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INHIBITION OF MITOGEN-ACTIVATED PROLIFERATION OF HUMAN LYMPHOCYTES "IN VITRO" BY HIGH CONCENTRATIONS OF GLUTAMINE

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ABSTRACT

We investigated the influence of high concentrations of glutamine and asparagine on "in vitro" cellular growth of lymphocytes stimulated with phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), a recognized test of cellular immunocompetence. Human peripheral lymphocytes were cultured in flat-bottomed 96-well microplates at 37 °C for 96 (PHA and Con A) or 144 hours (PWM) in the presence of a mitogen at different concentrations and either glutamine or asparagine supplemented at doses of 2, 4 or 8 mM. Lymphocyte reactivity, measured by the incorporation of tritiated thymidine into cellular DNA, was compared to identical cultures in the absence of supplemented amino acids (controls). We found that glutamine in doses of 2 mM and higher inhibited lymphocyte proliferation of mitogen-stimulated human lymphocytes, whereas asparagine caused no effect. These results demonstrate that, although necessary for cellular division in moderate amounts, glutamine in high concentrations has the reverse effect.

Key words: glutamine, lymphocyte proliferation, immunosuppression

INTRODUCTION

Lymphocytes play an important role in the immune response and their proliferation depends on the availability of amino acids for protein synthesis (1). Glutamine is an important component necessary for

cellular proliferation in a variety of cell systems including lymphocytes (2-4). Glutamine oxidation provides energy (5), and its carbon and nitrogen provide precursors for the synthesis of purine and pyrimidine nucleotides particularly in proliferating cells (6). Glutamine also provides nitrogen for the production of glucosamine, GTP and NAD. Therefore, the pathway of glutamine catabolism allows intermediates to be directed along different biosynthetic pathways. The high utilisation of glutamine by lymphocytes indicates that this amino acid plays an important role for the maintenance of immune function. The "in vivo" immunosuppression which accompanies clinical situations, such as sepsis (7) and cancer caquexia (8), has been attributed to plasma glutamine low levels. In this context, a recent report demonstrated that dietary administration of a combination of arginine and glutamine improves survival in septic mice (9).

Since to our knowledge the available data in the literature fundamentally demonstrate the effects of moderate amounts of glutamine (0.5 to 1.0 mM) on cell growth and the inability of lymphocytes to multiply in glutamine-deficient culture medium, in the present study we investigated the effect of high concentrations of glutamine on "in vitro" mitogen-activated lymphocyte proliferation, a well known test of cellular immunocompetence. The influence of asparagine on the same parameter was also studied.

MATERIAL AND METHODS

Lymphocytes

Samples of peripheral venous blood from adult healthy individuals were collected into heparinized tubes. The plasma was separated and

the mononuclear enriched population isolated by the Ficol-Hypaque gradient method (10). The cells were removed from the interphase and after washing three times with 5 % fetal calf serum (FCS, virus and mycoplasma screened, Cultilab) in RPMI/1640 with glutamine (Gibco), were centrifuged for 5 min at 400 g and finally suspended in RPMI/1640. The final concentration of the cell suspension was 2.0 x 10^6 cells/milliliter of medium. Viability of cells was determined by the Trypan blue test and was always higher than 95 %.

Response to mitogens

The details of the procedure have been already reported (11). Briefly, flat-bottomed 96-well microtitre plates (Falcon) were prepared by adding 50 ul of cell suspension (10^5 cells), 60 ul of medium containing either PHA (final concentrations in culture between 0.25 to 5.0%, v/v, Difco), Con A (final concentrations in culture between 12.5 to 125 ug/ml, Pharmacia) or PWM (final concentrations in culture between 0.1 to 1.0%, v/v, Welcome), 40 ul of autologous plasma and 50 ul of medium containing glutamine or asparagine (Sigma Chemical Co.) at concentrations varying from 2 to 8 mM. The total culture volume was 200 ul and contained in addition 100 IU/ml penicillin and 100 ug/ml streptomycin. Control cultures were prepared identically but without the supplemented amino acids. The amounts of glutamine and asparagine in controls were those found in the culture medium and plasma, i.e., approximately 2 mM and 0.4 mM, respectively. The plates were covered with plastic lids and wrapped in adhesive plate film to prevent dehydration. The cultures were incubated in a humidified atmosphere of 5 % CO2 in air at 37 °C for 96 hours (PHA and Con A) or 144 hours (PWM). Twenty hours before harvest 50 ul of medium containing 0.5 uCi of tritiated thymidine (TRA 306, Amersham, specific

activity 2 Ci/mmol) were added to each well. Cultures were harvested on glass fiber filters by a semi-automatic multiple harvester (Cambridge Technology Inc.). Filters were dried at 60 °C for 60 min or overnight at 37 °C. The area of the filter sheets which contained cells (discs) were pushed into plastic vials, 5 ml of scintillation fluid (toluene with 5 g/l POP and 100 mg/l POPOP) was added to each vial. Cell bound ^{3}H-thymidine was determined by counting in a LKB Liquid Scintillation Counter. In some cultures, cell viability was measured at the end of the incubation period by the Trypan blue dye exclusion test. Identical triplicate cultures were always performed and the median of each triplicate used in calculations. Results of counts per minute (CPM) were expressed as percentage of control cultures. Comparison between means of lymphocyte responsiveness was calculated by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test.

RESULTS and DISCUSSION

The effects of glutamine and asparagine, added separately to cultures at concentrations of 2, 4 or 8mM, on mitogen-induced response of lymphocytes were studied (Figures 1 and 2). The Figures display means and standard deviations of lymphocyte proliferation (tritiated thymidine uptake), expressed as percentage of controls (100 % growth), of cultures treated with PHA, Con A and PWM and supplemented withthe amino acids. Glutamine inhibited the rate of ^{3}H-thymidine incorporation into lymphocytes, while asparagine had no effect. Incorporation of tritiated thymidine was between 10,000 to 45,000 counts per minute depending on the mitogen and the dose used. Cell viability determined at the end of cultures supplemented with 8mM of glutamine or asparagine was similar to that of non-supplemented

cultures, reflecting no lymphocytotoxic activity of these amino acids (results not shown).

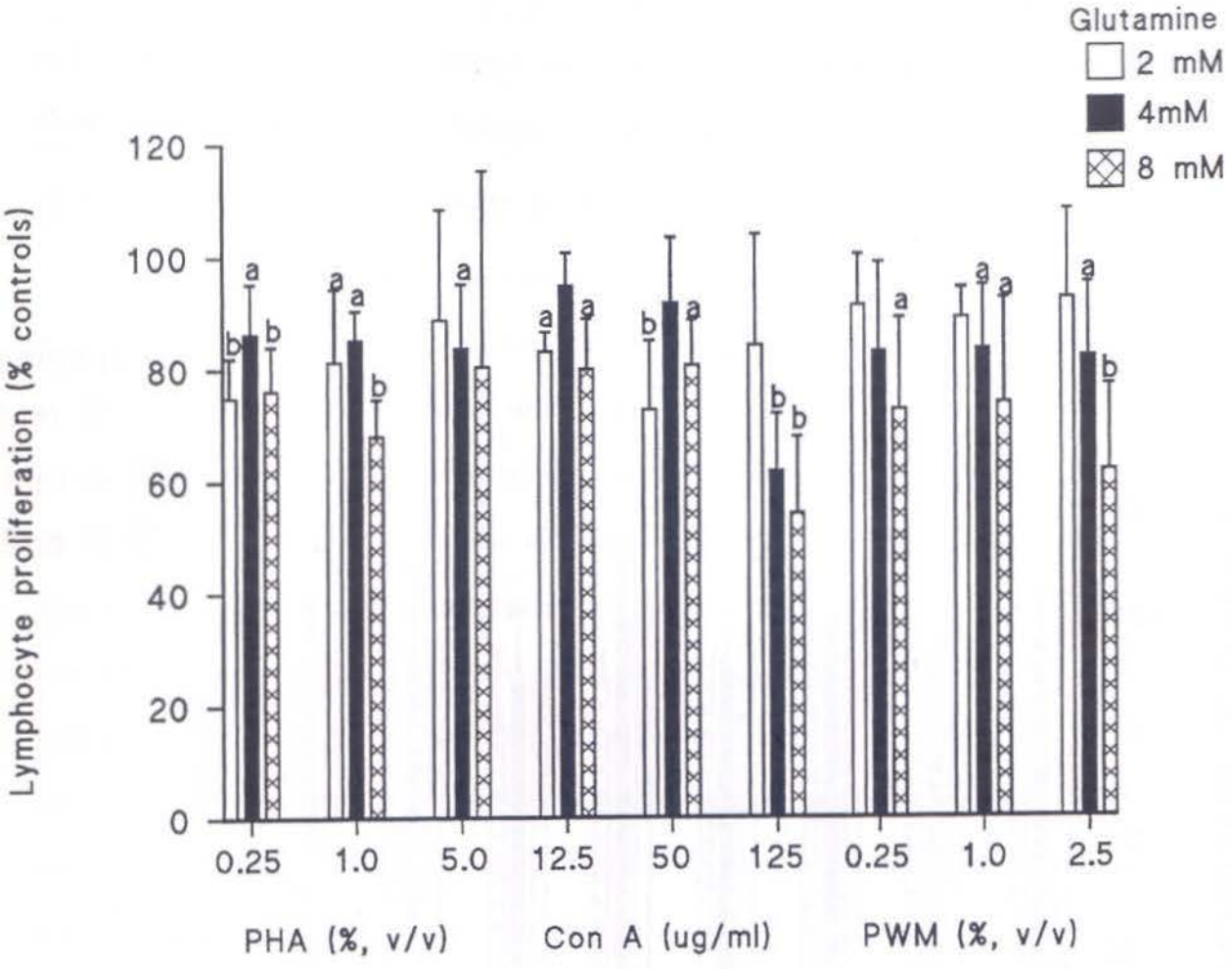

Figure 1. Effect of glutamine on the "in vitro" proliferative response of human peripheral lymphocytes stimulated by phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM). Means and standard deviations of four experiments (lymphocyte donors) performed in triplicate, expressed as percentage of tritiated thymidine incorporation of control cultures (100 % growth), are represented in the Figure. Differences between means of the various groups were calculated by ANOVA and by the Duncan multiple range test. Letters indicate significant differences between controls and the other groups. a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.01$.

In our experiments, ^{3}H-thymidine incorporation into DNA was used as an indicator of lymphocyte proliferation caused by PHA, Con A and PWM. We consider that this incorporation represents proliferation and not just DNA synthesis because there was an increase in the number of the cells at the end of the culture period (results not shown) and because the ^{3}H-incorporation was measured over a relatively long period (20 h)

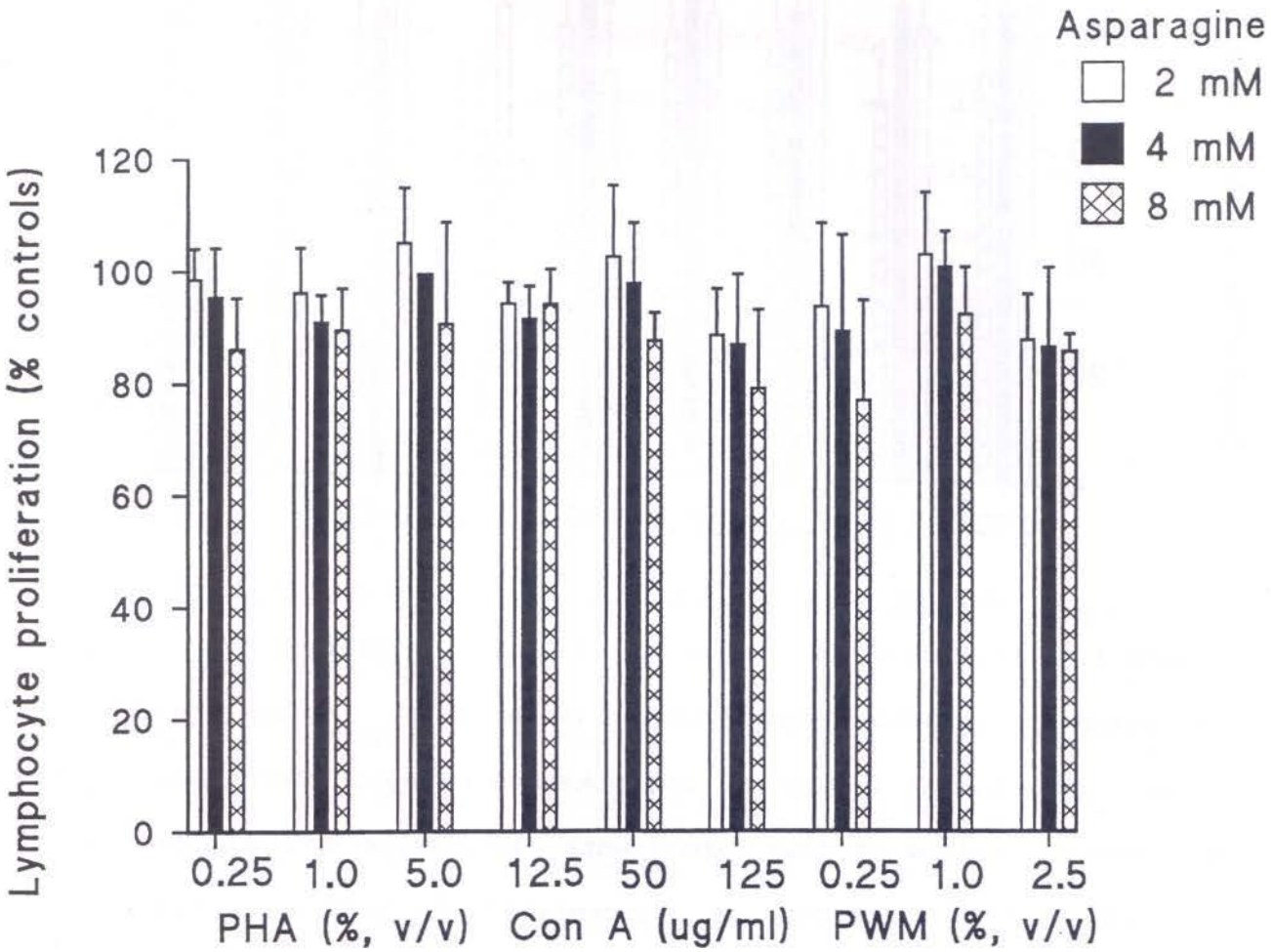

Figure 2. Effect of asparagine on the "in vitro" responsiveness of human peripheral lymphocytes stimulated by phytohemagglutinin (PHA), concannavalin A (Con A) and pokeweed mitogen (PWM). Means and standard deviations of four experiments (lymphocyte donors) performed in triplicate are displayed in the Figure. See Figure 1 for more details.

which is approximately the time for a complete cell cycle in lymphocytes. Previous investigators found that some amino acids affect the immune response "in vitro" and "in vivo", and this is interesting in view of our results (12-14).

We observed a diminution in lymphocyte responsiveness due to an excess of glutamine in the medium. Two millimolar of exogenous glutamine already caused the effect.

It is unlikely that the results reported in the present study are due to an interference of the amino acid with the used mitogens, since the detected effects were generally present with all mitogens and it is difficult for a given amino acid to react similarly with receptors of three distinct mitogens. The mechanism by which glutamine suppresses lymphocyte transformation is unknown, but may be due to ammonia formation from glutamine. On the one hand, lymphocytes contain high activities of glutaminase (15), and, on the other, it has been demonstrated that the glutamine toxic product ammonia strongly inhibits lymphocyte reactivity (16, 17). In this context, asparagine does not affect lymphocyte proliferation in vitro, because lymphocytes lack asparaginase activity and therefore virtually no ammonia is produced from this amino acid. An alternative explanation for the inhibitory activity of glutamine could be the formation of high amounts of glutamate. We have recently observed that glutamate suppresses in vitro mitogen-activated human lymphocyte blastogenesis (18), and this is in accordance with the observations of Drodge et al. (12) and Eck et al. (14), who found an in vivo suppression of the immune system by high plasma concentrations of glutamate in patients with malignant tumors.

Therefore, although essential for lymphocyte growth in the culture medium in concentrations approaching that of the human serum

(0.57 mM), our results indicate that greater glutamine concentrations are inhibitory to cellular proliferation.

On the other hand, low glutamine levels are found in sepsis (19), injury (20) and surgery (21) and are thought to contribute to the immunosuppression accompanying such situations. Therefore, the maintenance of plasma glutamine concentrations in such a group of patients very much at risk of immunosuppression is probably of benefit of maintaining immune function. It has been already observed that parenteral supplementation of glutamine have beneficial effects on the immune system of patients following bone marrow transplantation (22). In addition, dietary glutamine and arginine combination improves survival of septic mice (9).

In summary, although our results should be interpreted cautiously before further evidence is obtained, they show that high amounts of glutamine is immunosuppressive, rather than immunostimulatory. Thus, it is advisable that the recent trials aiming to improve the immune system of critically ill patients by glutamine administration should attempt to restore normal glutamine levels, avoiding high glutamine plasma levels which may possibly cause an opposite effect than that aimed.

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