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Glutamine and the immune system

Review Article

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Summary. Glutamine is utilised at a high rate by cells of the immune system in culture and is required to support optimal lymphocyte proliferation and production of cytokines by lymphocytes and macrophages. Macrophage-mediated phagocytosis is influenced by glutamine availability. Hydrolysable glutamine dipeptides can substitute for glutamine to support in vitro lymphocyte and macrophage functions. In man plasma and skeletal muscle glutamine levels are lowered by sepsis, injury, burns, surgery and endurance exercise and in the overtrained athlete. The lowered plasma glutamine concentrations are most likely the result of demand for glutamine (by the liver, kidney, gut and immune system) exceeding the supply (from the diet and from muscle). It has been suggested that the lowered plasma glutamine concentration contributes, at least in part, to the immunosuppression which accompanies such situations. Animal studies have shown that inclusion of glutamine in the diet increases survival to a bacterial challenge. Glutamine or its precursors has been provided, usually by the parenteral route, to patients following surgery, radiation treatment or bone marrow transplantation or suffering from injury. In most cases the intention was not to stimulate the immune system but rather to maintain nitrogen balance, muscle mass and/or gut integrity. Nevertheless, the maintenance of plasma glutamine concentrations in such a group of patients very much at risk of immunosuppression has the added benefit of maintaining immune function. Indeed, the provision of glutamine to patients following bone marrow transplantation resulted in a lower level of infection and a shorter stay in hospital than for patients receiving glutamine-free parenteral nutrition.

Keywords: Amino acids – Glutamine – Lymphocyte – Macrophage – Mononuclear cell – Cytokine – Infection

Introduction

Glutamine is the most abundant amino acid in the blood and in the free amino acid pool in the body. Glutamine can be synthesised in many cells and tissues of the body. However, only certain tissues are able to release significant amounts of glutamine. These include the lung, brain and skeletal muscle. Because of its large mass, skeletal muscle is considered to be the most important glutamine producer in the body. In skeletal muscle glutamine contributes approximately 60% of the total free amino acid pool and has a concentration of approximately 20 mM (Bergstrom et al., 1974; Garber, 1980; Lund, 1981). Once released from skeletal muscle, glutamine acts as an inter-organ nitrogen transporter (Lund and Williamson, 1985; Newsholme et al., 1989). Glutamine is as an energy source, a precursor for protein synthesis and donates nitrogen for the synthesis of purines, pyrimidines, nucleotides and amino sugars (Meister, 1956; Garber, 1980; Lund, 1981). The plasma glutamine concentration in the fed rat is approximately 1 mM while in the healthy adult human it is approximately 0.6 mM. Important users of glutamine include the kidney (Tizianello et al., 1982), liver (Haussinger, 1989), small intestine (Windmueller and Spaeth, 1974; Souba, 1991; Deutz et al., 1992a) and cells of the immune system (Newsholme et al., 1989; Calder, 1994a, 1995a).

Glutamine metabolism by cells of the immune system

The first enzyme in the pathway of glutamine utilisation is glutaminase:

$$\text{Glutamine} + H_2O \rightarrow \text{Glutamate} + NH_4^+$$

The activity of glutaminase is high in all lymphoid organs examined including lymph nodes, spleen, thymus, Peyer's patches and bone marrow (Ardawi and Newsholme, 1985), in lymphocytes isolated from rat lymph nodes, spleen and thymus and from human peripheral blood (Ardawi, 1988; Keast and Newsholme, 1990), in macrophages isolated from the mouse peritoneal cavity (Newsholme et al., 1986) and in rat neutrophils (Curi et al., 1997). Glutaminase activity increases in the popliteal lymph node in response to an immunological challenge (Ardawi and Newsholme, 1982).

Consistent with the high activity of glutaminase, glutamine is utilised at a high rate by cultured resting lymphocytes (Brand, 1985; Ardawi and Newsholme, 1983; Ardawi, 1988a; Brand et al., 1989; O'Rourke and Rider, 1989), macrophages (Newsholme et al., 1987; Newsholme and Newsholme, 1989) and neutrophils (Curi et al., 1997). Mitogenic stimulation of lymphocytes increases both glutaminase activity (Brand, 1985) and the rate of glutamine utilisation (Brand, 1985; Ardawi and Newsholme, 1983; Ardawi, 1988a; Brand et al., 1989; O'Rourke and Rider, 1989). The major products of glutamine utilisation by cultured lymphocytes and macrophages are glutamate, aspartate, lactate and ammonia, although alanine, lactate and pyruvate are also produced and some glutamine ($\leq$25%) is completely oxidised (Brand, 1985; Ardawi and Newsholme, 1983; Newsholme et al., 1987; Ardawi, 1988a; Brand et al., 1989; O'Rourke and Rider, 1989; Newsholme and Newsholme, 1989). These studies of glutamine metabolism by lymphocytes

and macrophages were performed using isolated cells in culture and it is possible that the high rate of glutamine utilisation and its limited oxidation are in some way a result of culture conditions (see discussion by Dejong et al., 1994; Calder, 1994b). However, although pre-surgery the porcine spleen released glutamine, there was net glutamine uptake by the spleen post-surgery which was accompanied by a 7-fold increase in ammonia release (Deutz et al., 1992b). Thus when the immune system is challenged (e.g. by surgery) at least one lymphoid organ (the spleen) dramatically increases its utilisation of glutamine.

The regulation of immune cell functions by glutamine

The high rate of glutamine utilisation by lymphocytes and macrophages and its increase when these cells are challenged suggests that provision of glutamine might be important to the function of these cells and so to the ability to mount an efficient immune response. Thirty years ago it was reported that addition of asparaginase or glutaminase to cultures of lymphocytes prevented the cells from proliferating (Hirsch, 1970; Simberkoff and Thomas, 1970). Furthermore, asparaginase treatment of animals leads to immunosuppression (Brambilla et al., 1970; Chakrabaty and Friedman, 1970; Ashworth and MacLennan, 1974; Kafkewitz and Bendich, 1983). The immunosuppressive effect of asparaginase was shown to be due to its ability to hydrolyse glutamine and so decrease its availability to the immune system (Ashworth and MacLennan, 1974; Durden and Distasio, 1981; Kafkewitz and Bendich, 1983). These observations suggest that a supply of glutamine is required for the immune system to function optimally. Several specific immunomodulatory actions of glutamine have now been reported.

Influence of glutamine on T-lymphocyte proliferation in vitro

Lymphocyte proliferation is the process of division in response to a mitogenic stimulus; in vivo this is most likely to be the presentation of processed antigen by an antigen presenting cell to the T-lymphocyte. In vitro T-lymphocytes can be stimulated to proliferate by using a variety of agents including the mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA). Most commonly lymphocyte proliferation is measured as the incorporation of a radioactively-labelled precursor (e.g. thymidine) into DNA. The proliferative response of rat (Ardawi and Newsholme, 1983; Szondy and Newsholme, 1989), mouse (Griffiths and Keast, 1990; Yaqoob and Calder 1997 [see Fig. 1]) and human (Parry-Billings et al., 1990a; Chuang et al., 1990) lymphocytes to T-cell mitogens is dependent upon the availability of glutamine: in the absence of glutamine these cells do not proliferate, but as the glutamine concentration in the culture medium increases lymphocyte proliferation increases. Lymphocyte proliferation increases greatly over the glutamine concentration range between 0.01 and 1 mM and appears to be maximal at normal physiological concentrations. Other amino acids, including glutamate, aspartate and arginine, cannot substitute for glutamine to support lymphocyte proliferation (Ardawi and Newsholme, 1983; Calder, 1995b). However, dipeptides

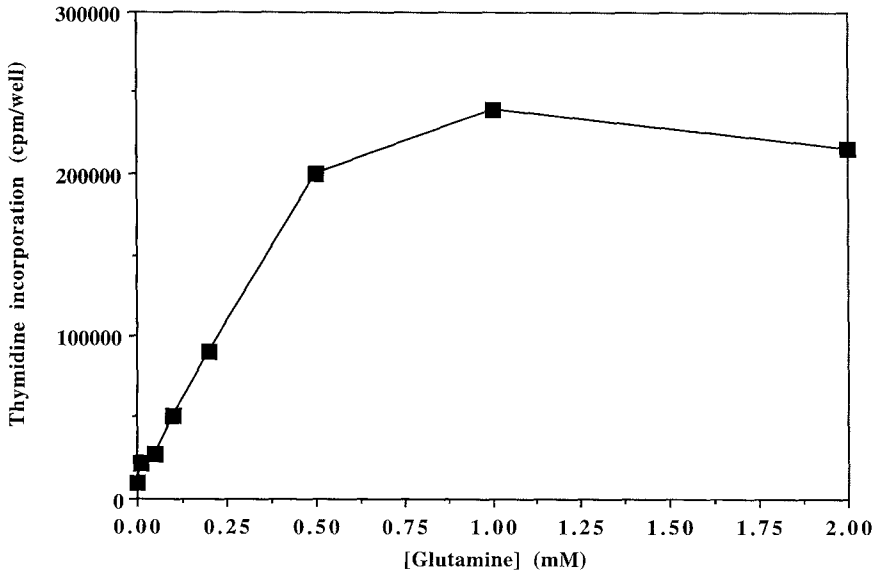

Fig. 1. Effect of glutamine on rat lymphocyte proliferation. Data are redrawn from Yaqoob and Calder (1997)

which contain glutamine (e.g. alanyl-glutamine) can act as a replacement for glutamine to support in vitro T-lymphocyte proliferation (Brand et al., 1989; Kweon et al., 1991).

In response to stimulation of T lymphocytes, there is enhanced transcription of genes for various cytokines and cytokine receptors; among these interleukin-2 (IL-2) and its receptor appear to be particularly important for T-lymphocyte proliferation and for T-lymphocyte-mediated regulation of the activity of other cells of the immune system (macrophages, natural killer cells, B-lymphocytes). The continued synthesis and secretion of IL-2 and the appearance on the cell surface of receptors for IL-2 are required if activated T-lymphocytes are to proliferate (Smith, 1988). Increased availability of glutamine enhanced IL-2 production by Con A-stimulated rat (Calder and Newsholme, 1992), mouse (Yaqoob and Calder, 1997) and human (Rohde et al., 1996a; Yaqoob and Calder, 1998) lymphocytes, and also increased expression of the IL-2 receptor on stimulated rat lymphocytes (Yaqoob and Calder, 1997). The latter study also reported that the proportion of $CD4^+$ lymphocytes increased with increasing concentration of glutamine in the culture medium (Yaqoob and Calder, 1997).

Influence of glutamine on B-lymphocyte differentiation in vitro

The differentiation of B-lymphocytes into antibody synthesising cells in vitro is glutamine dependent and increases greatly over the physiological range of glutamine concentrations (Crawford and Cohen, 1985). This effect of glutamine cannot be mimicked by glutamate or asparagine (Crawford and Cohen, 1985).

Influence of glutamine on macrophage functions in vitro

In contrast to lymphocytes which are rapidly dividing cells, macrophages are terminally differentiated cells which have lost their abilty to divide. However, they remain very active cells characterised by high rates of phagocytosis, protein secretion and membrane recycling. The level of cell surface expression of various molecules involved in phagocytosis and in intercellular interactions (major histocompatibility complex (MHC) II) by human blood monocytes was influenced by the concentration of glutamine in which the cells were cultured (Spittler et al., 1995, 1997). This was associated with increased function (i.e. increased phagocytosis of immunoglobulin G or complement opsonised particles and increased antigen presentation) with increasing glutamine availability (Spittler et al., 1995, 1997). Glutamine avaliabilty influenced the phagocytic uptake of unopsonised yeast cell walls (Parry-Billings et al., 1990a) and of opsonised sheep red blood cells (Wallace and Keast, 1992) by incubated murine macrophages. RNA synthesis by murine macrophages was found to be glutamine dependent (Wallace and Keast, 1992). Alanyl-glutamine can replace glutamine to support in vitro phagocytosis by rat macrophages (Kweon et al., 1991).

Influence of glutamine on neutrophil functions in vitro

Addition of glutamine to cultures of blood neutrophils taken from patients with burns or post-surgery improved the defective anti-microbial activity of those cells (Ogle et al., 1994; Furukawa et al., 1997).

Influence of glutamine on cytokine production in vitro

The influence of glutamine availabilty on the production of cytokines other than IL-2 by cultured rodent and human cells has been investigated.

Tumour necrosis factor-α (TNF-α) is the first cytokine released in response to bacterial endotoxin (or lipopolysacchide (LPS)). It is produced mainly by activated monocytes and macrophages. TNF activates neutrophils, monocytes and macrophages to initiate bacterial and tumour cell killing, increases adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulates T- and B-lymphocyte function, up-regulates major histocompatibility antigens and initiates the production of other pro-inflammatory cytokines such as IL-1 and IL-6. Thus, TNF is a mediator of both natural and acquired immunity and is an important link between specific immune responses and acute inflammation. In addition, TNF-α mediates the systemic effects of inflammation such as fever and hepatic acute phase protein synthesis. IL-1 appears to be the second cytokine released in response to inflammatory stimuli, including LPS and TNF, and it shares many of the pro-inflammatory effects of TNF. Again, IL-1 is produced mainly by activated monocytes and macrophages. There are two IL-1 species, α and β, which have similar biological activities and share cell surface receptors. IL-1 stimulates T- and B-lymphocyte proliferation and release of other cytokines (e.g. IL-2,

IL-6). IL-6 is produced by activated monocytes and macrophages in response to IL-1 and TNF. It has a wide range of activities, many of them shared with TNF and IL-1, including modulation of T- and B-lymphocyte function. Interferon-γ (IFN-γ) is a cytokine released by T-lymphocytes. It is a potent activator of monocytes, macrophages and natural killer cells inducing cytotoxicity and thus plays a key role in immunity towards bacteria and viruses.

Wallace and Keast (1992) showed that murine macrophages stimulated with LPS made increasing amounts of IL-1 as the supply of glutamine increased, while very recently Murphy and Newsholme (1999) reported similar enhancent of TNF-α production by rat macrophages with increasing glutamine availability.

IFN-γ production by human blood lymphocytes is enhanced with increasing availability of glutamine (Rohde et al., 1996a; Heberer et al., 1996; Yaqoob and Calder, 1998), with maximum production occurring at a concentration below 0.5 mM. In contrast to the observations with rodent macrophages, production of TNF-α, IL-1β and IL-6 by human blood monocytes (Rohde et al., 1996a; Yaqoob and Calder, 1998) and lymphocytes (Heberer et al., 1996) appears to be little affected by glutamine availability, although one study suggests otherwise for IL-6 production (Peltonen et al., 1997). IL-8 production by LPS stimulated human blood monocytes was markedly increased with increasing glutamine concentration (Murphy and Newsholme, 1999).

Influence of dietary glutamine on immune cell functions and cytokine production

Despite the large number of in vitro studies illustrating the immunoenhancing effect of glutamine, there are relatively few studies of the effectiveness of dietary glutamine. Three animal studies have now reported that enrichment of the diet with glutamine increases ex vivo T-lymphocyte proliferation (Shewchuk et al., 1997; Yoo et al., 1997; Kew et al., 1999). Shewchuk et al. (1997) reported that Con A-stimulated proliferation of spleen lymphocytes taken from tumour-bearing rats fed diets containing 257 g casein plus 20 g glutamine/kg was greater than that of those taken from rats fed 257 g casein/kg; the precise glutamine contents of these diets were not given but it can be estimated from the information provided that they contained approximately 20 to 30 g and 45 to 55 g glutamine/kg, respectively. In a recent study, spleen lymphocytes from mice fed for for two weeks on a diet containing 54.8 g glutamine/kg proliferated better in response to Con A than those from mice fed on a diet containing 19.6 g glutamine/kg (Kew et al., 1999); the glutamine-enriched diet also increased the proportion of $CD4^+$ lymphocytes in the spleen and increased the proportion of stimulated lymphocytes bearing the IL-2 receptor.

Until recently there has been little information about the effect of dietary glutamine on cytokine production. We have recently conducted two studies to investigate the effect of increasing the dietary supply of glutamine upon the ex vivo production of cytokines by murine macrophages and lymphocytes,

respectively. Mice were fed for two weeks on a diet which included 200 g casein/kg providing 19.6 g glutamine/kg, or a glutamine-enriched diet which provided 54.8 g glutamine/kg partly at the expense of casein. The production of all three cytokines investigated (TNF-α, IL-1β and IL-6) was greater for LPS-stimulated macrophages from mice fed the glutamine-enriched diet (Wells et al., 1999). IL-2 production was significantly greater for Con A-stimulated spleen lymphocytes from mice fed the glutamine-enriched diet (Kew et al., 1999). These two studies suggest that increasing the amount of glutamine in the murine diet enhances the ability of both macrophages and T-lymphocytes to respond to stimulation, at least in terms of cytokine production. These observations suggest that increasing the oral availability of glutamine could promote immune responses involving macrophage- or T cell-derived cytokines.

Plasma and muscle glutamine levels in trauma

One of the early responses to stress that occurs in skeletal muscle is the export of glutamine from the intracellular free amino acid pool. This lowers the intracellular glutamine concentration leading to protein breakdown and de novo synthesis of glutamine from other amino acids. Glutamine synthetase in skeletal muscle is upregulated by glucocorticoids (Max et al., 1988), and glucocorticoids increase glutamine efflux from skeletal muscle (Muhlbacher et al., 1984; Parry-Billings et al., 1990b). TNF-α also induces glutamine synthetase gene expression in cultured skeletal muscle cells (Chakrabarti, 1998).

Animal studies indicate that intramuscular and plasma glutamine concentrations are decreased in stress situations such as in sepsis (Parry-Billings et al., 1989; Ardawi and Majzoub, 1991) and cancer cachexia (Parry Billings et al., 1991) and following burn injury (Ardawi, 1998b); muscle glutamine concentration was also decreased in the wounded rat (Albina et al., 1987). Glucocorticoid treatment also decreases skeletal muscle and plasma glutamine concentrations (Muhlbacher et al., 1984; Parry-Billings et al., 1990b). In man plasma glutamine levels are lowered (by up to 50%) by sepsis (Askanazi et al., 1980; Roth et al., 1982; Milewski et al., 1982), injury (Askanazi et al., 1980), and burns (Stinnett et al., 1982; Parry-Billings et al., 1990a), following surgery (Askanazi et al., 1978; Lund et al., 1986; Parry-Billings et al., 1992a; Powell et al., 1994; Jensen et al., 1996), endurance exercise (Parry-Billings et al., 1992b; Rohde et al., 1996b; Castell et al., 1997) and athletic training (Keast et al., 1995; Hack et al., 1997) and in the overtrained athlete (Parry-Billings et al., 1992b). Furthermore, the skeletal muscle glutamine concentration is lowered by more than 50% in at least some of these situations (Askanazi et al., 1978, 1980; Roth et al., 1982; Milewski et al., 1982). These observations indicate that a significant depletion of the skeletal muscle glutamine pool is characteristic of trauma. The lowered plasma glutamine concentrations which occur are most likely the result of demand for glutamine (by the liver, kidney, gut and immune system) exceeding the supply, and it is proposed that glutamine be considered a conditionally essential amino acid during stress (Lacey and

Wilmore, 1990). It has been suggested that the lowered plasma glutamine contributes, at least in part, to the immunosuppression which accompanies such situations. Because of the apparent immunostimulatory actions of glutamine described above, it seems sensible to provide glutamine to patients following surgery, radiation treatment or bone marrow transplantation or suffering from injury, sepsis or burns.

Effect of exogenous glutamine on immune function and survival in animal models of infection and trauma

A number of animal studies have been performed to investigate the effect of glutamine on the ability to respond to infection. Glutamine-supplemented parenteral nutrition improved survival (75% vs. 25% in the control group receiving standard parenteral nutrition) in rats following caecal ligation and puncture (Ardawi, 1991). Likewise, intravenous glutamine improved survival (92% vs. 55% in the control group) following an intraperitoneal injection of live *Eschericia coli* into rats (Inoue et al., 1993). Parenteral administration of alanyl-glutamine into rats improved survival (86% vs. 44% in the control group) in response to intraperitoneally-infused *Eschericia coli* (Naka et al., 1996). Suzuki et al. (1993) fed mice for 10 days on diets containing casein or casein supplemented with 20g or 40g glutamine/kg and then innocculated them intravenously with live *Staphylococcus aureus*. Over the following 20 days, during which the mice were maintained on the same diets they had been fed prior to infection, 80% of the control animals died, while mortality was 60% in the 20g glutamine/kg group and 30% in the 40g glutamine/kg group. In addition to enhanced survival, these studies showed that glutamine improved nitrogen balance, diminished the sepsis-induced decrease in muscle glutamine concentration, and decreased muscle protein breakdown (Ardawi, 1991), increased plasma glutamine concentration (Inoue et al., 1993), increased intestinal function and/or integrity (Inoue et al., 1993; Naka et al., 1996), and enhanced muscle protein synthesis (Ardawi, 1991; Naka et al., 1996). These studies did not measure indices of immune function. However, Yoo et al. (1997) found that proliferation of blood lymphocytes from *Eschericia coli*-infected piglets was significantly higher if the piglets consumed a diet containing 40g glutamine/kg compared with a diet which did not contain glutamine. Furthermore, infusion of alanyl-glutamine into tumour-bearing rats increased the in vitro phagocytic capacity of alveolar macrophages (Kweon et al., 1991), while infusion into septic rats increased in vitro proliferation of mitogen-stimulated blood lymphocytes (Yoshida et al., 1992). Glutamine or alanyl-glutamine provided parenterally maintained the lymphocyte yield from Peyer's patches and intestinal integrity in mice given an intranasal innocculation of influenza virus (Li et al., 1998). These studies indicate that provision of glutamine either parenterally or enterally increases the function of various immune cells and that this might account for the enhanced resistance to infection observed in other studies.

In an animal model of haemorrhagic shock, standard parenteral nutrition decreased the ex vivo release of TNF-α and IL-6 by LPS-stimulated gut

mononuclear cells and spleen macrophages and was associated with injury to the gut mucosa and bacterial translocation into the mesenteric lymph nodes (Schroder et al., 1998). Inclusion of alanyl-glutamine and glycyl-glutamine in the parenteral regimen improved mucosal structure and prevented the fall in ex vivo IL-6, but not TNF-α, release (Schroder et al., 1998).

Provision of glutamine in trauma in man

The provision of glutamine or glutamine "precursors" (glutamine-containing dipeptides, N-acetylglutamine, α-ketoglutarate, branched chain amino acids), usually by the parenteral route, has been used in various trauma situations (Stehle et al., 1989; Hammarqvist et al., 1990; Lowe et al., 1990; Souba et al., 1990; Scheltinga et al., 1991; Ziegler et al., 1992; van der Hulst et al., 1993). In most cases the intention was not to stimulate the immune system but rather to maintain nitrogen balance, muscle mass and/or gut integrity. Nevertheless, the maintenance of plasma glutamine concentrations in such a group of patients very much at risk of immunosuppression might have the added benefit of maintaining immune function.

The provision of glutamine intravenously to patients following bone marrow transplantation resulted in a lower level of infection (12% of patients with clinical infections vs. 42% in the control group) and a shorter stay in hospital (29 $\pm$ 1 days vs. 36 $\pm$ 2 days) than for patients receiving glutamine-free parenteral nutrition (Ziegler et al., 1992). A later report by this group (Ziegler et al., 1998) showed that glutamine treatment resulted in greater numbers of total lymphocytes, T-lymphocytes and $CD4^+$ lymphocytes (but not B-lymphocytes or natural killer cells) in the bloodstream after the patients were discharged. The authors suggested that glutamine specifically enhances T-lymphocytes and that this might be responsible for the diminished infection rate observed.

Very low birthweight babies who received a glutamine-enriched premature feeding formula (providing 0.3 g glutamine/kg body weight per day) had a much lower rate of sepsis (11% vs. 31%) than babies who received a standard formula (Neu et al., 1997). In a study of patients in intensive care, glutamine provision decreased mortality compared with standard parenteral nutrition (43% vs. 67%) and changed the pattern of mortality (Griffiths et al., 1997). Neither of these studies reported immunological outcomes of the treatments. However, another study of patients in intensive care reported that enteral glutamine increased the blood lymphocyte CD4:CD8 ratio (Jensen et al., 1996). In a recent study, in which patients received nutrition (enteral glutamine vs. standard enteral feed) from within 48 hours of the trauma, there was a significant reduction in the 15-day incidence of pneumonia (17% vs. 45% in the control group), bacteremia (7% vs. 42%) and severe sepsis (4% vs 26%) in the glutamine group, although this was not associated with reduced mortality (Houdijk et al., 1998). Parenteral administration of glutamine into patients post-colorectal surgery increased mitogen-stimulated proliferation of blood lymphocytes (O'Riordain et al., 1994), suggesting that glutamine does

improve T-lymphocyte function is patients at risk of sepsis; glutamine did not affect ex vivo TNF or IL-6 production. In another study in post-operative patients those who received alanyl-glutamine parenterally had increased blood lymphocyte numbers, increased ex vivo production of cysteinyl leukotrienes by blood neutrophils and a shorter stay in hospital (Morlion et al., 1998).

Giving marathon runners a glutamine containing drink immediately and 2 hours after completing the race led to a significant reduction in the incidence of infections (19% vs. 52% in the control group) over the following week (Castell and Newsholme, 1997).

In addition to this direct immunological effect, glutamine, even provided parenterally, improves gut barrier function in patients at risk of infection (van der Hulst et al., 1993). This would have the benefit of decreasing the translocation of bacteria from the gut and so eliminating a key source of infection.

Concluding remarks

Glutamine depletion in vivo results in immunosuppression and many stress situations in man are associated with lowered plasma (and muscle) glutamine levels. Glutamine is used at a high rate by cells of the immune system and there is much evidence that key functions of these cells, tested in vitro, are dependent upon the provision of glutamine. Evidence is now emerging that glutamine supplied orally or intravenously has immunostimulatory actions. As such, administration of glutamine or its precursors should prove beneficial as a therapy for individuals whose immune system is compromised by stress. Nevertheless, more information is required about the influence of glutamine upon the action of the immune system in vivo, how this might differ between health and disease and how it might depend upon the route of glutamine administration. Importantly, the mechanism of action of glutamine within the immune system remains unresolved.

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