBMC stimulated with anti-CD3 or PHA. Data report mean ± SD of triplicates.

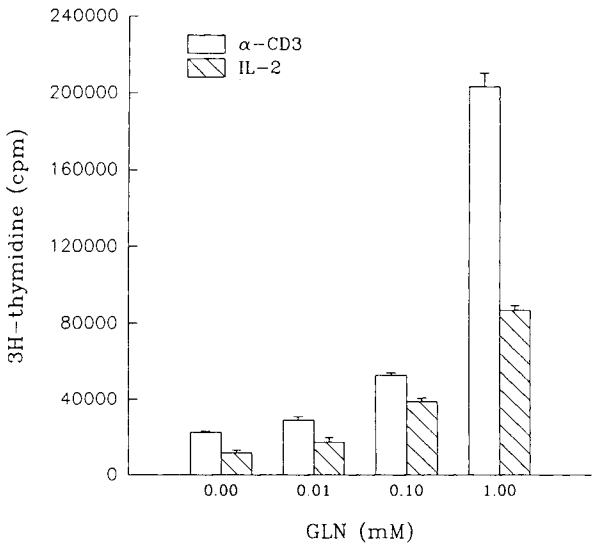

Fig. 7. Effects of glutamine on ^{3}H-thymidine incorporation by a CD4$^+$ T cell clone (ALP8) stimulated with anti-CD3 or IL-2. Data report mean ± SD of triplicates at the indicated concentrations.

into the S phase of the cell cycle [Cantrell and Smith, 1984]. Thus, the dependence of p55 IL-2R expression on the exogenous glutamine concentrations crucially affects the progression of T cells into the cell cycle.

The expression of the transferrin receptor, CD71, was also found to be reduced at low exogenous glutamine concentrations. Lymphocyte proliferation requires iron, which is taken up bound to transferrin through transferrin receptor–mediated endocytosis [Iscove and Melchers, 1978; Herzberg and Smith, 1987]. This receptor is expressed at low levels on the cell surface of resting lymphocytes, but expression increases upon activation prior to the S phase of cell cycling [Teixeira and Kühn, 1991; Reed et al., 1986; Lindsten et al., 1989]. The expression of the leucocyte-common antigen (L-CA or CD45), low molecular weight isoform CD45RO, a typical marker of "memory" cells [Sanders et al., 1988], was also dependent on the presence of glutamine in a dose-related manner. The CD45 molecule is a tyrosine phosphatase that is assumed to be involved in the regulation of intracellular signal transduction upon bind-

ing of specific ligands on its extracellular domain [Charbonneau et al., 1988; Ostergaard et al., 1989]. T cells require CD45 to enter the cell cycle in response to antigen [Pingel and Thomas, 1989; Draetta et al., 1988; LaSalle and Hafler, 1991]. Moreover, CD45RO is the physiological ligand of CD22 adhesion molecule on B cells, thus exerting an important role in cell–cell interactions [Stamenkovic et al., 1991]. Thus, the glutamine-dependent expression of cell surface activation markers involved in the regulation of T cell activation and T cell cycling could help to explain the impairment of T cell proliferation in the absence of adequate glutamine concentrations.

Cytokine protein production during the later phases of T cell activation as exemplified by the production of INF-γ was dose dependently related to the glutamine concentration in the culture medium, despite detection of cytokine mRNA in the absence of glutamine. This suggests post-transcriptional control since the intracellular concentration of glutamine may be sufficient to provide enough for protein synthesis to be saturated, considering the very low K_m for t-RNA amino acid synthesis. At least 0.5 mM exogenous glutamine was required for optimal IFN-γ production. INF-γ has been shown to play a pivotal role in the immune response against infectious challenges [Schreiber et al., 1985] and in the generation of effector cells with cytotoxic activity against tumor targets [Giovarelli et al., 1988]. In contrast, production of TNF-α, a catabolic and proinflammatory cytokine [Tracey et al., 1986; Beutler and Cerami, 1988], was only partially affected by the exogenous addition of glutamine. Similar amounts of TNF-α were released within the corresponding physiological range of 0.1 to 1 mM glutamine. One possible explanation for finding different patterns of IFN-γ and TNF-α secretions might be inherent with the different main cellular sources of these cytokines: T lymphocytes for INF-γ versus monocytes for TNF-α [Kelso, 1989].

Lymphocyte proliferation was confirmed to depend on exogenous glutamine upon PHA stimulation, as previously reported by others [Szondy and Newsholme, 1989; Parry-Billings et al., 1990; Ardawi, 1988]. We found that this also holds true for lymphocytes activated upon anti-CD3 mAb triggering of the T cell receptor complex. An increase of exogenous glutamine from 0.1 mM to 1 mM leads to a twofold enhancement of proliferative response of PBMC. To confirm T cell–specific dependency on glutamine supply, a $CD4^+$ T cell clone was tested, validating the above reported results, irrespective of the activating signal (anti-CD3 mAb, rhIL-2). Cell cycle analysis showed that the percentages of cycling cells correlated with the glutamine concentration. At least 0.5 mM exogenous glutamine is required for an efficient transition through the cell cycle.

Altogether these data help define the exogenous glutamine requirements in different activation steps of lymphocytes upon exposure to anti-CD3 mAb. An initial glutamine-independent and a later glutamine-dependent phase can be identified. These data are in agreement with those reported by Szondy and Newsholme, concerning rat lymphocyte proliferation upon Con A stimulation [Szondy and Newsholme, 1991]. According to Crabtree's classification, both these phases represent "early" activation events [Crabtree, 1989]. Steps, such as the entry into cell cycle and the expression of CD71 and CD45RO activation markers, clearly depend on an adequate glutamine supply. This "in vitro" study indicates that a glutamine concentration of 0.5 mM is a prerequisite for a complete and effective activation of human PBMC.

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